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Specific blockade of morphine- and cocaine-induced reinforcing effects in conditioned place preference by nitrous oxide in mice.

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Abbreviations

N₂O, nitrous oxide;

CPP, conditioned place preference;

ANOVA, analysis of variance;

i.p., intraperitoneally

s.c., subcutaneously

Abstract

Nitrous oxide (N₂O), a pharmacological active gas and an antagonist of NMDA receptors, has been reported to be effective in the treatment of alcohol and tobacco withdrawal syndrome. However, the neurobiological bases of N₂O effects are unknown. The aim of the present studies was to examine the effect of N₂O on acquisition and expression of morphine (10 mg/kg; s.c.)- and cocaine (20 mg/kg; i.p.)-induced conditioned place preference (CPP) in mice. Unbiased place conditioning method was used. Mice were exposed to N₂O during the conditioning phase (acquisition of CPP) or during postconditioning phase (expression of CPP). The same protocol was used to evaluate the impact of N₂O on locomotor activity, two-trial recognition task (memory), spontaneous alternation, sucrose consumption (anhedonic state), forced swim (depressive state) and elevated O-maze tests (anxiety state). In all these tests, mice were treated with morphine (10 mg/kg, s.c.) the first day, the following day mice were given saline. This sequence alternated during the next 4 days. Control animals received saline every day. The behavior of animals was evaluated on day 8. N₂O did not induce CPP but impaired the acquisition of morphine-induced CPP and blocked the expression of cocaine- and morphine- induced CPP. The effects of the gas were long lasting and persist 4 days following the exposure. Moreover no behavioral modifications in tests usually used to investigated emotional state as compared to control mice were observed in animals exposed to N₂O, ruling out an effect of this gas on attention, anxiety, depression, locomotion and anhedonia. These studies raise the possibility that N₂O could have a clinical interest in the management of morphine and cocaine addiction.

Introduction

Addiction is a chronic relapsing disease, but the development of pharmacotherapies has been largely neglected by the pharmaceutical industry. Although drugs of abuse have different initial targets and mechanisms of action, they share common effects on crucial neural circuits involving several neurotransmitters (e.g., dopamine, glutamate, γ -aminobutyric acid). Some medications that target these systems are under study, with varying degrees of success (review in Heidbreder, 2005). One strategy is the use of NMDA antagonists, which suppress or reduce development and expression of alcohol and opioid withdrawal (review in Heidbreder 2005).

Nitrous oxide (N_2O) is a pharmacologically active gas, with an action on the opioid system (Gillman and Lichtigfeld, 1998) and antagonist properties at the NMDA receptors level (Jevtovic-Todorovic et al. 1998a). Thus, N_2O may induced similar effects to those produced by NMDA antagonists, such as MK801 and ketamine (Jevtovic-Todorovic et al. 1998b).

N_2O is an inhalant drug that is commonly used in medical and dental practice for its anesthetic, analgesic, and anxiolytic effects (Franks et al. 1998, Jevtovic-Todorovic et al. 1998; Yamakura and Harris 2000). It is used as conscious sedation to control the anxiety in patients who undergo dental procedures (Kupietzky and Blumenstyk, 1998).

Previous clinical reports have proposed that N_2O could be effective in the treatment of alcohol or tobacco withdrawal syndrome (Gillman and Lichtigfeld, 1998; 2002). Moreover, several studies have indicated that N_2O treatment may have long-term effects and that treated patients are less susceptible to craving and relapsing episodes (Gillman and Lichtigfeld, 1990, 1994). However, these findings have not been confirmed by a recent clinical study comparing the effects of N_2O versus placebo (Alho et al., 2003). Very surprisingly, despite these clinical data, only few animal studies have been performed to evaluate the potential benefits and mechanisms of action of N_2O on treatment of drugs of abuse.

Thus the aim of this work was to determine whether N_2O 50% can be a potential treatment in drug addiction. We examined the rewarding properties of N_2O and its effects on acquisition and expression of morphine and cocaine induced conditioned place preference (CPP) in mice. Moreover, the motivational and emotional states of the animals were evaluated following repeated exposure to

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morphine and N₂O to exclude a possible interference due to behavioural alterations. Thus several behavioural experiments were performed, locomotor activity, two-trial recognition task (memory), sucrose consumption (anhedonic state), forced swim test (depressive state) and elevated O-maze (anxiety state).

Experimental procedures

Animals and drugs

Male CD-1 mice (Iffa Credo, France) weighing 22–24 g at the beginning of the experiments were housed in a room with 12 h alternating light/dark cycle and controlled temperature ($21 \pm 1^\circ\text{C}$). Food and water were available *ad libitum*. The animals were treated in accordance with the *NIH Guidelines for the Care and Use of Laboratory Animals* (1985) and in agreement with the local ethical committee. Morphine (Francopia, France) was administered subcutaneously (s.c.) at 10 mg/kg and was dissolved in saline 0.9%. Cocaine (Sigma, France) was injected intraperitoneally (i.p.) at 20 mg/kg in saline. N₂O at 50% (commercial name : Kalinox®) was provided by Air Liquide (France). The 50/50 mixture of N₂O and O₂, is commonly used in clinic for pain relief and thus is commercially available in the same tanks. 50% N₂O in rodents could be compared to 25% in human, according to the minimum alveolar anaesthetic concentration (MAC) ratio (Nakahara et al. 1997, Sun et al. 1996).

In all experiments, N₂O was infused through a single opening on the bottom of the hermetic chamber, with a constant flow rate, and all of the air in the chamber is displaced by N₂O during the time of the exposition. This is monitored by measuring the O₂ concentration in the chamber.

Place preference paradigm

A previously described unbiased place conditioning method was used (Valverde *et al.*, 2001). The place preference apparatus consisted of two main square conditioning compartments (15 ×15 ×15 cm) separated by a triangular central division. Two distinctive sensory cues differentiated the compartments: the wall coloring (black or stripes) and the floor texture (grid or smooth). The combination was as follows: black wall–grid floor and striped wall–smooth floor. The movement and location of mice were recorded by computerized monitoring software (Videotrack, Viewpoint, Lyon, France). Briefly, the protocol consisted of three phases (figure 1a) :

1. *Preconditioning phase.* Drug-naive mice had free access to both compartments for 20 min, and the time spent in each compartment was recorded.
2. *Conditioning phase.* This phase consisted of 6 days in which each conditioning chamber was closed. On the first conditioning day, mice were treated with morphine (10 mg/kg; s.c.) or cocaine (20 mg/kg; i.p.), and were placed after injection in one of the conditioning environments individually for 20 min. The following conditioning day, mice were given saline in the opposite compartment and this sequence alternated during the next 4 days. Control animals received saline every day.
3. *Postconditioning phase.* This phase took place 24 h after the final conditioning session and was carried out exactly as the preconditioning phase.

Mice were exposed to N₂O during the conditioning phase (acquisition of CPP) or during postconditioning phase (expression of CPP). In this latter experiment, CPP apparatus was placed in the hermetic chamber. Results are expressed in scores (mean \pm s.e.m.) calculated as the difference between postconditioning and preconditioning time spent in the drug-paired compartment.

Sensitization to conditioned rewarding effects of morphine in the place preference paradigm

Mice received injections of saline or morphine (10 mg/kg, s.c.) once daily for 5 days (figure 1b). Then after 3 days drug free, the place preference protocol started. Six groups of mice were constituted:

Control group (SS), mice received once daily injections of saline for 5 days, and were conditioned with saline.

Control-morphine group (SM), received once daily injections of saline for 5 days, and were conditioned with morphine (10 mg/kg, s.c.).

Control-morphine/N₂O group (SM), received once daily injections of saline for 5 days, and were conditioned with morphine (10 mg/kg, s.c.) and exposed to N₂O during the test..

Morphine-control group (MS), received once daily injections of morphine (10 mg/kg, s.c.) for 5 days, and were conditioned with saline.

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Morphine-morphine group (MM), received once daily injections of morphine (10 mg/kg, s.c.) for 5 days, and were conditioned with morphine (10 mg/kg, s.c.).

Morphine-morphine/ N₂O group (MM/N₂O), received once daily injections of morphine (10 mg/kg, s.c.) for 5 days, and were conditioned with morphine (10 mg/kg, s.c.) and exposed to N₂O during the test.

In all tests described below mice were treated with morphine (10 mg/kg, s.c.) the first day, the following day mice were given saline. This sequence alternated during the next 4 days. Control animals received saline every day. The behavior of animals was evaluated on day 7.

Locomotor activity

The locomotor activity of mice was measured in an actimeter composed of eight cages of transparent plastic of equal size (19 x 11 x 14 cm) under low illumination (5 lux). Displacements were measured by photocell beams located across the long axis, 20 mm (horizontal activity) and 60 mm (vertical activity) above the floor (Immetronic, Bordeaux, France). The sessions lasted 20 min. Mice were exposed 20 min to N₂O before the beginning of the session. Locomotor activity was expressed in scores (mean ± sem) as the total number of interruption of the photocell beams.

Two-trial recognition task

The Y-maze apparatus was made of wood with a black plastic floor and had three arms. Each arm was 30 cm long, 8 cm wide and 15 cm high. The movement and location of mice were recorded by computerized monitoring software (Videotrack, Viewpoint, France). A visit to an arm was recorded as soon as mouse enters in the arm.

After each trial, the floor of the maze was cleaned in order to minimize the influence of olfactory cues. The maze was placed in a room under dim illumination (light intensity on the floor of the maze arms: 10 lux), with masking noise. One hour before the beginning of the experiment, mice were placed in a quiet room adjacent to the experimental room, under the same light conditions. Briefly, the protocol consisted of two phases.

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1. Trial 1 (acquisition phase) : A guillotine door closed one arm of the Y-maze. The position of the closed arm was chosen randomly between arm no. 2 and arm no. 3. Mice were placed in arm no. 1, their head pointing away from the center of the maze, and they were allowed to visit the two arms of the maze during 5 min.

2. Trial 2 (evaluation of memory performance) : About 30 min after the first phase, the door was removed and animals had free access to all three arms during 5 min.

During this second trial, the number of visits and the time spent in the three arms were recorded, to evaluate whether animals discriminated the novel arm and visited it longer and/or more often than the two familiar arms.

Mice were exposed to N₂O during 20 min before the test. The results are expressed as the mean \pm s.e.m. of the time spent (s) in each arm.

Spontaneous alternation

The same Y-maze apparatus described above was used. Mice were placed in one arm of the maze and the sequence and number of arm entries were recorded over a period of 10 min. An arm visit was recorded when a mouse moved all four paws into the arm. Scoring of alternations consisted of the evaluation of response sequences, in which entering into the least recently visited arm, was considered an alternation response. The proportion of alternations was calculated by dividing the number of alternations by the total number of arm visits. The mice were exposed to N₂O during the test.

Sucrose consumption test

During this test, isolated mice were given, for 48 h, a free choice between two bottles, one with 2% sucrose solution and another with tap water. To prevent possible effects of side preference in drinking behavior, the position of the bottles was switched after 24 h. No previous food or water deprivation was applied before the test. The consumption of water and sucrose solution was estimated simultaneously in control and experimental groups by weighing the bottles. The preference for sucrose

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was calculated as a percentage of consumed sucrose solution of the total amount of liquid drunk. Mice were exposed 20 min to N₂O before giving bottles of sucrose.

Forced swim test

The test was based on that described by Porsolt *et al.* (1977). Each mouse was placed for 6 min in a vertical glass cylinder (10 cm x 15 cm) containing water to a depth of 9 cm at 21-23°C. After the first 2 min, the total duration of immobility was measured during a 4 min test. The mice were judged to be immobile when they remained floating passively in the water (the very small movements necessary to keep their head above water were not taken into account). Results were expressed as means of duration of immobility \pm SEM. Mice were exposed 20 min to N₂O before each session.

Elevated O-maze test

The O-maze (diameter, 46 cm; runway width, 5.5 cm) (Shepherd *et al.*, 1994) consisted of two open and two wall-enclosed sectors of equal size, elevated 40 cm above ground and was illuminated (100 lux) from the top. Animals were placed on one open sector in front of an enclosed sector. Animal behaviour was videotaped. The total number and duration of entries made in open and closed sectors were recorded during 4 min. Results are expressed as the ratio between the cumulative number of visits or time spent in the open parts of the maze considered as an aversive area and the whole number or duration of visits. Mice were exposed to N₂O during 20 min before the test.

Statistical analysis

One-way analysis of variance (ANOVA) was conducted using computer software (Statview, SAS institute Inc., Cary, USA) for comparison across the experimental conditions. When a significant difference among the treatments was obtained in the ANOVA, the Newman-Keuls's post hoc test was applied to define which group contributed to these differences. Significance was accepted with $P < 0.05$. In the experiment performed to evaluate the long term effects of N₂O on expression of morphine-

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and cocaine- induced CPP, a two-way ANOVA with repeated measures was performed followed by a one-way ANOVA.

Results

Conditioning Place Preference (CPP)

Comparison of preconditioning times spent in each compartment did not show any significant difference, indicating the unbiased characteristics of the experimental design (For example, for one experiment of CPP, the time was : 278.32 ± 7.07 in one compartment and 283.88 ± 11.78 in the other one, $F(1, 48) = 0.163$; $P = 0.687$). Moreover mice of the control group, which received saline during each of the conditioning sessions, exhibited no preference for either of compartments confirming that the time spent in one compartment was stable over repeated tested.

As illustrated in Figure 2 animals which received morphine at 10 mg/kg (s.c.) developed a significant preference for the drug-paired compartment. In contrast, animals exposed to N₂O during the conditioning phase exhibited no preference or aversion for the gas-associated compartment. Moreover it appears that acquisition of morphine-induced CPP was partially blocked when mice received morphine and were exposed at the same time to N₂O, $F(3, 91) = 9.882$, $P < 0.0001$.

In contrast, as shown in Figure 3a, expression of morphine-induced CPP (10 mg/kg; s.c.) was totally abolished by exposure to N₂O during the test, $F(3, 18) = 3.364$, $P = 0.0417$. Similarly, as illustrated in Figure 3b, N₂O was able to block expression of cocaine- (20 mg/kg; i.p.) induced CPP, $F(2, 24) = 7.524$, $P = 0.0029$. In a preliminary experiment we have shown that exposure of mice to medical air instead of N₂O did not modify the behaviour of animals. Thus morphine conditioned animals exposed to medical air expressed a significant CPP as compared to saline-treated mice (data not shown).

As illustrated in Figure 4a, the effects of N₂O on expression of morphine CPP were long lasting. A two-way ANOVA with repeated measures, revealed a significant “treatment effect”, $F(2, 32) = 5.121$, $P = 0.0052$ with no effect on the test day, $F(2, 32) = 0.528$, $P = 0.6664$. Similar results were obtained with cocaine (Figure 4b). Two-way ANOVA with repeated measures revealed a significant “treatment effect”, $F(2, 24) = 6.160$, $P = 0.0069$ with no effect on the test day, $F(2, 24) = 2.004$, $P = 0.1459$.

Figure 5 shows the CPP induced by morphine at 10 mg/kg (s.c.) in mice which have previously received once daily injections of morphine (10 mg/kg; s.c.) for 5 days before the beginning of CPP procedure. In animals with a prior history of morphine administration (MM group), the recording effect of morphine in the CPP was higher as compared to saline pretreated mice (SM group).

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Moreover, exposure to N₂O during the test totally blocked the expression of morphine induced CPP in SM group as well as in MM group, $F(5,79) = 9.458, P < 0.0001$.

Locomotor activity

The locomotor activity of saline and morphine mice was not modified by N₂O treatment. The total number of interruption of photocell beams measured during 20 minutes were : 305.69 ± 8.69 and 226.92 ± 17.00 for saline treated mice in absence and in presence of N₂O, respectively ; and 265.20 ± 7.25 and 230.73 ± 12.14 for morphine treated mice in absence and in presence of N₂O, respectively. ANOVA revealed no differences in the locomotor activity between the four groups, $F(3,50) = 1.663; P = 0.1863$ (data not shown).

Two-trial recognition task

As illustrated in figure 6a, during the 5 min trial 2 mice exposed to morphine, N₂O or morphine/ N₂O visited the novel arm longer than the two familiar ones as the saline group, $F(11, 107) = 1.305; P < 0.0001$.

Spontaneous alternation

There was no significant effect of morphine, N₂O or morphine/ N₂O on the alternation between arms (figure 6b) $F(3,36) = 1.527; P = 0.2240$.

Sucrose consumption test

ANOVA test performed on the consumption of solution yielded a significant 'drink effect', $F(7, 72) = 30.485; P < 0.0001$. Post-hoc Newman Keuls test revealed preference for sucrose consumption in all the group, and no difference between each group (Figure 7).

Forced swimming test

ANOVA test revealed no differences between the four groups, $F(3,32) = 1.260; P = 0.3046$. Thus the times of immobility (seconds) of mice were : 182.89 ± 8.58 and 193.22 ± 12.59 for saline treated mice

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in absence and in presence of N₂O, respectively ; and 152.56 ± 11.70 and 149.44 ± 15.87 for morphine treated mice in absence and in presence of N₂O, respectively (data not shown).

Elevated O-maze test

As shown in figure 8, for all groups mice spent more time in the closed arm, $F(7,62) = 29.083$, $P < 0.0001$. Regarding the time spent in the open arms, no difference was observed between the four groups of animals, $F(3,31) = 2.300$; $P = 0.0967$.

Discussion

The aim of these studies was to examine the effects of N₂O 50% on cocaine- and morphine-induced CPP. As illustrated in Figure 1, we showed that N₂O did not induce aversive or reinforcing effect using the conditioned place preference paradigm. These results pointed out the lack of rewarding effects of N₂O contrasting with previous reports, which demonstrated that nitrous oxide, at concentrations ranging from 15-70%, functions as a reinforcer in primates (Wood et al., 1977). However, in the only drug discrimination study found in the literature using N₂O substituted for the training drug, ethylketocyclazocine, a selective kappa opioid agonist, but not for morphine (Hynes and Hymson, 1984), indicating a kappa-like discriminative stimulus effect of N₂O. This observation could be consistent with the results of studies of N₂O in humans reporting dysphoric effects (hallucinations, confusion...). Nevertheless, in other studies pleasant effects in humans or no effects have been observed (Zacny and Galinkin, 1999). Our results obtained in mice seems to be consistent with the latter results. However, Ramsay et al. (2003) found a conditioned place aversion after 4 conditioning sessions of 40 min with 30% and 60% N₂O/O₂, and a preference with 8% N₂O/O₂ in Long-Evans rats. Several reasons may explain the apparent discrepancies. First, species differences (rats vs mice) cannot be excluded. Second, data analysis of CPP paradigm was highly different. Indeed, they used a biased paradigm and aversion or preference was evaluated in the same experimental group, comparing the time spent in the clear compartment of the CPP apparatus versus the striped compartment, and not in direct comparison with control. This method is more and more abandoned, researchers preferring the unbiased design in order to avoid some interpretational problems (review in Tzschentke, 1998).

In this study, we demonstrated that N₂O impaired the acquisition of morphine-induced CPP and more interestingly blocked the expression of cocaine- and morphine-induced CPP. The neuroanatomical bases of the mnemonic processes involved in CPP have received relatively little empirical investigation. However, the mechanisms determining place conditioning appear to follow Pavlovian conditioning principles, requiring an association between environmental cues and the effective state produced by the drug. Thus the development of CPP is a complex behavioural phenomenon including, among others, spatial learning processes. Thus it was essential to evaluate whether mice treated with N₂O showed an alteration of memory processes. Using the two-trial recognition task (Dellu et al. 2000) it clearly appeared that N₂O did not affect the recognition of the novel arm. Thus, the reduced acquisition of CPP did not seem to be caused by a direct alteration of mouse mnemonic capacity. Regarding the effects of N₂O on morphine- and cocaine- induced CPP, several hypotheses could be proposed. One could speculate that N₂O exposure altered the quality of the exploration occluding expression of CPP. To investigate this hypothesis, we evaluated the effects of N₂O on the spontaneous alternation behavior measured in the Y-maze, which could be used to assess attention, memory and/or related functions (Anisman et al., 1975). The results obtained demonstrate that N₂O did not modify spontaneous alternation behavior that may reflect a lack of perception and/or attention. Another hypothesis that may explain the abolition of CPP by N₂O could be related to modification of anxiety. Indeed, Dockstader and Van der Kooy (2001) have demonstrated that deficit in retrieval of morphine induced CPP was caused by anxiety, rather than to a specific learning or motivational deficit. To investigate this hypothesis we evaluated the behavioural modifications induced by N₂O in the elevated O-maze. In this animal model no difference in the basal behavior between mice exposed to medical air, and mice exposed to N₂O was observed, suggesting a lack of modification of the anxiety state of the animal in our experiment conditions. Moreover, N₂O is known to have an anxiolytic-like action in mice at 70% but not at 50% (Gries et al. 2005).

The emotional state of animals exposed to N₂O was also investigated. We compared the effects of the gas on the forced swim test, a model usually performed to evaluate antidepressant-like properties of drugs, and on sucrose consumption, that can reveal the responsiveness to reward. In both animal

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models, N₂O did not modify the behaviour of mice as compared to control groups. These results demonstrate that N₂O did not lead to anhedonic and depressant-like states.

Another very interesting result obtained is that N₂O prevents the expression of conditioned response to mice sensitized to morphine. These results go in the same way that the recent study of David et al. 2006, showing that N₂O at 70% block locomotor sensitization to amphetamine. They suggest that these effect is due to NMDA antagonist action of N₂O. Strikingly we showed that the effects of N₂O on morphine and cocaine induced CPP were long lasting and persist at least 4 days following the exposure. This reinforces the assumption suggesting a N₂O action on biochemical targets that remain to be identified. This is supported by the demonstration that an established morphine induced CPP can be permanently disrupted if protein synthesis is blocked, as the consolidation of an appetitive instrumental learning task (Hernandez et al., 2004; Milekic et al. 2006).

To our knowledge, this is the first experimental study demonstrating the effects of N₂O on the rewarding component of drug abuse (morphine and cocaine). In summary, we demonstrated that N₂O impaired the acquisition of morphine-induced CPP and blocked the expression of morphine- or cocaine-induced CPP. Moreover these effects are long lasting even after only one exposure of mice to the gas. It seems that these results are not a consequence of behavioural alterations that may biased the CPP test, as no modification in tests usually used to investigated emotional state of animals were observed. Despite the evidence for opiate receptor involvement in the analgesic effect of N₂O, opiate systems appear to be less likely to play role in its other effects, including those effects more directly related to its abuse. The subjective and psychomotor effects of N₂O in humans are not antagonized by naloxone even at high doses (Zacny et al.1994). these results were consistent with a report that N₂O does not have morphine-like discriminative stimulus effects in rats (Hynes and Hymson, 1984). The neurobiological mechanisms that could explain these results are under investigations in the laboratory. The NMDA antagonist properties of N₂O could play a role as direct or indirect impact on protein/peptide synthesis.

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- Alho, H., Methuen, T., Paloheimo, M., Seppa, K., Strid, N., Apter-Kaseva, N., Tiainen, J., Salaspuro, M. and Roine, R., 2003. Nitrous oxide has no effect in the treatment of alcohol withdrawal syndrome: a double-blind placebo-controlled randomized trial. *J Clin Psychopharmacol.* 23, 211-214.
- Anisman, H., Wahlsten, D. and Kokkinidis, L., 1975. Effects of d-amphetamine and scopolamine on activity before and after shock in three mouse strains. *Pharmacol Biochem Behav.* 3, 819-824.
- David, H. N., Anseau, M., Lemaire, M. and Abraini, J. H., 2006. Nitrous oxide and xenon prevent amphetamine-induced carrier-mediated dopamine release in a memantine-like fashion and protect against behavioral sensitization. *Biol Psychiatry.* 60, 49-57.
- Dellu, F., Contarino, A., Simon, H., Koob, G. F. and Gold, L. H., 2000. Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice. *Neurobiol Learn Mem.* 73, 31-48.
- Dockstader, C. L. and van der Kooy, D., 2001. Mouse strain differences in opiate reward learning are explained by differences in anxiety, not reward or learning. *J Neurosci.* 21, 9077-9081.
- Franks, N. P. and Lieb, W. R., 1998. A serious target for laughing gas. *Nat Med.* 4, 383-384.
- Gillman, M. A. and Lichtigfeld, F. J., 1990. Analgesic nitrous oxide for alcohol withdrawal: a critical appraisal after 10 years' use. *Postgrad Med J.* 66, 543-546.
- Gillman, M. A. and Lichtigfeld, F. J., 1994. The uses of analgesic nitrous oxide in neuropsychiatry. *S Afr Med J.* 84, 706.
- Gillman, M. A. and Lichtigfeld, F. J., 1998. Clinical role and mechanisms of action of analgesic nitrous oxide. *Int J Neurosci.* 93, 55-62.
- Gillman, M. A. and Lichtigfeld, F. J., 2002. Randomized double-blind trial of psychotropic analgesic nitrous oxide compared with diazepam for alcohol withdrawal state. *J Subst Abuse Treat.* 22, 129-134.
- Gries, D. A., Condouris, G. A., Shey, Z. and Houpt, M., 2005. Anxiolytic-like action in mice treated with nitrous oxide and oral triazolam or diazepam. *Life Sci.* 76, 1667-1674.
- Heidbreder, C. A. and Hagan, J. J., 2005. Novel pharmacotherapeutic approaches for the treatment of drug addiction and craving. *Curr Opin Pharmacol.* 5, 107-118.
- Hernandez, P. J. and Kelley, A. E., 2004. Long-term memory for instrumental responses does not undergo protein synthesis-dependent reconsolidation upon retrieval. *Learn Mem.* 11, 748-754.
- Hynes, M. D. and Hymson, D. L., 1984. Nitrous oxide generalizes to a discriminative stimulus produced by ethylketocyclazocine but not morphine. *Eur J Pharmacol.* 105, 155-159.
- Jevtović-Todorović V, Todorović SM, Mennerick S, Powell S, Dikranian K, Benschoff N, Zorumski CF, Olney JW. 1998. Nitrous oxide (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. *Nat Med.* 4, 460-463.
- Jevtović-Todorovic, V., Wozniak, D. F., Powell, S., Nardi, A. and Olney, J. W., 1998. Clonidine potentiates the neuropathic pain-relieving action of MK-801 while preventing its neurotoxic and hyperactivity side effects. *Brain Res.* 781, 202-211.
- Kupietzky, A. and Blumenstyk, A., 1998. Comparing the behavior of children treated using general anesthesia with those treated using conscious sedation. *ASDC J Dent Child.* 65, 122-127.
- Milekic, M. H., Brown, S. D., Castellini, C. and Alberini, C. M., 2006. Persistent disruption of an established morphine conditioned place preference. *J Neurosci.* 26, 3010-3020.
- Nakahara, T., Akazawa, T., Nozaki, J. and Sasaki, Y., 1997. Effect of nitrous oxide at sub-MAC concentrations on sevoflurane MAC in adults. *Masui.* 46, 607-12.
- Porsolt, R. D., Bertin, A. and Jalfre, M., 1977. Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther.* 229, 327-336.
- Ramsay, D. S., Watson, C. H., Leroux, B. G., Prall, C. W. and Kaiyala, K. J., 2003. Conditioned place aversion and self-administration of nitrous oxide in rats. *Pharmacol Biochem Behav.* 74, 623-633.

- Shepherd, J. K., Grewal, S. S., Fletcher, A., Bill, D. J. and Dourish, C. T., 1994. Behavioural and pharmacological characterisation of the elevated "zero-maze" as an animal model of anxiety. *Psychopharmacology (Berl)*. 116, 56-64.
- Sun, W.Z, Shyu, B.C., Shieh, J.Y., 1996. Nitrous oxide or halothane, or both, fail to suppress c-fos expression in rat spinal cord dorsal horn neurones after subcutaneous formalin. *Br J Anaesth*. 76, 99-105.
- Tzschentke, T. M., 1998. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog Neurobiol*. 56, 613-672.
- Yamakura, T. and Harris, R. A., 2000. Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels. Comparison with isoflurane and ethanol. *Anesthesiology*. 93, 1095-1101.
- Zacny, J.P, Coalson, D.W, Lichtor, J.L, Yajnik, S., Thapar, P., 1994. Effects of naloxone on the subjective and psychomotor effects of nitrous oxide in humans. *Pharmacol. Biochem. Behav*. 49, 573-578.
- Zacny, J. P. and Galinkin, J. L., 1999. Psychotropic drugs used in anesthesia practice: abuse liability and epidemiology of abuse. *Anesthesiology*. 90, 269-288.

Legends

Figure 1 : Summary of the experimental protocols used. a- place preference paradigm : mice were given saline, morphine (10 mg/kg s.c.) or cocaine (20 mg/kg i.p.). Mice were exposed to N₂O (50%) during the conditioning phase (20 min) or during the test. b- sensitization to conditioned rewarding effects of morphine : mice were treated with morphine 10 mg/kg i.p. or saline once daily, from day two to day six. After a period of three days mice were conditioned according to the place preference paradigm as described above (a).

Figure 2 : Effects produced by s.c. injection of saline, morphine (10 mg/kg), exposure to N₂O for 20 min or by injection of morphine with simultaneous exposure to N₂O in the conditioned place preference in mice. Each column represents the mean score \pm SE (n = 18-27 per group). * P< 0.05 and *** P< 0.001 compared with saline group, \neq P< 0.05 compared with the N₂O group and § P< 0.05 compared with morphine/ N₂O group, (Newman-Keuls test).

Figure 3 : a- Influence of N₂O upon the expression of conditioned place preference induced by morphine (10 mg/kg, s.c.) and b- cocaine (20 mg/kg, i.p.). Mice were exposed to N₂O during the test. Each column represents the mean score \pm SE (n = 18-27 per group). * * P< 0.01 compared with saline group and \neq P< 0.05 compared with the combination drug- N₂O group, (Newman-Keuls test).

Figure 4: Influence of N₂O upon the expression of conditioned place preference induced by morphine (10 mg/kg, s.c.) and cocaine (20 mg/kg, i.p.) on day 8 and day 12. Animals were only exposed to N₂O on day 8. Each column represents the mean score \pm SE (n = 8-10 per group). * P< 0.05 and * * P< 0.01 compared with saline group and \neq P< 0.05 compared with the morphine or cocaine group, (Newman-Keuls test).

Figure 5: Effect of N₂O upon the sensitization to the rewarding effects of morphine measured in the conditioned place preference. Each column represents the mean score \pm SE (n = 10-16 per group). *

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$P < 0.05$ and *** $P < 0.001$ compared with control (SS) group, § $P < 0.01$ compared with morphine-morphine (MM)/ N_2O group and \neq $P < 0.01$ compared with saline-morphine (SM) group, (Newman-Keuls test).

Figure 6 : a - Influence of N_2O upon the memory test of two-trial recognition test. Each column represents means \pm SE (n = 9-10 per group). * $P < 0.05$ and * * $P < 0.01$ compared with arms 1 and 2 group, (Newman-Keuls test). b - Influence of N_2O upon the spontaneous alternation. Each column represents the mean percent of alternation \pm SE (n = 10 per group, ANOVA test). Mice were exposed to N_2O during the test. Animals were pretreated with saline or morphine as described in materials and methods.

Figure 7 : Effect of N_2O in the sucrose consumption. Each column represents the mean percent intake of liquid \pm SE (n = 10 per group). ** $P < 0.01$ compared with top water groups (Newman-Keuls test). Animals were pretreated with saline or morphine as described in materials and methods.

Figure 8 : Behavioural effect of N_2O in elevated O-maze test. Each column represents the mean time \pm SE (n = 7-10 per group). * * $P < 0.01$ compared with time spent in open arms, (Newman-Keuls test). Animals were pretreated with saline or morphine as described in materials and methods.