Nitrous oxide persistently alleviates pain hypersensitivity in neuropathic rats: A dose-dependent effect

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**BACKGROUND:** Despite numerous pharmacological approaches, there are no common analgesic drugs that produce meaningful relief for the majority of patients with neuropathic pain. Although nitrous oxide (N2O) is a weak analgesic that acts via opioid-dependent mechanisms, it is also an antagonist of the N-methyl-D-aspartate receptor (NMDAR). The NMDAR plays a critical role in the development of pain sensitization induced by nerve injury.

**OBJECTIVE:** Using the chronic constriction injury of the sciatic nerve in male rats as a preclinical model of neuropathic pain, the first aim of the present study was to evaluate the lowest N2O concentration and the shortest time of N2O postinjury exposure that would produce persistent relief of neuropathic pain. The second aim was to compare the effects of N2O with gabapentin, a reference drug used in human neuropathic pain management.

**METHODS:** Changes in the nociceptive threshold were evaluated using the paw pressure vocalization test in rats.

**RESULTS:** Among the various N2O concentrations tested, which ranged from 25% to 50%, only 50% N2O single exposure for 1 h 15 min induced a persistent (minimum of three weeks) and significant (60%) reduction in pain hypersensitivity. A single gabapentin dose (75 mg/kg to 300 mg/kg, intraperitoneally) induced an acute (1 h to 1 h 30 min) dose-dependent effect, but not a persistent effect such as that observed with N2O.

**CONCLUSIONS:** These preclinical results suggest that N2O is advantageous for long-lasting neuropathic pain relief after sciatic nerve injury compared with other drugs used in humans such as gabapentinoids or NMDAR antagonists. The present preclinical study provides a rationale for developing comparative clinical studies.

**Key Words:** Central sensitization; Neuropathic pain; Nitrous oxide

Neuropathic pain involves not only a nociceptive process but also a transitional process (1), which is persistent and increases synaptic gain, thereby leading to persistent pain (2). Based on this concept, the recommendations of the European Federation of Neurological Societies guidelines do not include antinociceptive drugs as proposed by the WHO for cancer pain but, instead, focus on drugs acting as antiepileptics, antidepressants and lidocaine plasters as the first line of treatment (3). A promising therapeutic strategy is the use of N-methyl-D-aspartate receptor (NMDAR) antagonists based on evidence that the overactivation of NMDARs plays a critical role in the development of long-lasting sensitization of pain pathways induced by injury (4,5). However, NMDAR antagonists, such as ketamine or related compounds, often result in unacceptable side effects (6). Although novel NMDAR antagonists that selectively target the NR2B subunit have a superior therapeutic index with more limited side effects (7,8), they require long-term or repetitive treatments for sustained analgesic effect (6,9); this approach leads to patient discomfort and high costs because hospitalization is necessary for such a treatment.

Nitrous oxide (N2O) is a common analgesic acting via endogenous opioid release (10,11). However, several in vitro (12,13) and in vivo (14,15) studies have reported that N2O also acts as an NMDAR antagonist that may prevent or reduce pain sensitization (16). We have previously shown (17) that a single 50% N2O exposure for 1 h 15 min induced a persistent reduction in hyperalgesia-allodynia in a rat neuropathic pain model associated with a chronic constriction injury (CCI) at the sciatic nerve (18). Although several concerns regarding the deleterious effects of N2O have been raised in recent years, qualitative reviews of current controversies (19,20) have concluded that N2O have improved pain relief over time.
should remain an option in contemporary anesthesia. In patients receiving general anesthesia for major noncardiac surgery in the gas mixture for Anaesthesia (ENIGMA-II) trial, addition of N₂O to the gas mixture did not increase the risk for death, cardiovascular complications or the risk for surgical site infection (21). Moreover, the intratracheal N₂O led to a reduction in the risk for persistent postsurgical pain (PFSP) (22). However, this long-term beneficial effect in humans was obtained with a high concentration such as 70% N₂O/30% oxygen mixture; this dose induces profound sedative effects, limiting its use outside a hospital environment. Therefore, the first aim of our study was to determine the lowest N₂O concentration and the shortest time of N₂O post-surgical nerve tissue injury capable of inducing persistent relief in the CCI male rat model. The second aim was to compare the effects of N₂O with gabapentin, a reference drug used in humans to treat neuropathic pain.

METHODS

Animals

Experiments were performed on adult male Sprague Dawley rats (Charles River Laboratories, France) weighing 250 g to 300 g. The rats were housed in groups of four per cage with a 12 h light/12 h dark cycle (lights on at 07:00) at a constant mean (± SD) room temperature of 23±2°C. The animals had ad libitum access to food and water. All experiments were performed during the light period. Experiments were conducted according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Ethics Committee in Animal Experimentation of Bordeaux (CEEA30, Project Number 5012066-A), in an authorized laboratory (No. B-33-063-6) and under the supervision of the authorized researcher Ben Boujema (No. 3310009, delivered by the Ministère de l’Alimentation, de l’Agriculture et de la Pêche).

Neuropathic pain model

A peripheral mononeuropathy was produced on day 0 (D₀), using the CCI model (18). Rats were anesthetized with 1% to 3% isoflurane vaporized via a nose cone. The left common sciatic nerve was exposed by blunt dissection at the mid-thigh level, and four loose ligatures (4-0 chronic catgut) were tied around the nerve (identified as the injured hind paw). The muscle and skin were closed in layers and the wound site was covered with an antibiotic mixture of 2% fucidine (Léo, France) and Primyxine (oxytetracycline hydrochloride and polymyxin B sulfate, Chemineau, France). No surgery was performed on the right hind paw (the uninjured hind paw). From an ethical viewpoint and in the purpose of limiting the number of animals in pain experiments, no sham-operated animals were used in this experiment. To minimize differences in the procedure, all operations were performed by the same experimenter. Animals were given 24 h to recover after the operation.

Drugs

Gabapentin (75 mg/kg, 150 mg/kg or 300 mg/kg [Sigma-Aldrich, France]) was dissolved in physiological saline (0.9%) and administered by intraperitoneal injection (3 mL/kg body weight). Control animals received an equal volume of saline injections. The different N₂O concentrations were delivered (Air Liquide, France) via bottles containing premixed nitrous oxide, oxygen and nitrogen: N₂O:N₂:O₂ 12.5%/37.5%/50% (12.5% N₂O), N₂O:N₂:O₂ 25%/25%/50% (25% N₂O), N₂O:N₂:O₂ 35%/15%/50% (35% N₂O) and N₂O:N₂:O₂ 50%/50%/50% (50% N₂O). A bottle containing N₂O₂ 50%/50%/0% (Air) was used for the control group (Air Liquide, France). All gas exposure was performed in a Plexiglas chamber (42 cm × 26 cm × 26 cm) as previously described (16). Four rats were placed in each chamber. Fresh gases was fed into the chamber (4 L/min) through an inlet port and purged by a vacuum set. N₂O concentrations were monitored continuously to confirm premixed gas concentrations (VEO Multigas Monitor, Phaseion Medical Technologies, Sweden).

Mechanical test

The nociceptive threshold (NT) in handled rats was determined using a modification of the Randall-Selitto method (23): a paw pressure vocalization test consisted of constantly increasing pressure that was applied to the hind paw until the rat squeaked. A Basile analgesimeter was used (Apelex, France; stylus tip diameter 1 mm). A 600 g cut-off value was chosen to prevent tissue damage.

General procedures

Animals were acclimated to the animal care unit for four days on arrival to the laboratory. To avoid perturbation from experimental conditions that could affect measurement of the NT, the experiments were performed by the same experimenter under quiet conditions in a testing room located near the animal care unit. For two weeks before the experiment, the animals were weighed daily and handled gently for 5 min; animals were then placed into the test room for 2 h (from 09:00 to 11:00), where they were left to become accustomed to the various apparatuses. All experiments began at 10:00 during the light period. Rats were also acclimated to the plexiglas chamber for one week (15 min per day), with the gas inflow rate set at 4 L/min. NT measurements were taken for two days preceding the surgery (ie, on D₁ and D₁₋₁) and repeated on D₀ before tissue injury. Experiments were initiated only when no statistical change in the basal NT was observed for three successive days (D₀₋₁, D₀ and D₀₋₂, one-way ANOVA, P>0.05). The reference value of NT was chosen as the basal value before tissue injury for each hind paw on D₀. The rats were randomly assigned to different experimental groups. Nitrous exposures or gabapentin intraperitoneal injections were performed seven days post-CCI in all experiments because, as previously reported (17), a stable and homogenous hyperalgesia was obtained over this time.

Experimental protocols

Experiment 1: Delayed effects of a single N₂O exposure for 1 h 15 min at various concentrations (12.5%, 25%, 35% and 50% N₂O) on neuropathic pain

In this experiment, five groups of rats (each group n=8) were used: CCI rats exposed to air, 12.5% N₂O, 25% N₂O, 35% N₂O and 50% N₂O for 1 h 15 min. Gas exposures were performed seven days (D₀) after hind paw injury (D₀). The mechanical NT was evaluated for both the injured and uninjured hind paws once daily from D₀ to D₁₋₁ or D₁₋₂.

Experiment 2: Delayed effects of repeated daily N₂O exposures (1 h 15 min) for three days at various concentrations (12.5%, 25%, 35% and 50% N₂O) on neuropathic pain

In this experiment, five groups of rats (each group n=8) were used: CCI rats exposed to air, 12.5% N₂O, 25% N₂O, 35% N₂O and 50% N₂O for 1 h 15 min. Gas exposures were performed once daily on D₁, D₂ and D₃ after the hind paw injury (D₀). The mechanical NT was evaluated for both the injured and uninjured hind paws once daily from D₁ to D₃.

Experiment 3: Effects of a single (45 min) or repeated (4 × 45 min) daily 50% N₂O exposure on neuropathic pain

Two experiments were performed to evaluate the influence of time exposure on the NT. The first experiment included two groups of rats (each group n=8): CCI rats were exposed to either air or 50% N₂O for 45 min. Gas exposures were performed seven days (D₀) after the hind paw injury (D₀). The second experiment included two groups of rats (n=8): CCI rats received four consecutive exposures to air or 50% N₂O for 45 min. Gas exposures were performed daily on D₁, D₂, D₃ and D₄ after the hind paw injury (D₀). The mechanical NT was evaluated for both the injured and uninjured hind paws once daily from D₁ to D₄.

Experiment 4: Dose-effect study of gabapentin (75 mg/kg, 150 mg/kg and 300 mg/kg) injection on neuropathic pain

Four groups of rats were used in this experiment. One week after the nerve injury (D₁), rats were injected with either saline (n=8), 75 mg/kg of gabapentin (n=8), 150 mg/kg of gabapentin (n=8) or 300 mg/kg of gabapentin (n=8). The mechanical NT was evaluated every 30 min, 60 min and 90 min after gabapentin injection on D₁. The NT was also evaluated once daily until D₁₋₄.
Statistical analysis

All data are expressed as mean ± SD. One- and two-way ANOVA was used to assess the time effects of treatments on the NT and individual group comparisons. The Dunnett post hoc test was used to assess the differences between time points versus the reference value on D7 (ie, before the first gas exposure). The Newman-Keuls post hoc test was used for multiple comparisons among groups; P<0.05 was considered to be statistically significant.

RESULTS

As expected, sciatic nerve damage induced a significant NT decrease in all male rats (Dunnett's test *P<0.05 for comparison with D0 basal value) on the injured hind paw (left hind paw). A more moderate NT decrease was observed on the uninjured hind paw (right hind paw).

Experiment 1: Delayed effect of a single N2O exposure for 1 h 15 min at various concentrations (12.5%, 25%, 35% and 50% N2O) on neuropathic pain

No change in the NT decrease induced by sciatic nerve injury was observed in rats that were exposed to a single 12.5% or 25% N2O exposure (P>0.05) (Figures 1A and 1B).

A single 35% N2O exposure on D7 induced a 43% reduction in the NT decrease on the injured hind paw after 24 h on D8 (Dunnett's test *P<0.05, Figure 1C), and a complete reduction on the uninjured hind paw (Dunnett's test *P<0.05, Figure 1D).

Experiment 2: Delayed effects of repeated daily N2O exposures (1 h 15 min) for three days at various concentrations (12.5%, 25%, 35% and 50% N2O) on neuropathic pain

No change in the NT decrease was observed in rats subjected to air or 12.5% N2O exposure once daily on D7, D8 and D9 (Dunnett's test P>0.05 Figure 2A).

A series of three daily 25% N2O exposures for 1 h 15 min induced a partial reduction in the NT decrease on both the injured (Dunnett test *P<0.05, Figure 2B) and uninjured hind paws (Dunnett's test P<0.05 Figure 2C). This beneficial effect progressively decreased during the N2O postexposure period, and disappeared nine and 14 days after exposure in both the injured and uninjured hind paws, respectively.

In contrast, a series of three daily 35% N2O exposures for 1 h 15 min induced a reduction in the NT decrease on the injured hind paw (Dunnett test *P<0.05, Figure 2C). This reduction was maximal...
(44%) after the first exposure (D1) and was maintained during the three days of N2O exposure (D1 to D3). This beneficial effect progressively disappeared during the post-N2O exposure period. A complete reduction in the NT decrease on the uninjured hind paw was observed during the N2O exposure period (Figure 3A), and this effect progressively disappeared.

A series of three daily 50% N2O exposures for 1 h 15 min induced a sustained reduction in the NT decrease (58% to 66% on D2 to D3) on the injured hind paw (Figure 2D) and completely eliminated the NT decrease on the uninjured hind paw (Figure 3A) (Dunnett’s test P<0.05).

Experiment 3: Effects of a single (45 min) or repeated (4 × 45 min) daily 50% N2O exposure on neuropathic pain

A single 50% N2O exposure limited to 45 min on D1 induced a reduction in the NT decrease on the injured hind paw after 24 h on D2 (Dunnett test P<0.05, Figure 4A) and a partial (53.8%) reduction on the uninjured hind paw (Dunnett’s test P=0.05). This reduction was not persistent during the postexposure period; it completely disappeared on D3 on the injured hind paw (Dunnett test P=0.05) and on D3 on the uninjured hind paw (Dunnett test P=0.05).

When daily 50% N2O exposures were repeated for four days, the reduction in the NT on the injured hind paw decrease was maintained during the N2O exposure period from D2 to D4 (Dunnett’s test P<0.05, Figure 4B). In contrast to the N2O results, no delayed effect was observed 24 h after the gabapentin injection (D2 to D4).

A single intraperitoneal injection of 300 mg/kg of gabapentin on D1 induced a reduction in the NT decrease on the injured hind paw for 1 h 30 min (Dunnett test P<0.05, Figure 5C) and for 1 h in the uninjured hind paw (Dunnett test P<0.05, Figure 3B). No effect was observed during the postgabapentin injection period (D8 to D14) on either the injured or uninjured hind paws.

DISCUSSION

Two main findings can be drawn from the present preclinical dose-dependent effect study. The first shows that a single 50% N2O exposure for 1 h 15 min in male rats is necessary and sufficient for inducing a persistent alleviation of neuropathic pain induced by a sciatic nerve injury. The second finding indicates that a single gabapentin injection only induced an acute analgesic effect, not a persistent effect on pain hypersensitivity as observed following a single 50% N2O exposure.

A systemic literature review indicates that PPSP is common and is often reported as neuropathic (5,24). It is difficult to manage PPSP because the response to most drugs remains unpredictable despite attempts to develop a more rational therapeutic approach (25,26). Here, our approach using the CCI model mimicked neuropathic pain in patients who are often treated weeks or months after nerve injury, particularly after a surgical lesion. The safety of N2O has been questioned (19,27-30) in recent years. Animal and in vitro studies have shown that toxic effects only occur at extreme doses of N2O exposure (31), up to three times the maximum concentration used in this study at hyperbaric conditions (120% to 150% N2O), or for long duration, such as 50% for 72 h (32). NMDAR antagonists, such as MK-801, phencyclidine, and ketamine, or N2O exposure (31) have been used to demonstrate that the longer excitatory injury will be more likely to convert from reversible to irreversible neurotoxic reactions in neurons because NMDAR blockade is maintained for a longer period of time. Thus, it is critical to determine the lowest N2O concentration and the shortest time of N2O postinjury exposure capable of inducing persistent relief in the neuropathic pain model. Our study indicates that a 50% N2O exposure for 1 h 15 min induced a persistent (minimum of three weeks) and significant (60%) reduction in pain hypersensitivity in this experimental model of neuropathic pain. Some caution must be exercised with these results because no blinded approach and sham-operated rats were used in the present study. However, we previously reported (17) that no significant change in NT was observed in sham-operated animals (surgery and sciatic nerve...
Dose-dependent effect of N\textsubscript{2}O on neuropathic pain

Figure 5) Dose-effects of a single intraperitoneal gabapentin injection (A 75 mg/kg, B 150 mg/kg and C 300 mg/kg), in a male rat neuropathic pain model. One week after chronic constriction injury (CCI) of the sciatic nerve was performed on day 0 (D\textsubscript{0}). CCI rats were injected with various gabapentin concentrations. The nociceptive threshold (NT) was evaluated every 30 min for 1 h 30 min after gabapentin injection. The NT on the injured hind paw was evaluated once daily until D\textsubscript{14}. The NT was expressed as the mean ± SD. Dunnett test \textit{p}<0.05 for comparison with the D\textsubscript{7} value. White circles: air group (n=8); black inverted triangle: 75 mg/kg gabapentin group (n=8); black diamond: 150 mg/kg gabapentin group (n=8); and black square: 300 mg/kg gabapentin group (n=8). The shaded areas indicated the day of the intraperitoneal gabapentin injection.

The beneficial effects of a single exposure to 50\% N\textsubscript{2}O on neuropathic pain prompted us to compare the effects induced by a single gabapentin administration. This comparison was performed given that gabapentinoid drugs have been proposed by the European Federation of Neurological Societies guidelines (3) as the first line of treatment despite various side effects (35). Because spinal plasticity and sensitization play pivotal roles in neuropathic pain after peripheral nerve injury, most laboratory studies have focused on the actions of gabapentin in the spinal cord (36-42). However, some studies proposed that gabapentin also acts on supraspinal structures to stimulate the bulbospinal descending inhibition to alleviate neuropathic pain, but this is controversial. Gabapentin was proposed as an effective drug for alleviating chronic pain because it mimics the pharmacological effects of NMDAR antagonists, such as ketamine or memantine, in preclinical models (26). In the same neuropathic pain model, we previously demonstrated that gabapentin and other \(\alpha\)\(\beta\) ligands decrease presynaptic GABA release in the locus coeruleus, consistent with gabapentin-induced activation of noradrenergic neurons in the locus coeruleus and, thus, an increase in noradrenaline release in the spinal cord (40,53). Interestsingly, gabapentin induces more spinal noradrenaline release in spinal nerve ligation animals compared with control animals, likely due to noradrenergic sprouting in the spinal cord after exposure but not injured). This indicates that the long-lasting NT decrease observed in these studies is the result from nerve injury, not from surgery. A shorter 50\% N\textsubscript{2}O exposure such as 45 min of exposure, only induced a transient effect, whereas repeated daily exposure to 50\% N\textsubscript{2}O for 1 h 15 min for three days did not improve the relief of neuropathic pain hypersensitivity compared with the single 1 h 15 min exposure. Although repeated exposures to 25\% or 35\% N\textsubscript{2}O induced a 30\% to 40\% reduction in neuropathic pain within a few days, there was no persistent effect, ie, the NT values progressively returned to the values observed before N\textsubscript{2}O exposure.

These preclinical findings suggest that a single exposure to N\textsubscript{2}O may be an efficient strategy for alleviating neuropathic pain in humans. A useful index to make interspecies comparisons, particularly between rats and humans, is the minimum alveolar anesthetic concentration, ie, the concentration that prevents purposeful movement to supramaximal noxious stimulation in 50\% of subjects. To achieve the minimum alveolar anesthetic concentration, N\textsubscript{2}O exposure must be used at 105\% in humans and approximately 150\% in rats (33,34). If 105\% in humans is also equivalent to 150\% in rats for analgesia, then a single gabapentin intraperitoneal injection induced an acute dose-dependent effect within 2 h. However, in contrast to results reported (17) that ketamine did not induce the acute analgesic effects observed with gabapentin or N\textsubscript{2}O: it only induced a delayed effect, lasting for several days (21). The mechanisms of these two different treatments warrant discussion. As previously reported, N\textsubscript{2}O induced two types of effects on neuropathic pain (17). The first and well-known effect was an acute opioid-dependent analgesic effect that disappeared as soon as the N\textsubscript{2}O exposure ended (10). It has been proposed that the acute N\textsubscript{2}O-induced antinociceptive effect is mediated by indirect inhibition of the nociceptive afferent neurons and/or postsynaptic inhibition of the second-order neurons via an opioid release in the periaqueductal brainstem; this leads to the activation of the descending noradrenergic inhibitory pathways and the subsequent activation of GABAergic interneurons through \(\alpha\)\(\beta\) adrenoreceptors (10,11).

Recently, a second effect of N\textsubscript{2}O has been described, a delayed and persistent non-opioid-dependent alleviation of neuropathic pain hypersensitivity (17). One hypothesis is that NMDAR antagonistic properties of N\textsubscript{2}O may be responsible for this reduction in pain hypersensitivity because it mimics the pharmacological effects of NMDAR antagonists, such as ketamine or memantine, in preclinical models (26). In the same neuropathic pain model, we previously reported (17) that ketamine did not induce the acute analgesic effects observed with gabapentin or N\textsubscript{2}O: it only induced a delayed reduction in pain hypersensitivity that was limited to two days. Other mechanisms involving AMPA receptors (50), G-Protein-gated inward rectifying K\textsuperscript{+} channels (51), and the two-pore-domain K\textsuperscript{+} channel TREQ-1 cannot be excluded (52); these alternative mechanisms should be further explored.

On the part of gabapentin, it is well acknowledged that its efficacy mainly depends on its action at the \(\alpha\)\(\beta\) subunit of calcium channels that are up-regulated in primary afferents and the spinal cord after nerve injury (38). However, at the supraspinal level, it has been demonstrated that gabapentin and other \(\alpha\)\(\beta\) ligands decrease presynaptic GABA release in the locus coeruleus, consistent with gabapentin-induced activation of noradrenergic neurons in the locus coeruleus and, thus, an increase in noradrenaline release in the spinal cord (40,53). Interestingly, gabapentin induces more spinal noradrenaline release in spinal nerve ligation animals compared with control animals, likely due to noradrenergic sprouting in the spinal cord after...
spinal cord ligation (54). These results suggest that gabapentin reduces presynaptic GABA release by disinhibiting the descending noradrenergic inhibitory pathways. Although speculative, one hypothesis is that both the acute N₂O analgesic effects and short-term gabapentin effects observed in neuropathic pain have some common mechanisms via the activation of the descending noradrenergic inhibitory pathways.

Our study has demonstrated that exposure to 50% N₂O for 1 h 15 min completely reduced the sustained contralateral pain hypersensitivity observed in the lesioned hind paw in this neuropathic pain model. Interestingly, N₂O exposure always re-established the basal NT. This result strongly suggests that long-lasting N₂O effects on neuropathic pain were not analgesic effects per se, but resulted from an inhibition of central neuroplastic mechanisms; these mechanisms may have led to a pain hypersensitivity responsible for the hyperalgesia in both the lesioned and unlesioned hind paws triggered by the unilateral nerve injury.

CONCLUSIONS

The present study demonstrates that a single exposure to 50% N₂O may represent a new and interesting therapeutic approach for inducing persistent neuropathic pain relief, at least after spinal nerve injury, compared with other compounds used in clinical setting (ie, gabapentinoids, sodium channel inhibitors or NMDAR antagonists). It would be interesting to evaluate effects of N₂O exposure after trigeminal injury. The main advantage of N₂O is that it induces a persistent relief of neuropathic pain for several weeks as early as the first 50% N₂O exposure. As compared with other drug treatments used for relieving neuropathic pain, this gas exposure does not require long-term or repetitive treatments for obtaining sustained pain relief. This may represent a favorable benefit:risk:cost ratio. These results provide a rationale for testing this compound in clinical studies aimed at improving neuropathic pain.

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