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Neurobiology of Nitrous Oxide-Induced Antinociceptive Effects

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Abstract

Nitrous oxide (N_2O), or laughing gas, has been used for clinical anesthesia for more than a century and is still commonly used. While the anesthetic/hypnotic mechanisms of N_2O remain largely unknown, the underlying mechanisms of its analgesic/antinociceptive effects have been elucidated during the last several decades. Evidence to date indicate that N_2O induces opioid peptide release in the periaqueductal gray area of the midbrain leading to the activation of the descending inhibitory pathways, which results in modulation of the pain/nociceptive processing in the spinal cord. The types of opioid peptide induced by N_2O and the subtypes of opioid receptors that mediate the antinociceptive effects of N_2O appear to depend on various factors including the species and/or strain, the regions of the brain, and the paradigms of behavior testing used for the experiments. Among three types of descending inhibitory pathways, the descending noradrenergic inhibitory pathway seems to play the most prominent role. The specific elements involved are now being resolved.

Index Entries: Nitrous oxide; analgesia; antinociceptive effect; descending inhibitory pathway; opioid peptides; adrenoceptor; GABA.

Introduction

Nitrous oxide (N_2O), or laughing gas, has had an intriguing history from its discovery to modern clinical application. Although John Mayow of the 17th century is recognized as the first person who isolated "nitrous air" (a mixture of nitric oxide, nitrogen dioxide, and nitrous oxide), Joseph Priestly of the 18th century in England is usually regarded as the discoverer of N_2O. Sir Humphry Davy of England first reported the analgesic effect of N_2O in 1800, and Horace Wells of the United States in the 19th century has been credited as the first clinician who successfully applied its analgesic properties to surgical operations. For a thorough history of

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N_2O, *see* reviews by Frost *(1)* and by Wynne *(2)*. N_2O has been used in clinical practice for more than 100 years, and remains the most commonly used anesthetic agent.

Administration of N_2O to humans and animals causes relatively potent analgesic/antinociceptive effects and weak anesthetic/hypnotic effects. These effects are not strong enough for N_2O to be used by itself for surgical anesthesia except for minor operations such as dental procedures. Nevertheless, N_2O is often used for general anesthesia in combination with other drugs, because the addition of N_2O reduces the requirement of other analgesic and anesthetic agents. In addition, N_2O possesses sympathomimetic effects, which are beneficial to counteract the sympatholytic effects of the volatile anesthetic agents, e.g., isoflurane, sevoflurane, and halothane, which are usually co-administered with N_2O. Other benefits of using N_2O with another volatile anesthetic agent stem from its physical characteristics, i.e., low solubility. These issues have been well-characterized in the past and are described elsewhere in anesthesia textbooks.

While the anesthetic/hypnotic mechanisms of N_2O remain largely unknown *(3)*, the underlying mechanisms of its analgesic/antinociceptive effects have been rapidly elucidated during the past several decades. In this article, we have reviewed the literature on the antinociceptive effects of N_2O and their underlying mechanisms, aiming to clarify what we have learned so far and to help direct future investigations. It is beyond the scope of this manuscript to review the anesthetic/hypnotic mechanisms of N_2O, including the effects on dopaminergic neurons, benzodiazepine receptors, and N-methyl-D-aspartate (NMDA) receptors.

Overview of the Mechanisms of N_2O-Induced Antinociceptive Effects and Related Neuronal Pathways

An overview of the mechanisms of N_2O-induced antinociceptive effects and the involved neuronal pathways are briefly described first to help readers follow the context of this review. Evidence to date indicates that N_2O induces opioid peptide release in the periaqueductal gray area (PAG) of the midbrain leading to the activation of the descending inhibitory pathways, which results in modulation of the pain/nociceptive processing in the spinal cord (Fig. 1). It is not yet known how N_2O induces opioid peptides release in the PAG.

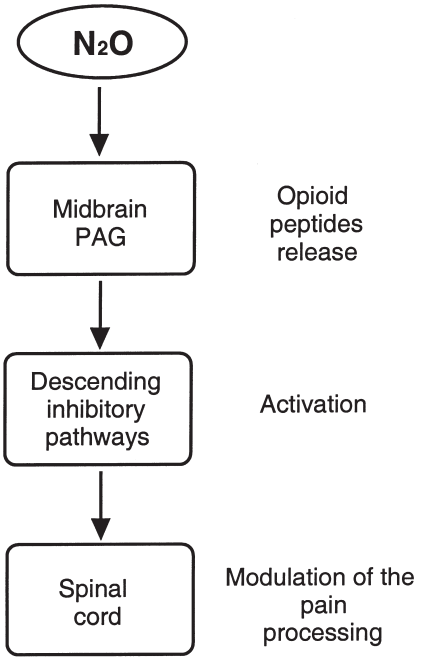

Fig. 1. A schematic overview summarizing the underlying mechanisms of N_2O-induced antinociceptive effects. Evidence to date indicates that N_2O induces opioid peptide release in the periaqueductal gray area (PAG) of the midbrain leading to the activation of the descending inhibitory pathways, which results in modulation of the pain/nociceptive processing in the spinal cord.

The descending inhibitory pathways are important components of the endogenous pain-modulating system, and the PAG plays the key role on integrating the ascending nociceptive input and the descending inhibitory output *(4–7)*. It is thought that descending inhibitory pathways are tonically inhibited

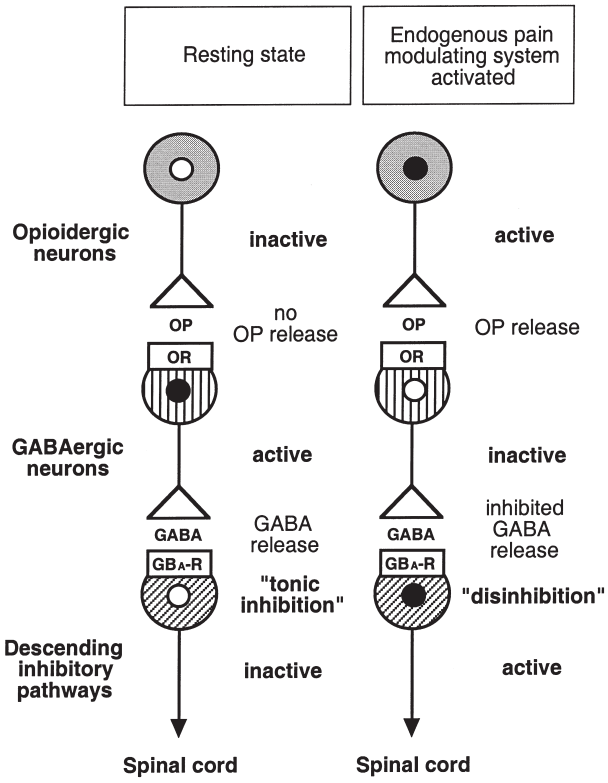

Fig. 2. A schematic representation of the possible role of the GABAergic neurons in the control of the descending inhibitory pathways. Open triangles indicate inhibitory synapses. Small closed circles indicate the nucleus of active cells, and small open circles indicate the nucleus of inactive cells. The descending inhibitory pathways are tonically inhibited under resting conditions by GABAergic inhibitory interneurons. Nociceptive input activates the descending inhibitory pathways by removing the tonic inhibition through activation of opioidergic inhibitory neurons; this is referred to as disinhibition. Abbreviations: GABA, γ-aminobutyric acid; GB_A-R, $GABA_A$ receptor; OP, opioid peptides; OR, opioid receptors.

under the resting conditions by inhibitory GABAergic interneurons in the PAG. Nociceptive input activates the descending inhibitory pathways by removing the tonic inhibition through other inhibitory neurons, e.g., opioidergic neurons, and this is referred to as disinhibition (Fig. 2). The PAG modulates nociceptive processing in the spinal cord mainly through the dorsolateral pontomesencephalic tegmentum (DLPT) and the rostral ventromedial medulla (RVM), which contain large populations of noradrenergic and serotonergic nuclei, respectively (Fig. 3). There are several projection neurons between the PAG, RVM, and DLPT, which regulate nociception by complex mechanisms *(8–10)*.

There are three major types of descending inhibitory pathways that project to the spinal cord from the brainstem: noradrenergic, serotonergic, and opioidergic inhibitory pathways *(4,5)*. Noradrenergic nuclei in the DLPT (A5, A6 or locus ceruleus, and A7), are the major source of the descending noradrenergic neurons. Serotonergic nuclei in the RVM (nucleus raphe magnus and the adjacent reticular formation) are the major source of the descending serotonergic neurons. Unlike noradrenergic or serotonergic neurons, the majority of opioidergic neurons that terminate in the dorsal horn of the spinal cord are local interneurons, although some opioidergic neurons originate from the A7 in the pons in rats *(11)*.

Involvement of the Opioidergic System in the Antinociceptive Effect of N_2O

Inhibition of N_2O-Induced Antinociceptive Effects by Opioid Receptor Antagonists

According to the literature *(12)*, a study by Seevers et al. in 1937 was the first to demonstrate the antinociceptive effects of N_2O *(13)*. The investigators used mechanical stimuli to demonstrate that N_2O caused a dose-dependent elevation of pain threshold in volunteers. Many investigators have since confirmed the antinociceptive effects of N_2O and their dose-dependency under various experimental conditions in humans and animals. Using the phenylquinone writhing test in CF-1 mice, Berkowitz et al. demonstrated in 1976 that N_2O

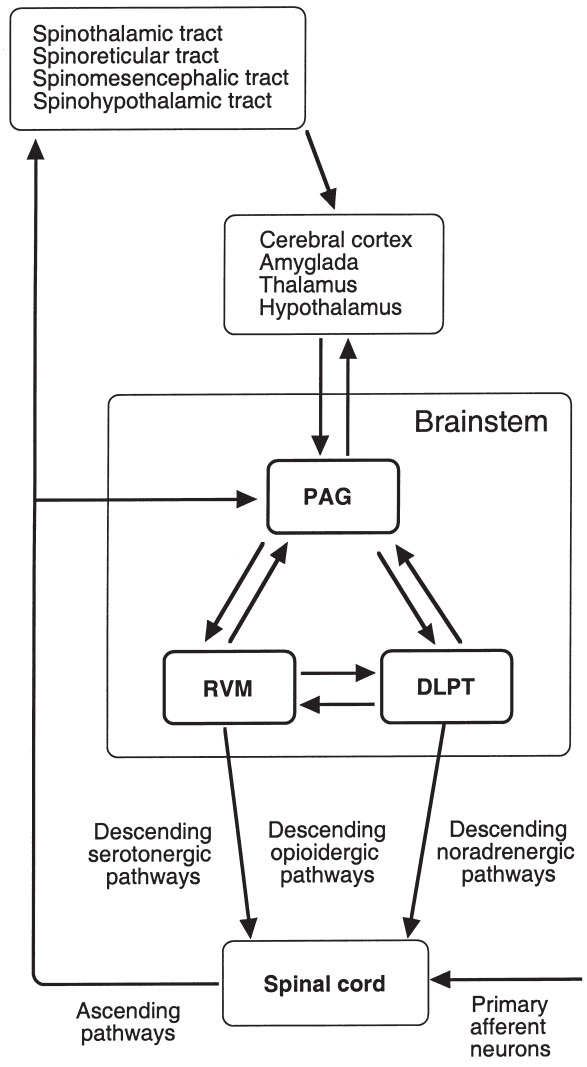

Fig. 3. A schematic representation of the endogenous nociceptive modulating system. The periaqueductal gray area (PAG) of the midbrain modulates the nociceptive processing in the spinal cord mainly through the dorsolateral pontomesencephalic tegmentum (DLPT) and the rostral ventromedial medulla (RVM), which contain a large population of noradrenergic and serotonergic nuclei, respectively. There are various projection neurons between the PAG, RVM, and DLPT, with complex regulatory mechanisms that are not yet fully understood. There are three major types of descending inhibitory pathways that are projecting to the spinal cord from the brainstem; noradrenergic, serotonergic, and opioidergic inhibitory pathways.

caused dose-dependent antinociceptive effects, which were inhibited by subcutaneous injection of opioid receptor antagonists, naloxone and naltrexone *(14)*. In addition, they showed that the antinociceptive effects of N_2O were reduced in animals that received morphine pretreatment (subcutaneous injection of morphine twice a day for 3–4 d). These experiments demonstrated for the first time that N_2O shares the common analgesic/antinociceptive actions with the opioids.

Inhibitory effects of opiate receptor antagonists against N_2O-induced antinociceptive effects were subsequently confirmed by many other investigators using various experimental paradigms in humans *(15,16)*, rats *(17–23)*, and mice *(24–30)*. At the same time, several investigators reported that opiate receptor antagonists showed no effect on the N_2O-induced antinociceptive effects in humans *(31–33)* or in rats *(34,35)*, or mixed results in humans *(36)*. Gillman et al. argued against these negative reports and suggested that naloxone had been administered inappropriately without considering its rapid decay in the brain after systemic administration *(37,38)*. Other than N_2O-induced antinociceptive effects, the opioid receptor antagonists did not block the brainstem blink reflex *(39)* and psychomotor effects *(40)* in humans and on the loss of righting reflex in mice *(41)*, while they did block the locomotor activity in mice *(42)*.

Opioid Peptides Release by N_2O

The most likely explanation for the opioid-like effects of N_2O is that N_2O induces endogenous opioid peptide release. While some studies failed to demonstrate these effects of N_2O *(43–45)*, two groups of investigators successfully demonstrated in mid-1980s that N_2O increased opioid peptide concentrations in the rat brain using radioimmunoassays. In 1985, Quock et al. reported that 60 min of 75% N_2O administration to the Sprague-Dawley rats caused a twofold increase in met-enkephalin, but no change in leu-enkephalin or β- endorphin, in cere-

brospinal fluid (CSF) *(46).* They also reported that the same treatment increased the concentrations of met-enkephalin in the brainstem, spinal cord, hypothalamus, and corpus striatum between 12 and 18% of the baseline levels, but not in the cerebral cortex or diencephalon *(47).* In 1986 and 1987, Zuniga et al. reported that 60 min of 60 or 80% N_2O administration to Sprague-Dawley rats increased β-endorphin concentrations in the mediobasal region of the hypothalamus, diencephalon, and PAG, but met-enkephalin and other opioid peptides were not examined in these studies *(48,49).* They subsequently confirmed their initial findings using an in vitro system *(50),* which is discussed later.

More recently, Finck et al. reported in 1995 that 66–75% N_2O administration to canines increased proenkephalin-derived opioid peptides (e.g., met-enkephalin), but not prodynorphin or proopiomelanocortin-derived opioid peptides (e.g., dynorphin and β-endorphin, respectively) in the third ventricular CSF using more sensitive and specific assay methods than those used in the aforementioned studies *(51).* These studies clearly indicate that N_2O induces opioid peptide release, but are not in agreement as to which of the opiate peptides are released. This may be dependent on the species or strain and the region of the brain, and requires further investigation.

Involvement of Opioid Receptors in the Antinociceptive Effects of N_2O in the PAG

As described earlier, the PAG (particularly the ventrolateral portion) plays a key role in integrating the ascending nociceptive input and the descending inhibitory output *(6,7).* In 1987, Zuniga et al. reported that kainic acid-induced lesioning of the ventral and caudal PAG almost completely attenuated the antinociceptive effects of N_2O on the tail and foot flick tests in the Sprague-Dawley rats *(49).* In 1994, Hodges et al. reported that unilateral microinjection of CTOP, a μ opioid receptor antagonist, into the PAG partially blocked the antinociceptive effects of N_2O in a dose-dependent manner on the hot plate test in the Sprague-Dawley rats *(21).* They also reported that administration of β-endorphin$_{1–27}$, a fragment of β-endorphin that competes with β-endorphin at the opioid receptors, showed a biphasic effect on the antinociceptive effects of N_2O. Specifically, β-endorphin$_{1–27}$ partially blocked the antinociceptive effects of N_2O in a dose-dependent manner up to 5.0 μg, but this effect declined as the dose was further increased *(49).* More recently, Fang et al. reported in 1997 that bilateral microinjection of naloxone (2.5 μg/0.5 μL saline), but not yohimbine (1.5 μg/0.5 μL saline), an α_2 adrenoceptor antagonist, into the ventrolateral PAG partially blocked the antinociceptive effects of N_2O in the tail flick test in Sprague-Dawley rats *(52).* These studies have provided direct evidence that the opioid receptors in the PAG mediate the antinociceptive effects of N_2O.

Opioid Receptor Subtypes that Are Involved in the N_2O-Induced Antinociceptive Effects

Quock et al. have conducted a series of experiments aiming to identify the opioid receptor subtypes that are involved in the N_2O-induced antinociceptive effects using opioid receptor subtype specific antagonists or antiserum against specific opioid peptides *(20,21,25–29,53–55).* Again, the identity of the specific opioid receptor subtypes mediating the antinociceptive effects of N_2O appears to be dependent on the species and/or strains and behavioral paradigm used for the experiments. In ICR and NIH-Swiss mice, the κ opioid receptor subtype seems to play a major role both at the supraspinal and spinal cord levels for the abdominal constriction test *(25,26,29,54,55).* In the Sprague-Dawley rats, κ and μ opioid receptor subtypes seem to play a major role at the supraspinal level on the tail flick test and the hot plate test, respectively *(20,21,53).* As discussed later, opioid receptors do not seem to be involved at the spinal cord level in the antinociceptive effects of N_2O in rats *(23).*

Involvement of Nitric Oxide in N_2O-Induced Opioid Peptide Release

In 1994, McDonald et al. reported that the antinociceptive effects of N_2O were blocked by nitric oxide synthase (NOS) inhibitors in the abdominal constriction test in the Swiss-Webster mice *(56).* This effect was completely reversed when L-arginine, but not D-arginine, was pre-administered intracerebroventricularly. Similar results were obtained on the hot plate test in the Sprague-Dawley rats *(56).* In contrast, NOS inhibitors did not block the antinociceptive effects of the exogenously administered opioids (e.g., morphine) on the abdominal constriction test in the Swiss-Webster mice *(56).* From these findings, the investigators have suggested that nitric oxide (NO) may play a key role in N_2O-induced opioid peptide release.

The same group of investigators subsequently demonstrated in Sprague-Dawley rats that NO was involved in met-enkephalin release in the spinal cord induced by intracerebroventricularly administered β-endorphin *(57).* This result has led the investigators to speculate that NOS inhibitors block N_2O-induced antinociceptive effects by inhibiting β-endorphin release at the supraspinal level, thus inhibiting met-enkephalin release in the spinal cord. While the first part of this speculation is supported by studies, the second part is inconsistent with findings suggesting that opioid receptors are not involved in N_2O-induced antinociceptive effects at the spinal-cord level in rats *(23).* Interestingly, the same group also reported that NO was involved in N_2O-induced benzodiazepine-like anxiolytic effects in Swiss-Webster mice *(58).* Thus, the involvement of NO in various effects of N_2O may be a common underlying mechanism rather than being specific for its antinociceptive effects. It is also possible that NO is involved in mediating the signaling pathways of both effects, as NO is involved in numerous reactions in the central nervous system (CNS) as a neurotransmitter.

Direct Effects of N_2O on Opioid Receptors

There is an argument that N_2O may directly interact with opioid receptors *(59).* However, results from in vitro receptor binding studies are controversial *(60–63);* thus, further investigation is needed to confirm such a direct action of N_2O.

Involvement of Descending Inhibitory Pathways in the Antinociceptive Effects of N_2O

Evidences from Surgical Experiments (Spinal-Cord Transection)

The involvement of descending inhibitory neurons in the antinociceptive effects of N_2O was first clearly demonstrated by an electrophysiological study using decerebrate nonanesthetized felines by Komatsu et al. in 1981 *(64).* These investigators showed that the inhibitory effect of N_2O on bradykinin (intra-arterial injection)-induced neural activity in the spinal cord was significantly reduced by transecting the spinal cord at the cervical level. Recent electrophysiological studies have confirmed this finding *(65,66).* More direct evidence comes from a recent study by Zhang et al., who showed that transection of the spinal cord at T3–T4 level completely eliminated the antinociceptive effects of N_2O on the tail flick test in Sprague-Dawley rats *(67).*

Descending Noradrenergic Inhibitory Neurons

Among three major types of descending inhibitory neurons, noradrenergic neurons have been shown to play an important role in mediating the antinociceptive effects of N_2O. In mid-1990s, two groups of investigators reported this for the first time. In 1995, Ohara et al. reported that intraperitoneal injection of yohimbine (an α2 adrenoceptor antagonist capable of crossing the blood-brain-barrier

[BBB]), but not L659-066 (an α2 adrenoceptor antagonist that does not cross BBB), almost completely blocked the antinociceptive effects of N_2O on the tail flick test in the Sprague-Dawley rats *(68).* In 1996, Guo et al. provided more direct evidence for the involvement of noradrenergic neurons in the antinociceptive effects of N_2O at the spinal cord level *(23),* finding that administration of α2 adrenoceptor antagonists (atipamezole, yohimbine, or N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline) intrathecally, but not intracerebroventricularly, blocked the antinociceptive effects of N_2O on the tail flick test in the Sprague-Dawley rats. The latter group investigators subsequently reported that 70% N_2O increased the norepinephrine concentration in the spinal cord more than four times that was seen at baseline using chronically implanted microdialysis probe in Sprague-Dawley rats *(67).* In the same study, they also reported that N_2O-induced norepinephrine release in the spinal cord was blocked by intraperitoneal injection of naltrexone. Furthermore, when norepinephrine in the spinal cord was chemically depleted by intrathecal injection of n-(2-chloroethyl)-n-ethyl-2-bromobenzylamine, N_2O no longer showed the antinociceptive effects in the tail flick test *(67).*

In 1998, Fukuhara et al. reported that the lesioning of locus ceruleus by electrical coagulation in Wistar rats attenuated the antinociceptive effects of N_2O in the tail flick test *(35).* More recently, Sawamura et al. reported that N_2O activated the pontine noradrenergic nuclei in Sprague-Dawley rats (A5, A6 or locus ceruleus, and A7) using c-Fos as an immunohistochemical marker of neuronal activation *(69).* When the rats were injected intracerebroventricularly with the mitochondrial toxin saporin coupled to the dopamine β-hydroxylase antibody, approx 70% of the noradrenergic cells in those nuclei were destroyed, and the animals no longer exhibited N_2O-induced antinociceptive effects in the tail flick test. (Dopamine β-hydroxylase is a norepinephrine synthesizing enzyme, which therefore exclusively localizes the lesion to noradrenergic neurons.) As discussed earlier, these noradrenergic nuclei are the major sites for mediating the descending noradrenergic inhibitory pathways *(9);* thus these findings provide further support for the involvement of descending noradrenergic inhibitory pathways in the antinociceptive effects of N_2O.

Descending Opioidergic Inhibitory Neurons

In Sprague-Dawley rats, Guo et al. reported that intrathecally administered naloxone did not inhibit N_2O-induced antinociceptive effects on the tail flick test, while administration of naloxone intraperitoneally (ip) and intracerebroventricularly (icv) showed the inhibitory effects *(23).* These results have clearly indicated that the involvement of opioid receptors in the antinociceptive effects of N_2O is at the supraspinal level but not at the spinal cord level, at least in rats. Contrariwise, Quock et al. reported that intrathecally administered κ opioid receptor antagonist, nor-binaltorphimine *(26,29),* or rabbit antiserum against dynorphins and met-enkephalin *(55)* blocked the antinociceptive effects of N_2O on the abdominal constriction test in mice. This controversy may be explained by species or experimental paradigm difference, but further investigation is needed for clarification.

Descending Serotonergic Inhibitory Neurons

In rats it appears that the descending serotonergic inhibitory pathways are unlikely to be involved in the N_2O-induced antinociceptive effects. A recent study using c-Fos as a marker of neuronal activation has shown that N_2O administration to the Fischer rats does not activate the serotonergic nuclei in the RVM *(70),* which play an important role in descending serotonergic inhibition *(4,5).* Furthermore, recent experiments from our laboratory have indicated that tropisetron, a 5-HT_3 receptor antagonist, does not block N_2O-induced c-Fos expression in the spinal cord in Fischer rats (unpublished data). An early report indicated

that 74% N_2O plus 1% halothane decreased the activity of tryptophan hydroxylase, the rate-limiting enzyme for serotonin synthesis, and reduced the rates of serotonin synthesis and utilization in the brain in Charles River rats, although it is not clear whether the effects are due to N_2O, halothane, or their combination *(71).* In mice, Muller et al. reported that in the Swiss-Webster strain 5-HT_3 receptor antagonist ICS-205930 blocked the antinociceptive effects of N_2O on the abdominal constriction test, while 5-HT_{1C}/5-HT_2 receptor antagonist, mianserin, potentiated the effects *(72).* Indeed, while the involvement of the descending serotonergic pathway in the opioid-induced antinociceptive effects has been advanced, there are recent studies challenging this mechanism *(73,74).*

Modulation of the Nociceptive Processing by N_2O in the Spinal Cord

Electrophysiological Studies

In the late 1960s and early 1970s, two groups of investigators studied the effects of N_2O in the feline spinal cord in electrophysiological experiments using the spinal cord-transected animals and decerebrated animals, and both groups reported that N_2O showed direct depressant effects on the spinal-cord neurons *(75–78).* This would seem to contradict the report by Komatsu et al., who showed that the inhibitory effect of N_2O on bradykinin (intra-arterial injection)-induced neural activity in the spinal cord was significantly reduced in the spinal cord-transected animals *(64).* This study has suggested the involvement of supraspinal input, i.e., descending inhibitory pathways, a hypothesis that has been supported by other similar studies *(65,66).* Another study using the spinal cord-transected felines has shown that N_2O depresses the spinal monosynapic reflexes but has less effect on the polysynaptic reflexes, which involve spinal interneurons *(79).* There is also a study showing that naloxone does not block the effects of N_2O on the firing response to nociceptive stimulation (intra-arterial bradykinin injection) in spinal cord-transected felines *(80).* An experiment in Wistar rats has also shown that naloxone pretreatment does not block the depressant effects of N_2O on somatosympathetic A- and C-reflexes *(81).* Taking all these reports together, the following interpretations can be drawn: 1) N_2O possesses both direct depressant effects on the spinal-cord neurons and indirect depressant effects through activating the descending inhibitory pathways, and 2) the opioidergic system is not involved in the direct effects of N_2O. It appears, however, that the direct effects of N_2O on the spinal cord neurons alone are not sufficient to produce the antinociceptive effects in most in vivo experimental models.

Actions of the Noradrenergic Neurons in the Spinal Cord

Activation of descending noradrenergic inhibitory neurons lead to the release of norepinephrine in the spinal cord *(67),* which result in modulation of the nociceptive processing through α adrenoceptors. In agreement with the accepted belief that α2 and α1 adrenoceptors mediate the inhibitory and excitatory neuronal activities, respectively, there are at least two neuronal systems that may be involved in the analgesic/antinociceptive effect of N_2O (Fig. 4). One mechanism is the direct presynaptic inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second order neurons via activation of α2 adrenoceptors. Support for the involvement of this mechanism comes from aforementioned studies using α2 adrenoceptor antagonists *(23,34).* Experiments using α2 adrenoceptor subtype knockout mice also have indicated that α2 adrenoceptors mediate the antinociceptive effects of N_2O *(69,82),* which will be discussed later.

Another possible mechanism is the indirect presynaptic inhibition of the nociceptive pri-

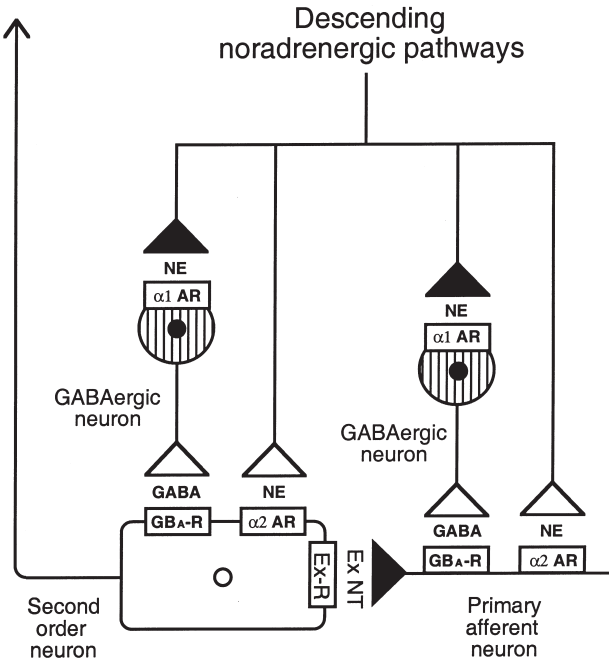

Fig. 4. Putative neuronal pathways in the spinal cord involved in the antinociceptive effects of N_2O. Closed triangles indicate excitatory synapses, and open triangles indicate inhibitory synapses. Small closed circles indicate the nucleus of cells activated by N_2O exposure, and a small open circle indicates the nucleus of a cell inactivated by N_2O exposure. There are at least two neuronal systems that may be involved: 1) direct presynaptic inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second-order neurons through activation of the α2 adrenoceptors, and 2) indirect presynaptic inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second-order neurons by the activation of GABAergic inhibitory interneurons through α1 adrenoceptors. Abbreviations: α2 AR, α2 adrenoceptor; Ex NT, Excitatory neurotransmitters; Ex-R, Receptors for excitatory neurotransmitters; GABA, γ-aminobutyric acid; GB_A-R, $GABA_A$ receptor; NE, norepinephrine.

mary afferent neurons and/or postsynaptic inhibition of the second-order neurons through the activation of inhibitory interneurons via α1 adrenoceptors (Fig. 4). A recent immunohistochemical study by Hashimoto et al. has shown that N_2O activates the GABAergic inhibitory interneurons in the spinal cord of Fischer rats *(83).* This mechanism is also supported by a recent anatomical study by Nuseir and Proudfit *(84)* and an electrophysiological study by Baba et al. showing that norepinephrine applied to the sliced spinal cord preparation from the Sprague-Dawley rats activates GABAergic inhibitory activity in the dorsal horn of the spinal cord through α1 but not α2 adrenoceptors *(85).* Furthermore, it has been demonstrated that intraperitoneally administered prazosin, an α1 adrenoceptor antagonist, blocked the antinociceptive effects of N_2O in the tail flick test in 129/svj mice, *(82).* Most recently, it has been reported that N_2O-induced c-Fos expression was co-localized with α1 adrenoceptors using double-staining methods and that N_2O-induced c-Fos expression was blocked by prazosin, an α1 adrenoceptor antagonist, but not by yohimbine *(86).* Recent experiments from our laboratory also have shown that the antinociceptive effects of N_2O on the plantar test are blocked by intraperitoneally administered either prazosin or yohimbine (unpublished data). Taken together, these findings indicate that both α2 and α1 adrenoceptors mediate the antinociceptive effects of N_2O at the spinal cord level in rats. It appears that both adrenoceptors are necessary to produce the antinociceptive effects, and a lack of either one results in a loss of antinociceptive effects.

α2 Adrenoceptor Subtypes Involved in N_2O-Induced Antinociceptive Effect

As described earlier, findings to date indicate that α2 adrenoceptors are partly involved in mediating the antinociceptive effects of N_2O in the spinal cord. To date, three α2 adrenoceptor subtypes have been identified and cloned; α2A, α2B, and α2C *(87).* No subtype-specific α2 adrenoceptor agonists or antagonists are yet available; thus investigators have used genetically modified mice to investigate subtype-spe-

cific effects. Using D79N transgenic mice, which have a dysfunctional α2A adrenoceptor subtype gene *(88),* it has been shown that the α2A subtype is not responsible for the antinociceptive effects of N_2O on the tail flick test *(82).* Experiments using knockout mice for each subtype *(89,90)* have shown that the α2B subtype, but not α2A or α2C subtypes, is responsible for mediating the antinociceptive effects of N_2O in the tail flick test and the hot plate test *(69).* One must be aware, however, that distribution of α2 adrenoceptor subtypes in the spinal cord is species-dependent; thus the results from knockout mice may not necessarily extrapolate to other species *(91).*

Where is the Initial Site of Action by N_2O?

The mechanisms by which N_2O induces opioid peptides release in the PAG remain unclear. Possible mechanisms are: 1) direct activation of opioidergic neurons that innervate the PAG, 2) direct activation of opioidergic neurons within the PAG, and 3) indirect activation of opioidergic neurons within the PAG through excitatory neurons from other sites (Fig. 5). Released opioid peptides in the PAG then inhibit the GABAergic neurons, which disinhibit either the excitatory neurons within the PAG that are part of the descending inhibitory pathways or the descending noradrenergic neurons in the DLPT (discussed later). It is also possible that activated opioidergic neurons in the PAG disinhibit the GABAergic neurons in the DLPT.

In 1987, Zuniga et al. demonstrated in an in vitro system that dispersed cells from the basal hypothalamus of the Sprague-Dawley rats induced β-endorphin by N_2O, but other opioid peptides were not examined *(50).* It is known that the basal hypothalamus contains the dense opioidergic neurons (pro-opiomelanocortin neurons) that project to the PAG *(92).* This finding supports the theory that N_2O directly activates the pro-opiomelanocortin neurons in the hypothalamus, which results in

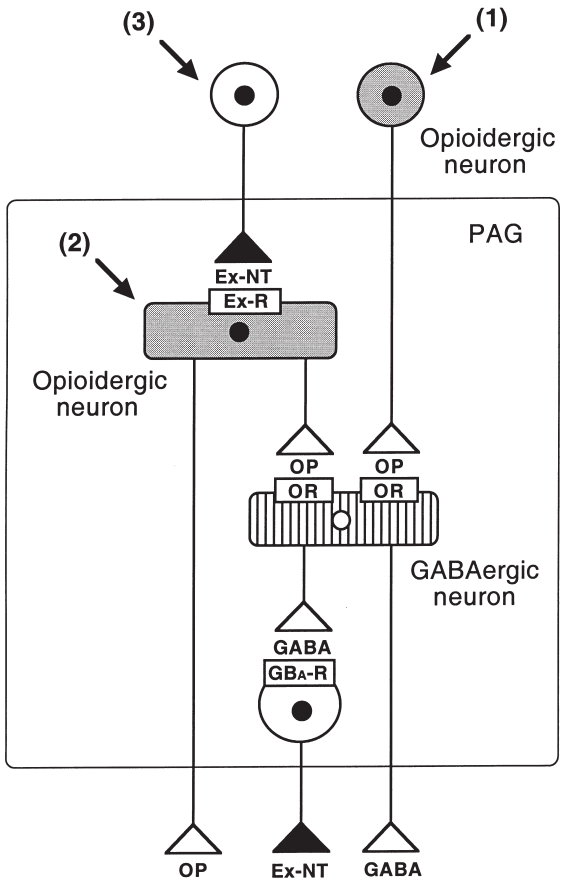

Fig. 5. Putative neuronal pathways in the PAG involved in the antinociceptive effects of N_2O. Closed triangles indicate excitatory synapses, and open triangles indicate inhibitory synapses. Small closed circles indicate the nucleus of cells activated by N_2O exposure, and a small open circle indicates the nucleus of a cell inactivated by N_2O exposure. There are three possible mechanisms: 1) direct activation of opioidergic neurons that innervate the PAG, 2) direct activation of opioidergic neurons within the PAG, and 3) indirect activation of opioidergic neurons within the PAG through excitatory neurons from other sites. Released opioid peptides in the PAG then inhibit the GABAergic neurons, which results in disinhibiting either the excitatory neurons within the PAG that are the part of descending inhibitory pathways or the descending noradrenergic neurons in the DLPT. It is also possible that activated opioidergic neurons in the PAG results in disinhibiting the GABAergic neurons in the DLPT. Abbreviations: Ex NT, Excitatory neurotransmitters; Ex-R, Receptors for excitatory neurotransmitters; GABA, γ-aminobutyric acid; GB_A-R, $GABA_A$ receptor; OP, Opioid peptides; OR, Opioid receptors.

release of β-endorphin in the PAG. In 1997, Gyulai et al. reported a positron emission tomography (PET) human study on the effects of 20% N_2O against 48°C tonic heat stimulus applied on the left forearm *(93).* The stimulation produced cerebral activation measured as regional cerebral blood flow in the contralateral thalamus, anterior cingulate, and supplementary motor area. Addition of N_2O during the stimulation abolished cerebral activation in these areas, but produced activation in the contralateral infralimbic and orbitofrontal cortices. These findings support the theory that N_2O produces the antinociceptive effects through activating the descending inhibitory pathways. The investigators also suggested that the infralimbic and orbitofrontal cortices may be the possible sites of action by N_2O, although these sites could be involved in other effects of N_2O rather than its antinociceptive effects *(93).*

Most recently, Ohashi et al. examined the c-Fos expression in the brain in Fischer rats and found that N_2O induced c-Fos expression in the following nuclei: amygdaloid nuclei of the cerebrum, mediodorsal nuclei of the thalamus, ventromedial nuclei, and dorsomedial nuclei of the hypothalamus, ventrolateral PAG of the midbrain, and noradrenergic nuclei of the pons *(70).* Most of these nuclei are involved in the endogenous pain-control systems *(4,5)* and, therefore, these findings are consistent with the theory that N_2O produces the antinociceptive effects by activating the descending inhibitory pathways. These findings also suggest that the initial sites of action of N_2O may be at a higher level than the PAG.

Other Issues Related to the Antinociceptive Effects of N_2O

Strain Differences on the Antinociceptive Effect of N_2O

Variation in human response to N_2O has been widely known since the beginning of its use in clinical applications. In 1993, Quock et al. demonstrated the mouse strain differences in response to the antinociceptive effects of N_2O on the acetic acid abdominal-constriction test *(94).* The investigators examined eight inbred and two outbred strains and reported descending order of responsiveness as, A/J, C57BL/6ByJ, C57/6J, BALB/cByJ, C3H/HeJ, Swiss-Webster, CXBK/ByJ, ICR, CBA/J, and DBA/2J. The same group also reported strain differences in mice in response to N_2O withdrawal seizures, which did not correlate with those of N_2O-induced antinociceptive effects *(95).* Thus, these investigators have suggested that the underlying mechanisms of antinociceptive and withdrawal responses are different. Using quantitative trait loci analysis, the same group of investigators has reported that several loci are strongly associated with high sensitivity to the antinociceptive effects of N_2O *(96),* although the significance of their findings is yet to be interpreted. A recent study also showed marked strain differences in rats in response to the antinociceptive effects of N_2O on the tail flick test *(97).* While the Fischer strain showed strong antinociceptive effects and did not develop acute tolerance, the Lewis strain showed no antinociceptive effects by N_2O (Fig. 6; discussed later). Wistar-Kyoto and Brown-Norway strains showed moderate and weak antinociceptive effects, respectively, and both strains developed acute tolerance after 30 min of N_2O exposure. Four outbred strains tested in the study, i.e., Sprague-Dawley (B&K Universal), Sprague-Dawley (Charles-River), Wistar, and Long-Evans strains showed similar responses as seen in Wistar-Kyoto strain *(97).* The significance of these strain differences are discussed below.

Acute Tolerance to the Antinociceptive Effect of N_2O

Many investigators have shown that the antinociceptive effects of N_2O diminish over time during continuous administration in humans *(98–102)* and in animals *(17,23,34,82,97,103,104).* This biologic phenomenon is referred to as "acute tolerance," which also has been seen in other effects of N_2O, e.g.,

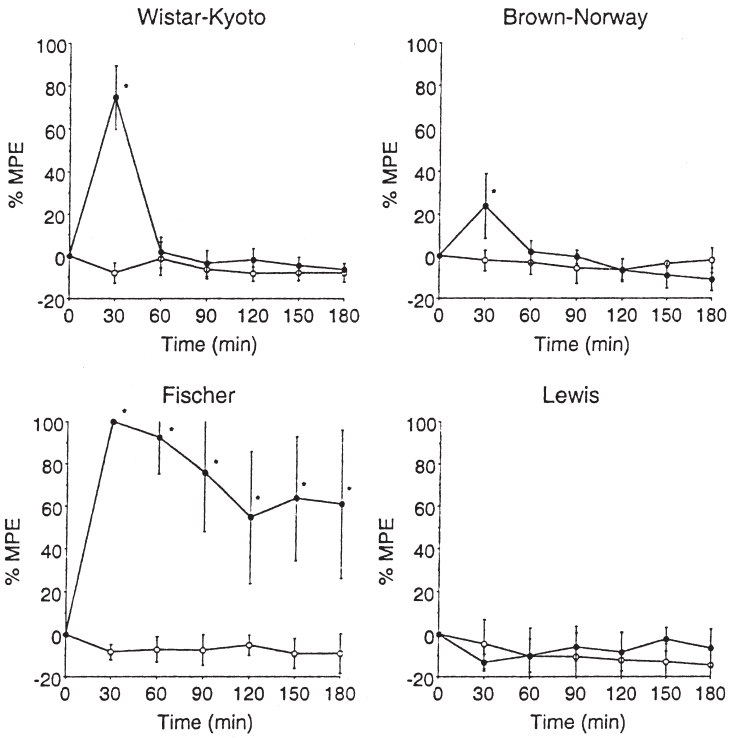

Fig. 6. Time course of the antinociceptive effects of N_2O on the tail flick test in four different inbred strains of rats. Open circles indicate control groups (exposed to the air), and closed circles indicate N_2O groups (75%). %MPE = Percent of maximum possible effect (mean ± S.D., $n = 4$). *$p < 0.05$ vs control. The Fischer strain shows strong antinociceptive effects and does not develop acute tolerance, but the Lewis strain shows no antinociceptive effects by N_2O. Wistar-Kyoto and Brown-Norway strains show moderate and weak antinociceptive effects, respectively, and both strains develop acute tolerance after 30 min of N_2O exposure. Adapted with permission from Fender et al. *(97).*

EEG activity in humans *(105)* and felines *(106)*, anticonvulsant action in felines *(107)*, loss of righting reflex in mice *(108)*, and N_2O-induced hypothermia in rats *(109)*. There have been reports that Wister rats do not develop acute tolerance in the tail flick test *(110)*, but this may be explained by the strain differences, as described earlier. In 1984, Rupreht et al. suggested that acute depletion of opiate peptides in the CNS causes acute tolerance to N_2O, based on the finding that maintaining high levels of enkephalin with intracerebroventricularly injected phosphoramidon, an enkephalinase inhibitor, prevented the development of acute tolerance for the antinociceptive effects of N_2O in a modified Randall-Selitto pressure nociception test in the Wistar rats *(104)*.

The marked strain differences in the development of acute tolerance to N_2O-induced antinociceptive effects in the tail flick test in rats *(97)* provides further supportive evidence for the theory that the antinociceptive effects of N_2O are mediated by opioid peptide release. For example, while the Fischer strain shows strong antinociceptive effects by N_2O and does not show acute tolerance, the Lewis strain shows no antinociceptive effects by N_2O *(97)*. It has been shown that these two strains differ markedly in behavioral responses to other drugs (including morphine, alcohol, and

cocaine) and demonstrate marked differences in catecholamine and opiate peptides synthesis in various regions of the brain *(111–115).* For example, the Lewis strain has lower basal levels of endogenous opioid peptide, which do not increase following morphine administration *(114).* Thus, it is suggested that the lesser amount of endogenous opioid peptides in the brainstem of the Lewis strain is insufficient to activate the descending noradrenergic inhibitory neurons. In contrast, the Fischer strain has an abundance of opioid peptide, which produces powerful antinociceptive effects to N_2O and confers resistance to the development of tolerance because the store is not easily depleted.

Chronic Tolerance to the Antinociceptive Effects of N_2O

As mentioned earlier, Berkowitz et al. reported in 1976 that the antinociceptive effects of N_2O on the phenylquinone writhing test were reduced in the CF-1 mice that received morphine pre-treatment (subcutaneous injection of morphine twice a day for 3–4) *(14).* At the same time, the antinociceptive effects of morphine were not reduced in mice or in the Long-Evans and Sprague-Dawley rats that received 75% N_2O for 14–18 h *(103).* The same group of investigators also found that chronic exposure to N_2O reduced the antinociceptive effects of subsequently administered N_2O in the CF mice in the phenylquinone writhing test (after 24 h of 75% N_2O exposure) and in the Sprague-Dawley rats in the tail flick test (after 18 h of 70% N_2O exposure) *(17).* Furthermore, they demonstrated that 18 h (but not 30 min) of 80% N_2O exposure to the Sprague-Dawley rats decreased the brainstem opioid receptor density approx 20% without changing receptor affinity *(116).* It is well-known that chronic administration of opioids result in development of chronic tolerance. Thus, these findings are consistent with the theory that the antinociceptive effects of N_2O are due in part to endogenous opioid peptides release.

Antinociceptive Effects of N_2O in Newborn

While activation of the descending noradrenergic neurons plays a pivotal role on the N_2O-induced antinociceptive effects, several lines of investigation in rats have shown that these neurons are not functionally mature at birth *(117,118).* Consequently, N_2O may not be an effective analgesic/antinociceptive agent in the newborn. In agreement with this speculation, a recent study has demonstrated that N_2O does not show antinociceptive effects in the tail flick test in the Sprague-Dawley rats until 3–4 wk of age *(119).* This initial finding has been confirmed by a recent study showing that the antinociceptive effects of N_2O in the formalin test similarly do not fully develop until 3–4 wk of age in Fischer rats *(120).* It has also been shown using c-Fos as a marker of neuronal activation that N_2O does not activate the spinal-cord neurons until 2 wk of age in Fischer rats *(121).* Although the sequence of events that take place during development of the CNS in rats and humans are not precisely comparable, the rat at birth is thought to be anatomically equivalent to that of human fetuses at 24 wk gestation *(122).* At one, two, and three wk after birth in rats, the CNS becomes equivalent to that of the full-term neonate, 1-yr-old, and toddler stage in humans, respectively *(122).* Thus, N_2O might not be efficacious as an analgesic agent for humans in early childhood. A well-designed clinical investigation is needed to resolve this clinically important point.

Effects of Volatile Anesthetics on N_2O-Induced Antinociceptive Effects

In 1994, Goto et al. incidentally found that 0.9% halothane, a volatile anesthetic agent that is commonly co-administered with N_2O in clinical practice, blocked the antinociceptive effects of 75% N_2O in the formalin test in the Sprague-Dawley rats *(122).* The same group also showed that 0.9% halothane and 1.1% isoflurane, another commonly used volatile

anesthetic agent, blocked the antinociceptive effects of 75% N_2O in the tail flick test in the same strain of rats *(123).* A more recent study also has reported a similar finding using a combination of 30% N_2O and 0.2–0.4% sevoflurane, the newest volatile anesthetic agent, on the cold-induced pain on the arm in humans *(124).* Although the underlying mechanisms of these effects remain unclear, these findings may be explained by general inhibitory effects of the volatile anesthetic agents on the neuronal transmission. Under certain concentrations, they may inhibit the activity of the descending inhibitory neurons to counteract the effects of N_2O. Interactions between N_2O and the volatile anesthetic agents are complex in terms of both antinociceptive effects and other anesthesia-related effects, which require further investigation.

Putative Pathways that are Mediating the Antinociceptive Effects of N_2O

Based on the currently available evidence discussed earlier and the neurobiology literature, putative pathways involved in the antinociceptive effects of N_2O are shown in Fig. 7 and are explained below. The rat is selected as a model because much more information is available for neuronal projections within the CNS than in mouse. The parenthesized letters throughout refer to sites of action indicated in Fig. 7.

The Initial Sites of Action by N_2O, and Opioid Peptides Release in the PAG

The initial sites of action of N_2O remain unknown, but there are at least three possible mechanisms of opioid peptides release by N_2O in the PAG: 1) direct activation of opioidergic neurons that innervate the PAG (A), 2) direct activation of opioidergic neurons within the PAG (B), and 3) indirect activation of opioidergic neurons within the PAG through excitatory neurons from other sites (C). The most likely candidates for the opioidergic neurons, which innervate the PAG and are activated by N_2O (A), are the pro-opiomelanocortin neurons that are concentrated in the basal hypothalamus *(92).* The candidates for the excitatory neurons, which are activated by N_2O and lead to the activation of opioidergic neurons in the PAG (B), are substance P (SP) and/or neurotensin neurons *(5).* For example, in rats the ventrolateral PAG contains the largest population of SP neurons, and it has been shown that SP microinjection into the PAG causes antinociceptive effects that can be blocked by naloxone, which are reviewed elsewhere *(5,6).* The PAG receives significant inputs from various areas of the brain including the somatosensory cortex, amygdala, hypothalamus, and brainstem *(5,6),* but where and how N_2O activates the SP neurons are yet to be determined. Lastly, the opioidergic neurons are rich within the PAG, so it has been suggested that a significant number of the neurons mediating the antinociceptive effects are intrinsic *(125),* which supports the third possibility (C).

Activation of Descending Noradrenergic Inhibitory Pathways

N_2O-induced opioid peptide release in the PAG results in activation of the descending noradrenergic inhibitory pathways via the noradrenergic nuclei in the DLPT. Among them, the A7 is the most likely candidate to be mediating the antinociceptive effects of N_2O because a majority of the noradrenergic neurons in the A7 projects to the laminae I-IV in the spinal cord that are the sites of nociceptive processing *(126).* In contrast, the activation of the locus ceruleus and A5 appear to be involved less in the antinociceptive effects, if at all, according to their projections into the spinal cord. For example, the locus ceruleus neurons mostly project to the laminae VII-VIII (ventral horn) and the laminae IX-X (motor neurons) *(127),* although strain differences of such neuronal projections have been reported *(128–131).* Those from A5 project to the laminae IV-VII and X and intermediolateral cell col-

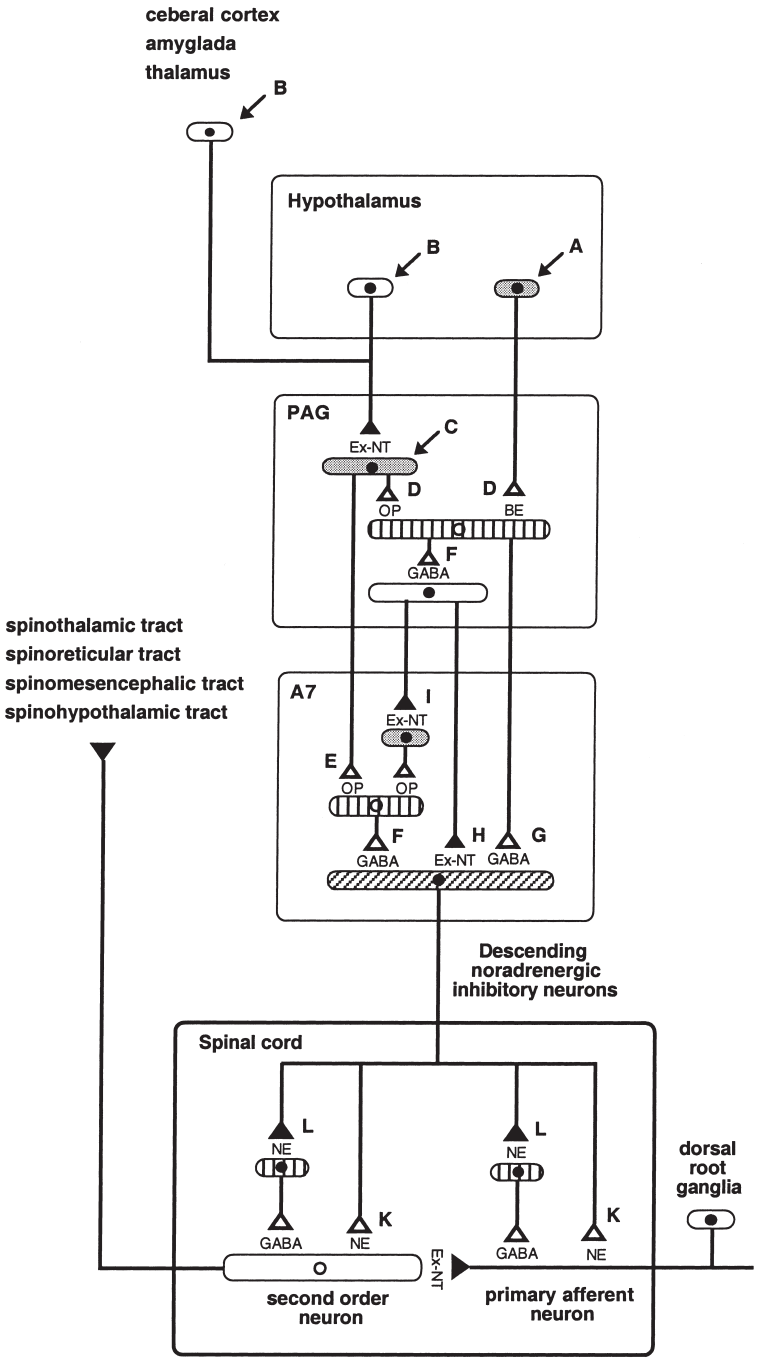

Fig. 7. Overall putative neuronal pathways involved in the antinociceptive effects of N_2O in rats. Closed triangles indicate excitatory synapses, and open triangles indicate inhibitory synapses. Small closed circles indicate the nucleus of cells activated by N_2O exposure, and small open circles indicate the nucleus of cells inactivated by N_2O exposure. Abbreviations: BE, β-Endorphin; Ex NT, Excitatory neurotransmitters; GABA, γ-aminobutyric acid; NE, Norepinephrine; OP, Opioid peptides; A-L represent putative mechanisms (*see* the text).

umn *(132).* These laminae are less involved in mediating the nociceptive processing. According to the literature on the noradrenergic nuclei, it is more likely that the locus ceruleus is involved in the hypnotic effects of N_2O *(133–135),* and A5 in the effects on the cardiovascular functions *(136,137).*

While the opioid peptides released in the PAG are most likely to inhibit the GABAergic neurons (D), it is also possible that N_2O activates the opioidergic neurons in the PAG, which results in inhibiting the GABAergic inhibitory interneurons in A7 through opioid receptors (E), because some enkephalinergic neurons in the ventrolateral PAG have been shown to directly project to A7, where the target cells have been identified as non-noradrenergic neurons *(9,138).* The GABAergic inhibitory interneurons in the PAG and A7 are active under resting conditions, and thus inhibit the descending noradrenergic inhibitory neurons (F); so-called tonic inhibition. Inhibition of the GABAergic inhibitory interneurons by N_2O-induced opioid peptides results in activation (disinhibition) of the descending noradrenergic inhibitory pathways. There are several possible pathways for this mechanism: 1) the GABAergic inhibitory interneurons directly project to the noradrenergic neurons in the A7 (G), 2) the GABAergic inhibitory interneurons innervate the excitatory neurons that project to the noradrenergic neurons in A7 (H) *(139),* or 3) to the opioidergic inhibitory interneurons in A7 (I). The opioidergic inhibitory interneurons in the latter then innervate the GABAergic inhibitory interneurons in the A7 (J). These pathways within the PAG and A7 remain mostly speculative for N_2O-induced antinociceptive effects, thus each possibility needs to be examined in further investigations.

Modulation of the Nociceptive Processing in the Spinal Cord

Activation of descending noradrenergic inhibitory neurons leads to the release of norepinephrine in the spinal cord, which results in modulation of the nociceptive processing via α adrenoceptors. There are at least two neuronal systems that may be involved: 1) direct presynaptic inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second-order neurons through activation of the α2 adrenoceptors (K) *(23,34),* and 2) indirect presynaptic inhibition of the nociceptive primary afferent neurons and/or postsynaptic inhibition of the second-order neurons by the activation of GABAergic inhibitory interneurons through α1 adrenoceptors (L) *(83–85).* A possibility for the involvement of other pathways involving such receptors as serotonergic and opioidergic receptors is not yet fully eliminated but appears unlikely, at least in rats. On the other hand, several types of primary afferent neurons and neurotransmitters are involved in the nociceptive neurotransmission in the spinal cord, and the effect of N_2O on each type should be different. Further investigation is needed to clarify these issues.

Summary

Evidence to date indicates that N_2O induces opioid peptides release in the periaqueductal gray area of the midbrain leading to the activation of the descending inhibitory pathways, which results in modulation of the pain/nociceptive processing in the spinal cord. It is not yet known how N_2O induces opioid peptides release in the periaqueductal gray area. The types of opioid peptides induced by N_2O and the subtypes of opioid receptors that mediate the antinociceptive effects of N_2O appear to depend on various factors including species and/or strain, the region of the brain, and paradigm of behavior testing used for the experiments. Among the three main types of descending inhibitory pathways, the descending noradrenergic inhibitory pathway seems to play a major role in the antinociceptive effects of N_2O. The precise neuronal pathways remain to be elucidated by further investigation.

Acknowledgments

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