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

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Le soulagement persistant de l’hypersensibilité à la douleur par le protoxyde d’azote chez des rats neuropathiques : un effet proportionnel à la dose

N europathic 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 gabapentin, a reference drug used in human neuropathic pain, 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 a superior therapeutic index with more limited side effects (7,8), therefore 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 gabapentin, a reference drug used in human neuropathic pain, 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.

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should remain an option in contemporary anesthesia. In patients receiving general anesthesia for major noncardiac surgery in the gas mixture for Anesthesia (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 intraoperative N₂O led to a reduction in the risk for persistent postsurgical pain (PPSP) (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 (CEEA50, 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% fusidic acid (Leo, France) and Primycin (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 (these sham-operated animals. To minimize differences in the procedure, performed in the present study because the authors previously reported (17) that no significant change in nociceptive threshold was observed in the uninjured hind paw once daily from D–2 to D21. 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 D₂ and D₃) and repeated on D₄ before tissue injury. Experiments were initiated only when no statistical change in the basal NT was observed for three successive days (D₇, D₈ and D₉, 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 D₇. The mechanical NT was evaluated for both the injured and uninjured hind paws once daily from D₇ to D₉ or D₁₄.

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 (Apedex, 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 then 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 D₂ and D₃) and repeated on D₄ before tissue injury. Experiments were initiated only when no statistical change in the basal NT was observed in the three successive days (D₇, D₈ and D₉, 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 D₇. The mechanical NT was evaluated for both the injured and uninjured hind paws once daily from D₇ to D₉ or D₁₄.

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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 the 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.

A single 50% N2O exposure on D7 induced a sustained reduction (57% to 66%) in the NT decrease on the injured hind paw from D8 to D12 (Dunnett's test P<0.05, Figure 1D) and a complete reduction on the uninjured hind paw.

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's test P<0.05, Figure 2B) and uninjured hind paws.

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 (D7) and was maintained during the three days of N2O exposure (D7 to D10). 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 D7 to D10) on the injured hind paw (Figure 2D) and completely eliminated the NT decrease on the uninjured hind paw (Figure 3A) (Dunnnett’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 D7 induced a reduction in the NT decrease on the injured hind paw after 24 h on D8 (Dunnnett test P<0.05, Figure 4A) and a partial (53.8%) reduction in the NT decrease on the injured hind paw after 24 h on D8 to D21 (P<0.05). This reduction was not persistent during the postexposure period; it completely disappeared on D14 on the injured hind paw (Dunnnett test P=0.05, Figure 4A) and on D14 on the uninjured hind paw (Dunnnett test P=0.05).

When daily 50% N2O exposures were repeated for four days, the reduction in the NT on the injured hind paw was maintained during the N2O exposure period from D1 to D4 (Dunnnett’s test P<0.05, Figure 4B) but disappeared one day later on D14 (Dunnnett test P>0.05).

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

No change in the NT decrease was observed in rats that received saline or the lowest gabapentin dose (75 mg/kg) on D7 on either the injured (Dunnnett test P>0.05, Figure 5A) or uninjured hind paws (Dunnnett test P>0.05, Figure 3B).

A single intraperitoneal injection of 150 mg/kg of gabapentin on D7 induced a partial reduction in the NT decrease on both the injured and uninjured hind paws for 1 h after gabapentin injection (Dunnnett test P>0.05, Figure 5A).
50% N₂O in rats is proportional to 35% N₂O in humans. This result
in humans is also equivalent to 150% in rats for analgesia, then
used at 105% in humans and approximately 150% in rats (33,34). If
exposure. Although repeated exposures to 25% or 35% N₂O induced a
neuropathic pain hypersensitivity compared with the single 1 h 15 min
values observed before N₂O exposure.

These preclinical findings suggest that a single exposure to N₂O 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, N₂O 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 50% N₂O in rats is proportional to 35% N₂O in humans. This result means that a reduction in the risk of persistent postsurgical pain does not require a high N₂O concentration exposure, such as the 70% used in the ENIGMA trial; in that trial (22), intraoperative N₂O led to a reduction in the risk for chronic pain in patients by more than one-half, with a median follow-up of 4.5 years. The margin of safety may be increased by limiting patients to a lower %N₂O exposure for 1 h 15 min.

The beneficial effects of a single exposure to 50% N₂O 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 (44-46). Similarly, a single dose of 150 mg of pregabalin is highly effective against neuralgia associated with thoracotomy (47).

These findings led us to evaluate the effects of a unique systemic administration via an intraperitoneal injection. Our study indicates that a single gabapentin intraperitoneal injection induced an acute dose-dependent effect within 2 h. However, in contrast to results obtained with a single 50% N₂O exposure for 1 h 13 min, there was no persistent effect. The limited effect of gabapentin is in agreement with results of a recent study (48) showing that an intrathecal pregabalin infusion for four weeks in rats produced analgesia only as long as the drug was administered, without blocking the emergence of persistent pain once the infusion ended. Moreover, the use of gabapentin induces a high risk for dizziness, edema and somnolence in humans (49). From a translational perspective, these results suggest that a single 50% N₂O exposure may be more advantageous than a single gabapentin administration to induce a sustained relief of neuropathic pain in humans as it is used in surgical patients. Clinical trials must be performed to confirm such a therapeutic effect, especially in postoperative patients because peripheral nerve lesions are a major cause of chronic pain after surgery.

The mechanisms of these different treatments warrant discussion. As previously reported, N₂O 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 N₂O exposure ended (10). It has been proposed that the acute N₂O-induced antinociceptive effect is mediated by indirect inhibition of the nociceptive afferent neurons and/or postynaptic 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 α₂δ adrenergic receptors (10,11).

Recently, a second effect of N₂O has been described, a delayed and persistent non-opioid-dependent alleviation of neuropathic pain hypersensitivity (17). One hypothesis is that NMDAR antagonistic properties of N₂O 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 N₂O; 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⁺ channels (51), and the two-pore-domain K⁺ channel TREK-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 α₁β 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 α₁β 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 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% N₂O exposure such as 45 min of exposure, only induced a transient effect, whereas repeated daily exposure to 50% N₂O 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% N₂O 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 N₂O exposure.

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**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 (D₀). 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 D₄. The NT was expressed as the mean ± SD. Dunnett test *P<0.05 for comparison with the D₀ 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.
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 \( N_2O \) 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% \( N_2O \) for 1 h 15 min completely reduced the sustained contralateral pain hypersensitivity observed in the unlesioned hind paw in this neuropathic pain model. Interestingly, \( N_2O \) exposure always re-established the basal NT. This result strongly suggests that long-lasting \( N_2O \) effects on neuropathic pain were not analgesic effects per se, but resulted from an inhibition of central neuroplasticity 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% \( N_2O \) 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 \( N_2O \) exposure after trigeminal injury. The main advantage of \( N_2O \) is that it induces a persistent relief of neuropathic pain for several weeks as early as the first 50% \( N_2O \) 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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