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Enkephalin release and opioid receptor activation does not mediate the antinociceptive or sedative/hypnotic effects of nitrous oxide

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Abstract

In previous studies using Fos expression as a marker of neuronal activation, we showed that nitrous oxide (N_2O) activates bulbospinal noradrenergic neurons in rats and that destruction of these neuronal pathways leads to loss of N_2O antinociceptive action. Based on previous rat studies it has been proposed that these noradrenergic neurons are activated through opioid receptors through the release of endogenous opioid ligands in the periaqueductal gray. Using mice with a disrupted preproenkephalin gene (Penk2 $-/-$) and the opioid receptor antagonist naltrexone, we investigated the role of enkephalinergic mechanisms and opioid receptor activation in the behavioral and bulbospinal neuron responses to N_2O in mice. The antinociceptive response to N_2O was investigated using the tail-flick, hot-plate, and von Frey assays, the sedative/hypnotic response was measured using rotarod and loss of righting reflex, and bulbospinal neuronal activation was assessed with pontine Fos immunostaining. No differences were observed between wild-type and Penk2 $-/-$ mice for the antinociceptive, sedative/hypnotic, and pontine neuronal activation effects of N_2O. Similarly, naltrexone did not block N_2O-induced antinociception, sedation, or hypnosis. We conclude that neither enkephalin nor opioid receptors participate in either the antinociceptive or the sedative/hypnotic actions of N_2O in mice.

Keywords: Nitrous oxide; Opioid; Analgesia; Anesthesia; Fos immunoreactivity

1. Introduction

Nitrous oxide (N_2O) has been used to provide anesthetic conditions suitable for the performance of surgery for more than 150 years. Because it is relatively impotent, it cannot be used as a sole anesthetic agent except when administered under hyperbaric conditions; therefore, it is most often used as an adjunctive general anesthetic agent for surgical procedures. Nitrous oxide is also used alone as an analgesic for obstetric, dental and neonatal/pediatric procedures.

Several investigators have reported that systemic administration of high-dose naloxone or naltrexone (5–20 mg/kg) partially blocks N_2O antinociception in rats and mice (Berkowitz et al., 1976; Moody et al., 1989; Quock and Graczak, 1988; Zuniga et al., 1987), and low-dose naloxone (0.06 mg/kg) has been reported to partially block N_2O analgesia in man (Yang et al., 1980). Furthermore, there is unilateral cross-tolerance between morphine and N_2O (Berkowitz et al., 1976). The periaqueductal gray region of the midbrain plays an important role in descending analgesia systems (Willis and Westlund, 1997) and has been proposed as a site of N_2O analgesic action based on the observations that lesioning of the periaqueductal gray blocks N_2O analgesia (Zuniga et al., 1987), and that injections of opioid receptor antagonists directly into the periaqueductal gray also inhibit N_2O analgesia (Fang et al., 1997; Hodges et al., 1994). Furthermore, neurochemical studies have demonstrated that met-enkephalin concentrations in the cerebral spinal fluid of rats and dogs increase after N_2O exposure (Finck et al., 1995; Quock et al., 1985). In addition, spinal cord transection blocks N_2O inhibition of nociceptive spinal input and its antinociceptive effect (Miyazaki et al., 1999; Zhang et al., 1999).

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Based on these observations, it has been suggested that the opioidergic system is needed for the analgesic response to N_2O.

We wondered whether N_2O evokes release of enkephalin in the periaqueductal gray with the subsequent activation of a descending inhibitory system. The current investigation examined the role of opioid receptors and enkephalin in N_2O analgesia, utilizing genetically modified mice with a disruption of the preproenkephalin gene resulting in enkephalin-deficient mice (Konig et al., 1996). The possible contributions of opioid receptors and enkephalin to the N_2O sedative/hypnotic response were also investigated. Also, the role of enkephalin in N_2O-induced activation of the bulbospinal neurons involved in antinociception was evaluated.

2. Materials and methods

These experiments were reviewed and approved by our institute's Subcommittee on Animal Studies and were in accordance with the provisions of the Animal Welfare Act, the PHS Guide for the Care and Use of Laboratory Animals, and VA Policy. Adult (20–30 g) male $Penk2^{Tm1pig}/Penk2^{Tm1pig}$ (hence forth referred to as Penk2 −/− mice), which have a disruption of the preproenkephalin gene resulting in enkephalin-deficient mice (Konig et al., 1996), and their wild-type controls, all on a congenic C57BL/6 background, were used for these experiments. Mice were housed in a temperature and humidity controlled environment and were maintained on a 12-h light/dark cycle. Food and water were available ad libitum.

2.1. Behavioral testing

Tail-flick and hot-plate testing was performed as previously described (Sawamura et al., 2000). The investigator was blinded and the experimental groups were mixed together during each testing session to ensure identical gas exposure conditions. Using a heating blanket, the paw and tail temperatures were maintained within 0.5 °C of 30 °C. Tail-flick latencies were determined from the mean of two consecutive latencies using a tail-flick apparatus (Columbus Instruments, Columbus, OH). The light stimulus intensity was pre-set at an intensity that elicited a mean latency of 3.7–3.5 s in room air with a cut-off time of 10 s. Hot-plate latencies were determined from the mean of two consecutive latencies on a hot-plate device set at a constant 55 °C surface temperature (Columbus Instruments). The latency was determined when the mouse first licked a hindpaw; the cut-off time for this test was 30 s.

Mechanical nociceptive withdrawal responses were measured with calibrated von Frey fibers (North Coast Medical, San Jose, CA) applied over the dorsum of the right hindpaw as previously described (Kingery et al., 2000). Each fiber was applied three consecutive times, pushing down on the hindpaw until the mouse withdrew its paw or the fiber bowed. Four graduated fibers were used sequentially (15, 33, 60, and 90 g), for a total of 12 consecutive fiber applications. The withdrawal threshold was the smallest fiber size, which evoked at least two withdrawal responses during three consecutive applications with the same fiber.

The loss of righting reflex was measured by placing the mouse on its back and determining if the animal could right itself. Rectal temperature was monitored and maintained within 0.5 °C of 36.5 °C with a heating pad. The calculation of the ED_{50} for loss of righting reflex was determined for each mouse, based on interpolation of the gas concentrations which bracketed the righting response. For the measurement of the sedation response, mice were placed on a rotarod (IITC, Life Sciences Instruments, Woodland Hills, CA) turning at 10 rpm. The mice were trained to remain on the rod for 60 s. Drug-naive Penk2 −/− mice and wild-type controls were indistinguishable in their ability to remain on the rod. Each mouse was tested once for its ability to remain on the rod at one of three exposure conditions (air, 35% and 70% N_2O).

2.2. Normobaric gas exposures

Behavioral studies were performed in a Plexiglas chamber (91-cm long, 48-cm wide, and 38-cm high) with a sliding door for insertion of the animals. The investigator's forearms could be inserted through two circular openings on the side of the chamber, which were sealed with rubber flap iris diaphragm air seals. Antinociceptive testing was always performed after 30 min N_2O exposure since we had previously demonstrated a maximal antinociceptive effect in mice at this time interval (Guo et al., 1999). Fresh gas flow (rate varied between 3 and 10 l/min) was introduced into the chambers via an inflow port; a fan was used to achieve adequate mixing within the chamber, and gases were purged by vacuum. Oxygen concentration in the chamber was maintained between 25% and 30% atmospheres absolute, and the N_2O concentration was maintained at 70% atmospheres absolute. An airway gas monitor (Model 254, Datex, Helsinki, Finland) was used to continuously monitor the concentrations of N_2O, oxygen and carbon dioxide in the chamber.

2.3. Hyperbaric gas exposures

Loss of righting reflex testing was performed inside a large (3.66 m diameter, 4.58 m height) clinical hyperbaric chamber, with the entire Plexiglas gas exposure chamber and the investigator inside the hyperbaric chamber. The gas mix inside the gas exposure chamber was kept at 70% v/v of N_2O and 30% v/v of oxygen. The chamber pressure was increased in 0.3 atm increments to generate 91%, 112%, and 133% atmospheres absolute of N_2O over

a 90-min testing session. After a 15-min exposure at each N_2O% atmospheres absolute, the loss of righting reflex testing was performed.

2.4. Immunohistochemistry

After 90-min exposure to 70% atmospheres absolute N_2O, the mice were transcardially perfused with a fixative and their brains were removed and post-fixed. The brainstem was sliced into 40-μm-thick sections with a cryotome, and every third section of the brainstem (from caudal periaqueductal gray to rostral medulla) was retained. Sections were stained using antibodies for Fos and tyrosine hydroxylase, (TH, a catecholamine synthesizing enzyme) as previously described (Sawamura et al., 2000). Using light microscopy, the Fos positive neurons were identified by dense black staining of the nucleus and the TH positive neurons were identified by yellow-orange staining of the cytoplasm. The brainstem regions were located according to a rat brain atlas (Paxinos and Watson, 1986). For each region, all the Fos positive nuclei were counted for each section and the four sections with the highest counts were pooled, giving a count which was the sum of all the Fos stained neurons in those four sections. Fos positive neurons in the noradrenergic nuclei were only counted in cells doubled stained with TH positive cytoplasm. The investigator performing the Fos counting was blinded to treatment groups.

2.5. Statistical analysis

All data are presented as the mean ± S.E.M., and differences are considered significant at a p value less than 0.05. The immunohistochemistry quantitation data were analyzed using unpaired t-tests. The tail-flick and hot-plate latencies were compared between Penk2 −/− and wild-type mice using paired t-tests. The von Frey thresholds and loss of righting reflex ED_{50}s were compared between the Penk2 −/− and wild-type mice using the Mann–Whitney U-test. Figures examining naltrexone inhibition of N_2O analgesic effects show the mean latency or threshold changes as the percentage of the maximum possible effect %MPE; %MPE = ([post-gas − baseline]/[cut-off − baseline]) × 100.

2.6. Experimental protocols

2.6.1. N_2O antinociception in enkephalin-deficient mice

The antinociceptive action of N_2O on the tail-flick, hot-plate, and von Frey assays was compared in the Penk2 −/− enkephalin-deficient mice and their wild-type controls. Baseline latencies and thresholds were determined in the gas chamber under room air conditions; thereafter, the mice were removed from the chamber which was then equilibrated with the test gas mixture (70% N_2O). After 30 min, the mice were placed back in the gas chamber and exposed to the test gas mixture for 30 min. The nociceptive testing was then repeated.

2.6.2. Naltrexone effect on N_2O and morphine antinociception in wild-type mice

This experiment examined the effect of the opioid receptor antagonist naltrexone on N_2O-induced antinociception in wild-type mice. Baseline tail-flick latencies and von Frey thresholds were determined in the gas chamber under room air conditions; thereafter, the mice were removed from the chamber which was then equilibrated with the test gas mixture (70% N_2O). The mice were injected with naltrexone (5 or 20 mg/kg, i.p.) and after 25 min, the mice were placed back in the gas chamber and exposed to

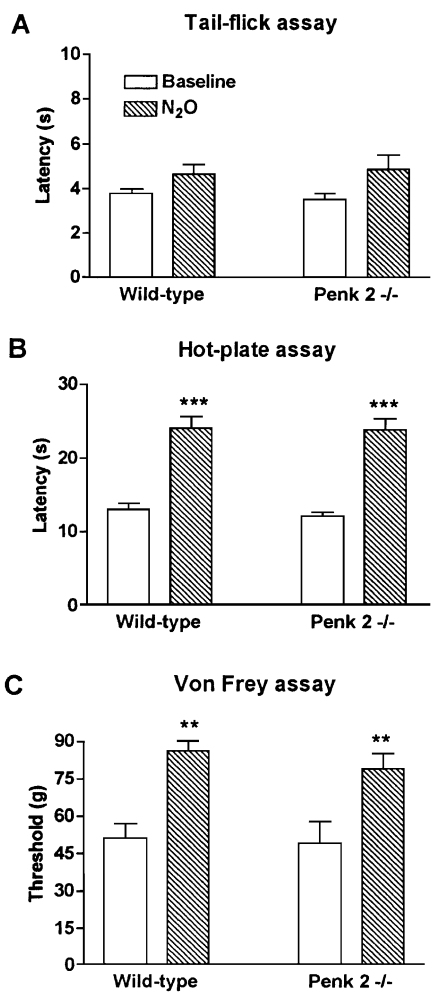

Fig. 1. The antinociceptive effects of nitrous oxide (N_2O) in wild-type and enkephalin-deficient Penk2 −/− mice. Baseline nociceptive thresholds were measured in air and after 30 min N_2O (70% atmospheres absolute, ATA) exposure. There was no significant difference in baseline threshold of wild-type ($n = 8$) and Penk2 −/− ($n = 8$) mice for any assay. (A) Tail-flick latency was not significantly increased in either group of mice after N_2O exposure. There were no differences between wild-type and Penk2 −/− mice in their N_2O antinociceptive responses on the hot-plate (B) and von Frey (C) assays. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. air.

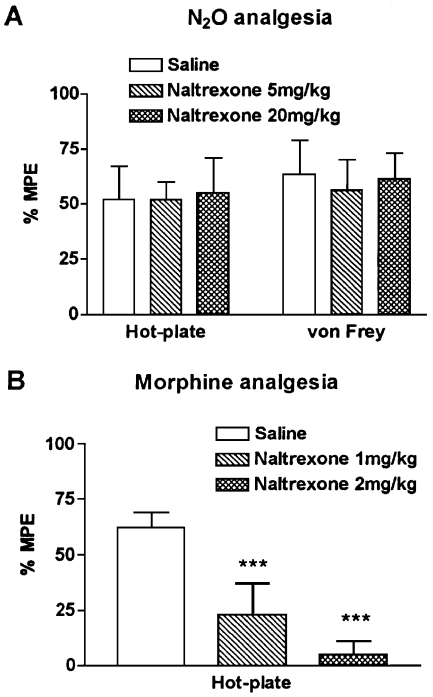

Fig. 2. (A) The opioid receptor antagonist naltrexone (5 and 20 mg/kg, i.p.) did not inhibit N_2O antinociception on the hot-plate and von Frey assays. (B) Low-dose naltrexone (2 mg/kg, i.p.) completely blocked morphine (10 mg/kg, i.p.) antinociception on the hot-plate assay. N_2O-induced changes in nociceptive latencies or thresholds are shown as the percentage of the maximum possible effect (%MPE) ($n = 8$ for each cohort). $^{***}p < 0.001$ vs. saline.

the test gas mixture for 30 min. The nociceptive testing was then repeated. Another experiment examined naltrexone inhibition of morphine antinociception in wild-type mice. After baseline hot-plate latencies were determined the mice were injected with naltrexone (1 or 2 mg/kg, i.p.) and then 5 min later were injected with morphine (10 mg/kg, i.p.). After 55 min, the nociceptive testing was repeated.

2.6.3. N_2O-induced sedation and hypnosis; role of enkephalins and opioid receptors

To determine if enkephalin release or opioid receptors contribute to N_2O sedation, we used the rotarod assay. Rotarod testing was performed in Penk2 $-/-$ mice, in their wild-type controls, and in wild-type mice injected with naltrexone. Baseline latencies were determined in the gas chamber under room air conditions; thereafter, the mice were removed from the chamber which was then equilibrated with the test gas mixture (either 35% or 70% atmospheres absolute of N_2O). One group of wild-type mice were injected with naltrexone (20 mg/kg, i.p.). After 30 min, the mice were placed back in the gas chamber and exposed to the test gas mixture for 30 min. The rotarod testing was then repeated. The hypnotic effect of N_2O was evaluated in Penk2 $-/-$ mice, in wild-type controls, and in wild-type mice injected with naltrexone. After injecting naltrexone (20 mg/kg, i.p.) in one group of wild-type mice, all mice were placed in a hyperbaric chamber and the loss of righting reflex ED_{50} was determined.

2.6.4. Role of enkephalin release in N_2O-induced activation of bulbar analgesic pathways

The immediate early gene product, Fos protein, is a widely used biochemical marker of sustained neuronal activation. These experiments measured N_2O-induced Fos expression in the periaqueductal gray and parabrachial neurons, discrete pontine regions involved in descending antinociception. We postulated that Penk2 $-/-$ mice may have reduced N_2O evoked Fos expression in these antinociceptive pathways, indicating a mechanism for enkephalin mediation of N_2O antinociception. Fos and TH double-staining in the brainstem were used to determine if N_2O exposure activated the catecholaminergic neurons in the A5, locus coeruleus, and A7 nuclei. Prior to the Fos experiment, Penk2 $-/-$ and wild-type mice were habituated to the experimental conditions to minimize background Fos expression induced by the stimulus of a novel environment. For 7 consecutive days, the mice were taken

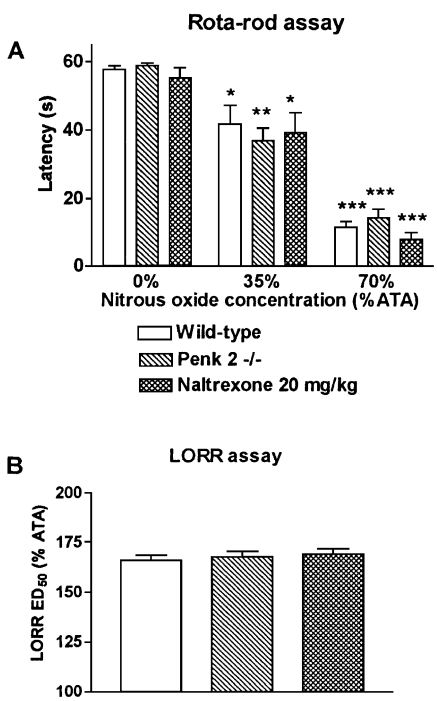

Fig. 3. (A) N_2O exposure (35% ATA and 70% ATA, for 30 min) caused mice to fall off the rotarod. N_2O sedative effect on the rotarod assay was equally effective in the wild-type mice, Penk2 $-/-$ mice, and wild-type mice injected with naltrexone ($n = 8$ each cohort). (B) The hypnotic effect of N_2O, as measured by the concentration (%ATA), which caused a loss of righting reflex in half the mice (LORR ED_{50}). There were no differences in the hypnotic effects of N_2O on the wild-type mice, Penk2 $-/-$ mice, and wild-type mice injected with naltrexone ($n = 12$ each cohort). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. air.

to the laboratory and individually placed in the Plexiglas test chamber (open to room air) for 90 min. Gas exposures of N_2O or air were performed on mice individually housed in an air tight cylindrical Plexiglas chamber (20 cm in diameter, 30 cm in height) for 90 min.

3. Results

3.1. N_2O antinociception was intact in enkephalin-deficient mice

There were no differences in baseline tail-flick and hot-plate latencies and von Frey thresholds between the wild-type and Penk2 −/− mice (Fig. 1). Fig. 1A illustrates that neither wild-type or Penk2 −/− mice had a significant N_2O antinociceptive effect for the tail-flick assay. N_2O antinociception was significant for the hot-plate assay and the von Frey assay (Fig. 1B,C). There were no differences between wild-type and Penk2 −/− mice in the antinociceptive effects of N_2O, indicating that enkephalin release does not mediate N_2O antinociception.

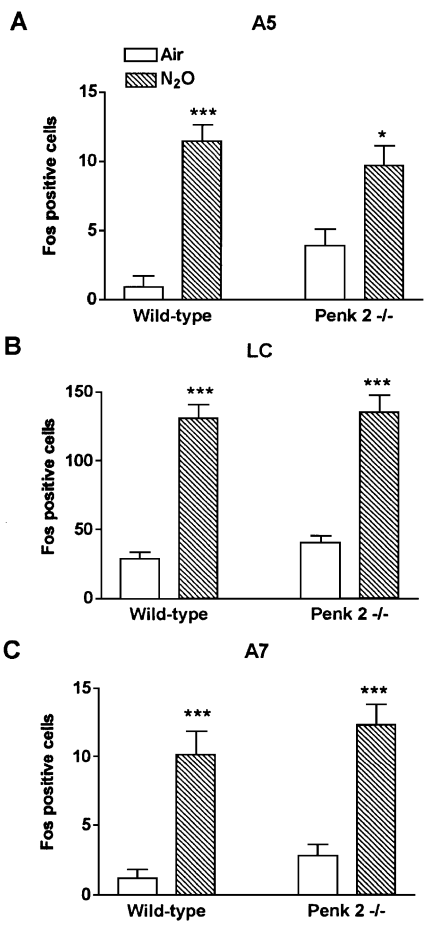

Fig. 4. Effect of N_2O on Fos induction in the neurons of the A5, locus coeruleus (LC), and A7 noradrenergic nuclei. Wild-type and Penk2 −/− mice were exposed for 90 min to N_2O ($n = 6$ each cohort) or room air ($n = 6$ each cohort), then transcardially perfused and the brains removed. Pontine sections were immunostained for Fos and tyrosine hydroxylase (TH), and the stained neurons in each region were counted section by section; the four sections with the highest counts were summed for each mouse. Nitrous oxide exposure increased Fos expression equally in the wild-type and Penk2 −/− mice in the noradrenergic neurons of the (A) A5, (B) LC, and (C) A7 nuclei. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. air.

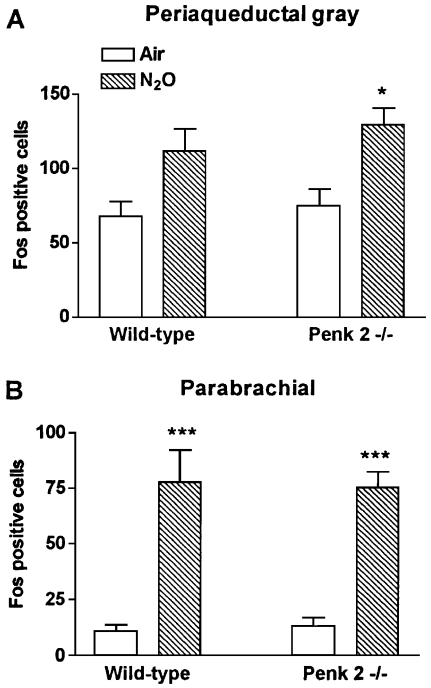

Fig. 5. N_2O-induced Fos expression in the neurons of the periaqueductal gray and parabrachial regions of the pons. Wild-type and Penk2 −/− mice were exposed to N_2O ($n = 6$ each cohort) or room air ($n = 6$ each cohort). Pontine sections were stained for Fos and positive neurons counted. Nitrous oxide exposure increased Fos expression equally in the wild-type and Penk2 −/− mice in both the (A) periaqueductal gray and (B) parabrachial regions. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. air.

3.2. N_2O antinociception was not antagonised by naltrexone

The opioid receptor antagonist naltrexone (5 and 20 mg/kg, i.p.) did not inhibit the N_2O antinociceptive effect for either the hot-plate assay or von Frey assay (Fig. 2A). Low-dose naltrexone (2 mg/kg, i.p.) completely blocked the morphine (10 mg/kg, i.p.) antinociceptive effect for the hot-plate assay (Fig. 2B). These data indicate that N_2O antinociception is not mediated via opioid receptor activation.

3.3. N_2O-induced sedation and hypnosis was intact in enkephalin-deficient mice and was not antagonised by naltrexone

N_2O evoked a concentration-dependent sedative effect on the rotarod assay (Fig 3A). There was no difference in

the sedative effect of N_2O between wild-type, Penk2 $-/-$, and wild-type mice treated with naltrexone (20 mg/kg, i.p.). Fig. 3B illustrates that the hypnotic efficacy of N_2O did not differ among the three groups of mice (loss of righting reflex ED_{50} was $167 \pm 3\%$ for wild-type mice, $168 \pm 3\%$ for Penk2 $-/-$ mice, and $170 \pm 3\%$ for wild-type mice treated with naltrexone). These data indicate that N_2O-induced sedation and hypnosis is not mediate via enkephalin release or opioid receptor activation.

3.4. N_2O activated pontine periaqueductal gray neurons, parabrachial neurons and noradrenergic neurons in enkephalin-deficient mice

These experiments examined N_2O-induced Fos expression in the pontine regions, which mediate descending analgesia. Fig. 4 illustrates that both wild-type and Penk2 $-/-$ mice exposed to N_2O exhibited an increase in Fos positive neurons in periaqueductal gray and parabrachial regions. TH-stained noradrenergic neurons in the A5, locus coeruleus, and A7 brainstem nuclei also expressed increased Fos immunoreactivity after exposure to N_2O, and this increase was equally robust in both wild-type and Penk2 $-/-$ mice (Fig. 5). Representative Fos staining in the periaqueductal gray and the locus coeruleus is shown following room air or N_2O exposure in Penk2 $-/-$ mice (Fig. 6). These data demonstrate that enkephalin release does not mediate N_2O-induced activation of the pontine cell groups inhibiting nociception.

4. Discussion

N_2O-induced antinociception, sedation, and hypnosis were intact in the enkephalin-deficient mice providing evidence that this endogenous ligand is not required for the behavioral effect of N_2O in mice. While met-enkephalin levels increase in the cerebral spinal fluid of rats after exposure to N_2O (Finck et al., 1995; Quock et al., 1985), the tissue concentrations of met-enkephalin in the brainstem and spinal cord of rats are either increased or unchanged after N_2O exposure (Morris and Livingston, 1984; Quock et al., 1986). Contrastingly, N_2O exposure had no effect on cerebral spinal fluid concentrations of met-en-

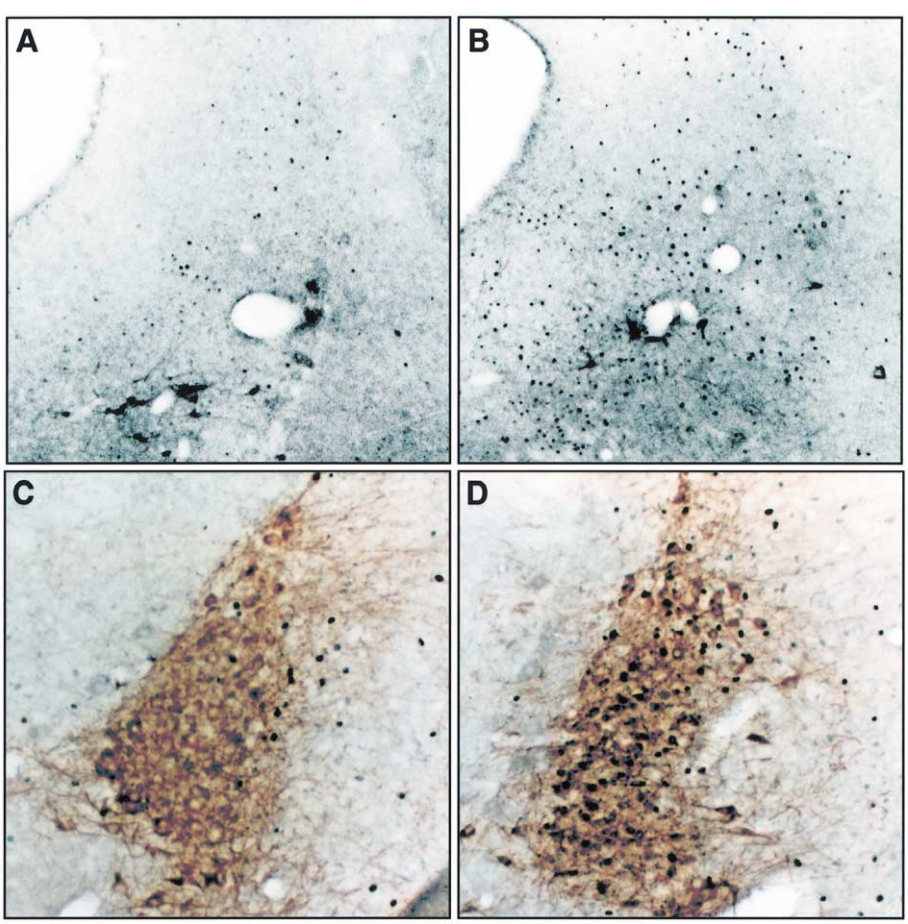

Fig. 6. Fos immunostained neurons in the periaqueductal gray region of Penk2 $-/-$ mice. (A) Representative section in mouse exposed to air. (B) Increase in Fos stained neurons after N_2O exposure. TH (light brown) and Fos (blue) staining in LC noradrenergic neurons in Penk2 $-/-$ mice. (C) Representative section in mouse exposed to air. (D) Marked increase in double-stained neurons after exposure to N_2O.

kephalin or beta-endorphin in humans (Evans et al., 1985). Future investigations utilizing transgenic mice deficient in beta-endorphin or specific opioid receptors may further clarify the role of the opioidergic analgesic system in N_2O antinociception.

Previous studies in rats, mice, and humans have demonstrated partial or complete antagonism of N_2O antinociception using opioid receptor antagonists (Berkowitz et al., 1976; Fang et al., 1997; Hodges et al., 1994; Moody et al., 1989; Quock and Graczak, 1988; Yang et al., 1980; Zuniga et al., 1987). Conversely, this investigation demonstrated that naltrexone did not block the antinociceptive effects of N_2O in mice. These disparate findings may be attributable to differences in species or mouse strains, different nociceptive assays or end-points for the same assay, and different concentrations and exposure times for N_2O-induced antinociception. Our negative results are in agreement with human investigations (Levine et al., 1982; Willer et al., 1985; Yagi et al., 1995; Zacny et al., 1999) and with studies in rats (Fukuhara et al., 1998; Ohara et al., 1997; Shingu et al., 1981) demonstrating opioid antagonists have no effect on N_2O antinociception. These corroborative studies also used different species, nociceptive assays and end-points, and different concentrations and exposure times for N_2O than we utilized in the current study.

The preponderance of human experimental data indicates that N_2O analgesia cannot be blocked with naloxone (0.01–0.4 mg/kg, i.v.). The doses of naloxone that have been reported to partially block N_2O antinociception in rats and mice are 100–2000 times greater then the doses used to reverse opioid-induced respiratory depression in man. High-dose naloxone may act as an opioid receptor agonist (Kumar et al., 1988; Reisine and Pasternak, 1996), which may explain the disparate results between the human and some of animal studies.

One possible explanation for the discrepant results in the animal studies is that 30 min exposure to 70% N_2O causes increased motor activity in rats and mice. Great care must be taken to ensure the animals are withdrawing from noxious stimulus during the nociceptive assay and not simply spontaneously moving their limbs or tails (Sawamura et al., 2000). Under these circumstances, it is crucial that the investigator performing the testing be blinded to the experimental groups, which was done in the current study.

Another possible explanation for the conflicting data regarding the effects of naloxone on N_2O analgesia in animal studies may be due to the fact that just two to four repeated exposures of an animal to an agent that induces endogenous analgesia (such as N_2O, stress, or acupuncture) can generate a conditioned antinociceptive response to the visual cues of the testing apparatus; this conditioned antinociceptive response is naloxone reversible (Bossut and Mayer, 1991; Watkins et al., 1982). Studies using repeated nociceptive testing of the same mice at various time points during N_2O exposure run the risk of developing an opioid-mediated conditioned analgesic response after two to four tests. One published negative study in rats observed no naloxone effect on N_2O tail-flick antinociception after 30-min exposure (two tail-flick tests performed during N_2O inhalation), but after 120 min N_2O exposure (five tail-flick tests performed during N_2O inhalation), there was a borderline naloxone inhibitory effect on N_2O tail-flick antinociception (Fukuhara et al., 1998). Since we previously determined that the hot-plate assay could only be used once in mice, in the current investigation, all experiments were performed on naïve mice with no prior N_2O exposure, thereby obviating possible naltrexone-sensitive conditioned antinociceptive responses.

The use of the immediate early gene product, Fos protein, as a biochemical marker of sustained neuronal activation in the brainstem catecholaminergic neurons has been validated using a wide range of stimuli (Hahn and Bannon, 1999; Jordan, 1998; Monnikes et al., 1997). The time course of its appearance (i.e., within 60 min of its provocation) ideally suits its use in these experiments (Presley et al., 1990). Previously, we demonstrated that N_2O evoked Fos expression in pontine noradrenergic neurons in rats, bulbospinal cell groups which mediate descending analgesia (Sawamura et al., 2000). We postulated that N_2O activated neurons in the periaqueductal gray regions, evoking release of enkephalin, which inhibit GABAergic interneurons (Chiou and Huang, 1999; Vaughan et al., 1997) that tonically suppress firing in noradrenergic neurons (Kawahara et al., 1999); the purported disinhibition may allow increased spontaneous firing of the noradrenergic bulbospinal neurons involved in spinal antinociception (Kingery et al., 1997). The intrathecal injection of an α_2 adrenoceptor antagonist blocks N_2O antinociception (Guo et al., 1996). Transection of the spinal cord or the selective destruction of the pontine catecholamine containing neurons also blocks the antinociceptive property of N_2O, indicating that descending noradrenergic spinal pathways are involved (Miyazaki et al., 1999; Sawamura et al., 2000; Zhang et al., 1999). Furthermore, exposure to N_2O induces Fos expression in the noradrenergic pontine neurons and provokes the release of norepinephrine at the level of the dorsal horn of the spinal cord (Sawamura et al., 2000; Zhang et al., 1999). While we have yet to investigate the mechanisms mediating N_2O activation of the pontine noradrenergic neurons, we speculate that N_2O blockade of NMDA receptors (Jevtovic-Todorovic et al., 1998) on GABAergic neurons abolishes tonic inhibition of the noradrenergic descending analgesic pathways.(Kawahara et al., 1999; Paquet and Smith, 2000) Collectively, these data indicate that N_2O activates a descending noradrenergic pathway, which stimulates α_2 adrenoceptors in the spinal cord through the released norepinephrine. Because we found no difference between Penk2 $-/-$ and wild-type mice in the N_2O evoked expression of Fos in the periaqueductal gray region, the parabrachial region, and in TH positive neurons in the

noradrenergic nuclei (A5, locus coeruleus, and A7) of the pons, we have excluded a possible role for enkephalin release in N_2O-induced activation of the noradrenergic pontine neurons and hence N_2O antinociception.

In conclusion, we have demonstrated that enkephalin release and naltrexone-sensitive opioid receptors do not contribute to the antinociceptive and sedative/hypnotic effects of N_2O in mice, and that enkephalin is not required for the activation of the noradrenergic pontine neurons during N_2O exposure.

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