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Comparison of N_2O- and chlordiazepoxide-induced behaviors in the light/dark exploration test☆

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Abstract

Earlier research has demonstrated similarities in the behavioral effects of nitrous oxide (N_2O) and benzodiazepine (BZ) drugs such as chlordiazepoxide (CP). The present research was conducted to compare the behavioral effects of N_2O and CP in mice in the light/dark exploration test. When challenged with either N_2O or CP, mice exhibited significant dose-dependent increases in the time spent in the light compartment and also in the number of transitions between the light and dark compartments. Pretreatment with BZ receptor antagonist flumazenil (FLU), the $GABA_A$ receptor antagonist SR-95531 or the selective neuronal nitric oxide (NO) synthase (nNOS) inhibitor 7-nitroindazole (7-NI) all antagonized anxiolytic effects of N_2O and CP. Based on these findings, it was concluded that N_2O and CP evoke similar behavioral effects in the light/dark exploration test that are similar in their interaction with BZ and $GABA_A$ receptor antagonists. There also appears to be a specific role for nNOS in generating the NO involved in mediation of these effects.

Keywords: Nitric oxide; Nitrous oxide; Light/dark exploration test; Benzodiazepine; Chlordiazepoxide; Mice

1. Introduction

The anesthetic gas nitrous oxide (N_2O) has been demonstrated to possess a significant anxiolytic effect that can be clinically exploited to reduce anxiety and fear in patients in dental and other situations (Allen, 1979; Smith and Beirne, 1985). Previously, we have demonstrated that the anxiolytic effects of N_2O parallel those of benzodiazepines (BZ) in several animal models of experimental anxiety. Moreover, the anxiolytic effects of N_2O are sensitive to antagonism by BZ receptor blockers and are significantly attenuated in BZ-tolerant animals (Czech and Quock, 1993; Emmanouil et al., 1994; Quock et al., 1992, 1993). Based on these observations, we have hypothesized that the behavioral effects of N_2O might be mediated by BZ receptors. Whether N_2O acts directly or indirectly upon these receptors remains to be seen.

Earlier research of the mechanism of action of BZ has revealed that BZs facilitate transmission at inhibitory GABAergic synapses (Zorumsky and Isenberg, 1991). We have also implicated nitric oxide (NO), a unique and ubiquitous gas with neurophysiological functions (Bredt and Snyder, 1992; Garthwaite et al., 1988; Snyder and Bredt, 1991), in the behavioral effects of both N_2O and BZs (Caton et al., 1994; Quock and Nguyen, 1992). In this study, we assessed the influence of BZ receptor blockade, $GABA_A$ receptor blockade and selective inhibition of neuronal NO synthase (nNOS) on the anxiolytic effects of N_2O and chlordiazepoxide (CP). We chose as our anxiety model the light/dark exploration test, which is based on both the aversive response of mice to illumination and the spontaneous exploratory behavior in such an environment (Crawley, 1981, 1985; Crawley and Goodwin, 1980).

2. Methods

2.1. Animals

Male NIH Swiss mice, 18–22 g body weight, were purchased from Harlan Sprague–Dawley Laboratories

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(Indianapolis, IN) and used in these experiments, which were approved by an institutional animal care and use committee. Mice were housed five per cage in the Wegner Hall Vivarium with access to food and water ad libitum. The facility is maintained on a 12-h light/dark cycle (lights on 07:00–19:00 h) under standard conditions (22 ± 1°C room temperature, 33% humidity). Mice were kept in the holding room for at least 4 days following arrival in the facility. Each animal was used only once, then discarded.

2.2. Apparatus

The light/dark exploration box (450 mm length × 270 mm width × 270 mm height) was constructed of acrylic (Abbott Plastics, Rockford, IL). An acrylic divider with a 75 × 75 mm opening at floor level divided the box into a light compartment (three-fifths of the total length) and a dark compartment (two-fifths of the total length). The walls of the light and dark compartments were made of black and white acrylic, respectively. Behavioral observations and assessments were generally performed between 10:00 and 14:00 h. During all experiments, the light compartment was illuminated by two 40-W white light fluorescent tubes mounted 180 mm directly overhead. In this paradigm, animals were individually placed in the center of the light compartment of the box, facing away from the divider, then observed for 5 min. The time spent in the light chamber of the box as well as the number of transitions between the light and dark compartments were recorded for each mouse. A mouse was considered to have entered the new area when all four legs crossed the threshold into the compartment.

2.3. Drugs

The following drugs were used in this research: N_2O, USP, O_2, USP and compressed air, USP (all from A&L

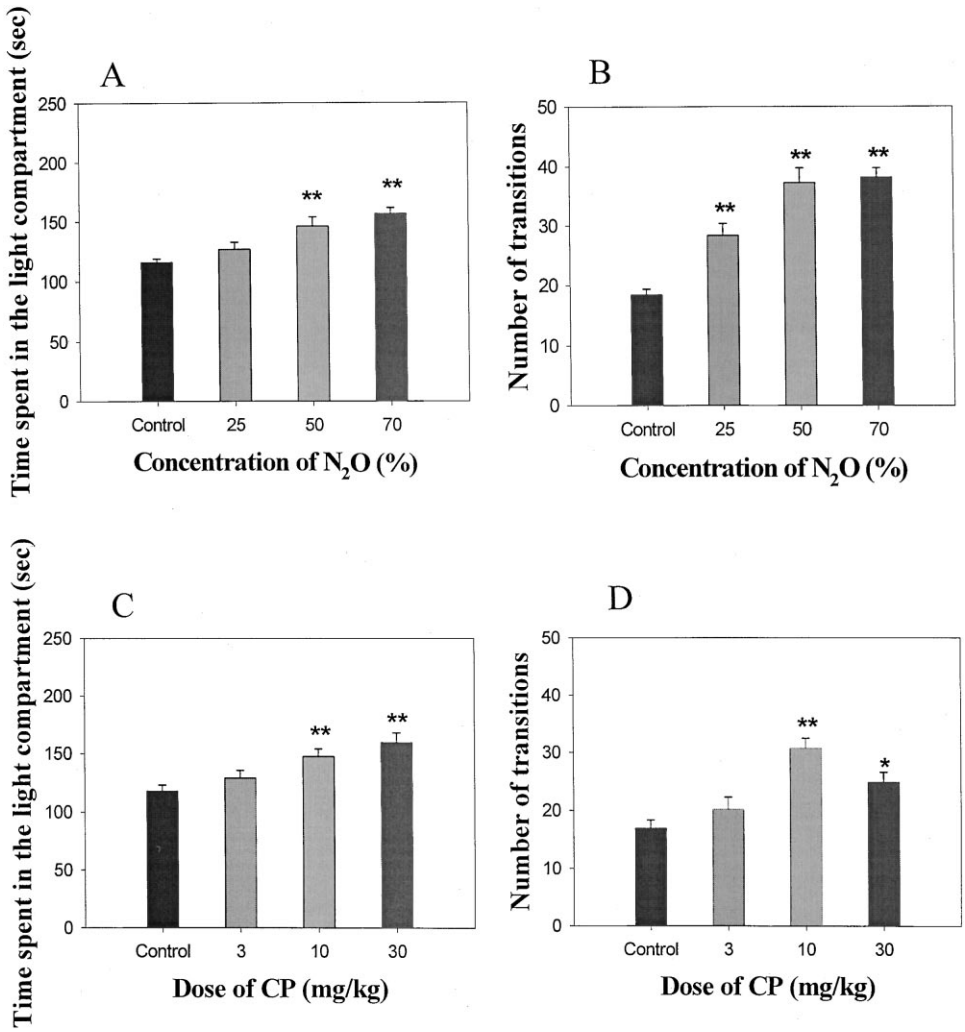

Fig. 1. Behavioral effects of N_2O (Frames A and B) and CP (Frames C and D) in the light/dark exploration test. The height of each bar represents the mean and each vertical bar represents the S.E.M. of 12–15 mice (Frames A and B) and 15–20 mice (Frames C and D). Significance of difference: $*P<.05$ and $**P<.01$, compared with the room-air control (one-way ANOVA followed by a post-hoc Dunnett's t test).

Welding, Spokane, WA); CP hydrochloride (Sigma, St. Louis, MO); flumazenil (FLU; Roche, Nutley, NJ); 2-[3-carboxypropyl]-3-amino-6-[4-methoxyphenyl]pyridazinium bromide (SR-95531) and 7-nitroindazole (7-NI; Research Biochemicals International, Natick, MA).

The gases N_2O and O_2 were delivered into the light/dark box via a length of polyethylene tubing using a portable N_2O/O_2 dental sedation system (Porter, Hatfield, PA). The proportions of N_2O and O_2 were varied within a total inflow rate of 10 l/min to achieve the different test concentrations of N_2O (i.e., 2.5 l/min N_2O + 7.5 l/min O_2 = 25% N_2O; 5.0 l/min N_2O + 5.0 l/min O_2 = 50% N_2O; 7.0 l/min N_2O + 3.0 l/min O_2 = 70% N_2O). A POET II anesthetic monitoring system (Criticare, Milwaukee, WI) was used to ascertain that desired N_2O/O_2 atmospheres had been attained within the filling time.

CP was prepared in 0.9% physiological saline and administered intraperitoneally at doses of 3, 10 or 30 mg/kg 30 min prior to testing. FLU was suspended in 0.3% Tween 80 and administered subcutaneously at a dose of 10 mg/kg 30 min prior to testing. SR-95531 was prepared in 0.9% physiological saline and administered in an intracerebroventricular dose of 1.0 ng/mouse 30 min prior to testing. 7-NI was suspended in peanut oil (Nabisco, East Hanover, NJ) and administered subcutaneously at a dose of 50 mg/kg 30 min prior to testing. CP, FLU and 7-NI were delivered in injection volumes of 0.1 ml/10 g body weight. SR-95531 was delivered in an intracerebroventricular injection volume of 4 μl/mouse. Whenever a group of animals was injected with pretreatment drug, other groups received the same volume of vehicle (VEH). Pretreatment drug doses were determined in preliminary experiments or taken from the scientific literature. The dose, pretreatment time and selection of peanut oil as the VEH for the 7-NI were based on the literature (Przedborski et al., 1996).

2.4. Intracerebroventricular injection

SR-95531 was delivered directly into the lateral cerebral ventricle of mice that were lightly anesthetized with halothane, USP (Halocarbon Laboratories, River Edge,

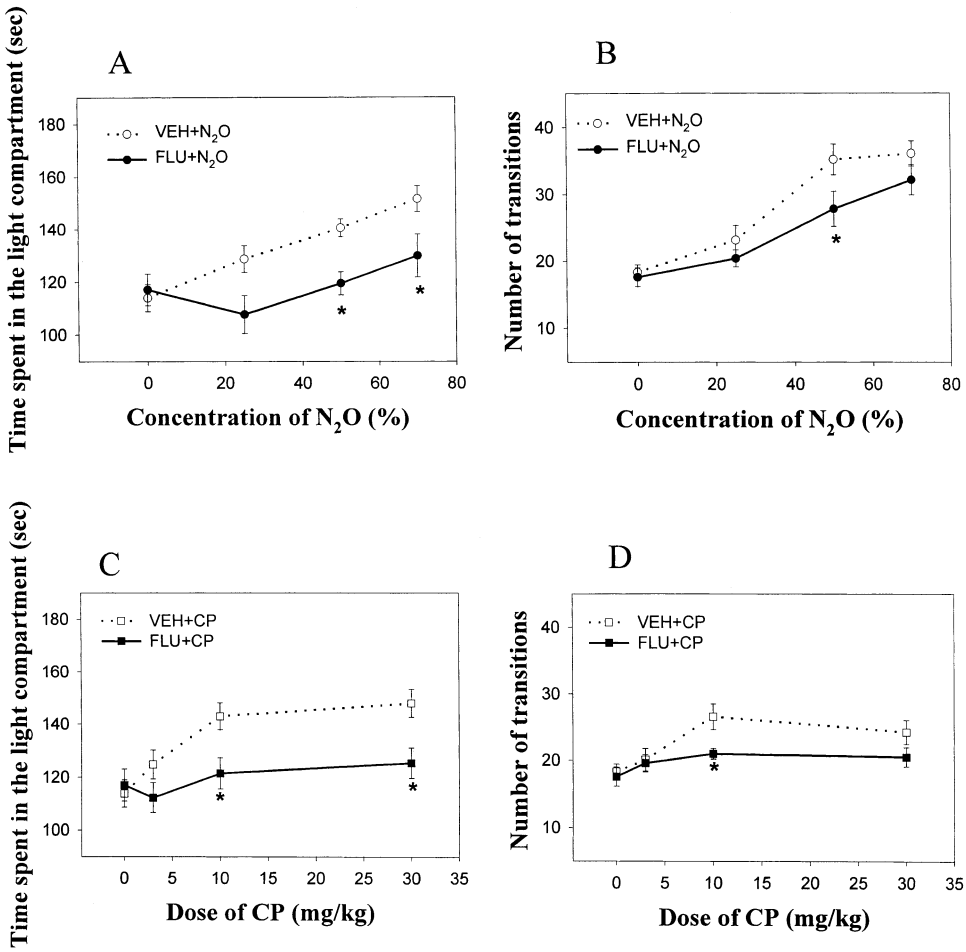

Fig. 2. Influence of BZ receptor blockade on behavioral effects of N_2O (Frames A and B) and CP (Frames C and D). The data are expressed as the mean ± S.E.M. of 12 mice per group. Significance of difference: * $P < .05$, compared with the VEH + N_2O or CP control (one-way ANOVA followed by a post-hoc Newman–Keuls test).

NJ) anesthetized mice, using a modification of the method of Haley and McCormick (1957). An incision was made in the scalp with a sharp scalpel, and the skin was pulled back to expose the calvarium. The injection was made using a microsyringe (Hamilton, Reno, NV) at a point on the calvarium 1 mm lateral and 2 mm caudal to bregma to a depth of 2.5 mm from the skull surface. The microinjection volume was 4.0 μl.

2.5. Statistical analysis of the data

The results are shown as mean ± S.E.M. Differences between mean were analyzed by one-way ANOVA and a post-hoc Dunnett's test. Differences among mean were analyzed using one-way ANOVA with the different treatments as the independent factor. When ANOVA showed significant differences, pairwise comparisons between means were tested by a post-hoc Newman–Keuls test. In all analyses, the null hypothesis was rejected at the .05 level.

3. Results

3.1. Behavioral effects of N_2O and CP in the light/dark exploration test

N_2O and CP evoked similar dose-related behavioral effects in the light/dark exploration test (Fig. 1A–D). One-way ANOVA revealed that treatment with N_2O (50% and 70%) and CP (10 and 30 mg/kg) significantly elevated the time spent in the light compartment ($P<.001$ for both N_2O and CP treatment) and the number of transitions in the light/dark exploration test ($P<.001$ for both N_2O and CP treatment). The only variance was that mice treated with 30 mg/kg CP exhibited locomotion and transition activity somewhat lower than that produced by 10 mg/kg (Fig. 1D).

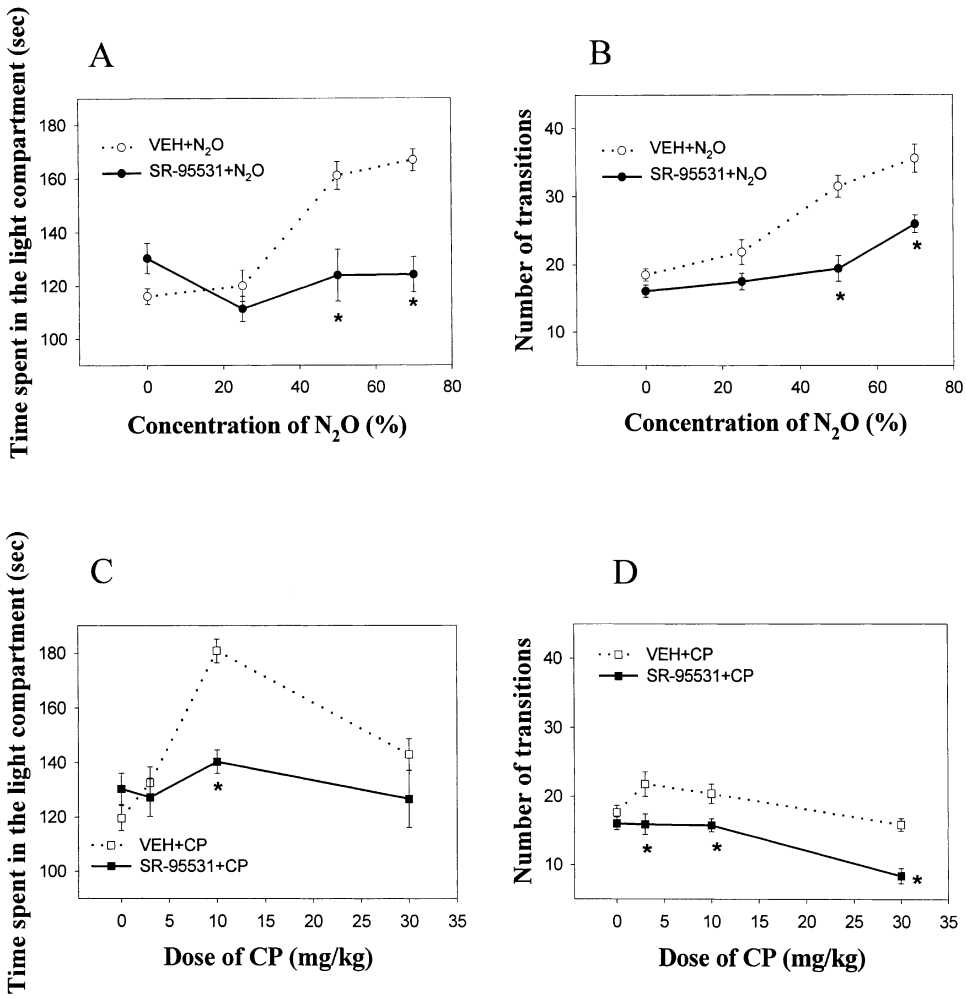

Fig. 3. Influence of $GABA_A$ receptor blockade on behavioral effects of N_2O (Frames A and B) and CP (Frames C and D). The data are expressed as the mean ± S.E.M. of 12–15 mice (Frames A and B) and 15 mice (Frames C and D). Significance of difference: * $P<.05$, compared with the VEH + N_2O or CP control (one-way ANOVA followed by a post-hoc Newman–Keuls test).

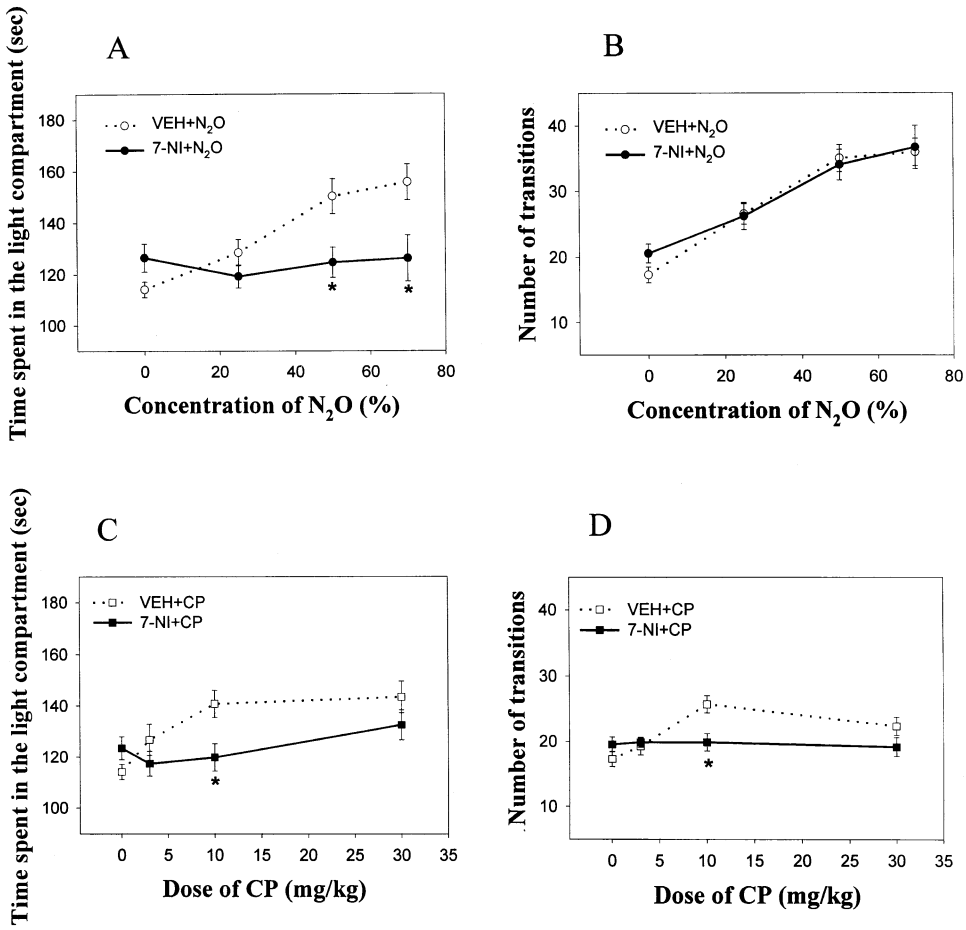

Fig. 4. Influence of nNOS inhibition on behavioral effects of N_2O (Frames A and B) and CP (Frames C and D). The data are expressed as the mean ± S.E.M. of 12–15 mice (Frames A and B) and 15–20 mice (Frames C and D). Significance of difference: * $P < .05$, compared with the VEH + N_2O or CP control (one-way ANOVA followed by a post-hoc Newman–Keuls test).

3.2. Influence of BZ receptor blockade on behavioral effects of N_2O and CP

As shown in Fig. 2A–D, subcutaneous pretreatment with the BZ receptor antagonist FLU (10 mg/kg) significantly antagonized the effects of N_2O and CP, as reflected by reduction of the drug-induced increases both in time spent in the light compartment and number of transitions between light and dark compartments. Treatment with FLU alone had no effect on VEH-treated mice.

3.3. Influence of $GABA_A$ receptor blockade on behavioral effects of N_2O and CP

Fig. 3A–D show that intracerebroventricular pretreatment with the $GABA_A$ receptor antagonist SR-95531 (1.0 ng/mouse) significantly reduced the N_2O- and CP-induced increases in the time spent in the light compartment and number of light/dark intercompartmental transitions. The already reduced transitions induced by CP at 30 mg/kg were further reduced by SR-95331 (Fig. 3D). Pretreatment with SR-95531 alone had no effect on VEH-treated mice.

3.4. Influence of nNOS inhibition on behavioral effects of N_2O and CP

As shown in Fig. 4, subcutaneous pretreatment with the selective nNOS inhibitor 7-NI (50 mg/kg) antagonized N_2O- and CP-induced increases in the time spent in the light compartment but not the number of transitions between compartments. 7-NI significantly reduced CP-induced transitions only at the 10 mg/kg dose but had no effect on N_2O-induced transitions at any concentration. 7-NI alone did not elicit any appreciable effect in the light/dark exploration test.

4. Discussion

Previous research has determined that N_2O evokes behavioral effects in mice that are strikingly similar to those produced by BZs (Czech and Quock, 1993; Emmanouil et al., 1994; Quock et al., 1987, 1992, 1993). It is now recognized that the pharmacological effects of BZs originate from facilitation of transmission at inhibitory GABAergic synapses (Zorumsky and Isenberg, 1991). Furthermore,

preliminary studies from our laboratory indicate similar behavioral effects evoked by BZ and GABA agonists in the mouse-elevated plus-maze (Elfline et al., 1998). The current research was intended to further compare the effects of BZs and N_2O following blockade of $GABA_A$ and BZ receptor. Another objective was to determine the effects of BZs and N_2O in the presence of a selective inhibition of nNOS. To confirm the universality of the role of NO in mediating behavioral effects of N_2O, the current research was conducted in a different experimental paradigm than earlier studies.

The light/dark exploration test is based on rodents' natural aversion to bright light and wide-open spaces (Crawley, 1981, 1985; Crawley and Goodwin, 1980). With some limitations — i.e., strain sensitivity and inability to detect anxiogenic activity (Crawley, 1985; Kilfoil et al., 1989) — this test has been validated pharmacologically, behaviorally and physiologically as a model of experimental anxiety. According to this paradigm, rodents typically spend more time in the smaller darkened compartment and avoid, for the most part, the larger, brightly illuminated area. Anxiolytic drugs will produce a dose-related elevation in the amount of time spent in the light compartment and also increase the number of transitions between the light and dark compartments. On the other hand, anxiogenic drugs (i.e., BZ receptor inverse agonists) increase the amount of time spent in the dark compartment but fail to increase the number of light/dark transitions (Crawley, 1985; Kilfoil et al., 1989).

When challenged with increasing doses of CP, mice exhibited significant dose-dependent increases in the time spent in the light compartment as well as the number of intercompartmental transitions. The increase in transitions induced by 30 mg/kg CP was lower than that produced by 10 mg/kg. Previous unpublished observations from our laboratory show the effects of CP to produce dose-related effects in the shape of an inverted U, suggesting increased sedation in response to high doses of CP. Comparable to administration of BZs, exposure to N_2O (50% or 70% by inhalation) exhibited a similar behavioral effect, which also significantly increased the time spent in the light compartment and in the number of intercompartmental transitions by a dose-dependent manner. This is consistent with the observations of Czech and Green (1992), who exposed mice to 25–75% N_2O, but not that of Fung et al. (1993), who used extremely low-level exposure to N_2O (i.e., 1000–2000 ppm, which corresponds to 0.1–0.2%).

The twofold increase in the number of transitions under N_2O is consistent with earlier reports that N_2O lacks sedative and anesthetic properties in mice and, in fact, produces opioid receptor-mediated locomotor stimulation (Dorris and Truong, 1993; Hynes and Berkowitz, 1979). On the other hand, one earlier study suggested that N_2O exposure reduces locomotion (Fung et al., 1993); however, concentrations used in this investigation were extremely low, i.e., 1000–2000 ppm (0.05–0.1%).

Both N_2O- and CP-induced increases in time spent in the light compartment and in transitions were reduced by pretreatment with FLU, which lends support to the hypothesis that N_2O-induced behavioral effects are mediated by BZ receptors. This is in agreement with previous observations of antagonism of N_2O-induced behavioral effects by BZ receptor blockade (Czech and Quock, 1993; Emmanouil et al., 1994; Quock et al., 1992, 1993).

The $GABA_A$ receptor antagonist SR-95531 was also effective in attenuating the effects of N_2O and CP, which implicates $GABA_A$ receptors in the behavioral effects of N_2O and CP. Inhibition of CP by SR-95531 was also observed in preliminary studies in our laboratory (Elfline et al., 1998). This finding is consistent with the concept that the BZ drug-induced effects are mediated through inhibitory GABA neurotransmission (Zorumsky and Isenberg, 1991).

NOS inhibitors are useful tools in investigating the physiological or pharmacological significance of NO. Commonly used L-arginine-derived NOS inhibitors such as N^G-L-nitro arginine (L-NOARG), which was utilized by our laboratory in earlier research (Caton et al., 1994; Quock and Nguyen, 1992), can inhibit endothelial NOS as well as nNOS and may induce circulatory changes that may, in turn, affect central functions, including behavior. 7-NI has been reported to be a selective inhibitor of nNOS in vivo (Moore et al., 1993). Przedborski et al. (1996) also showed that administration of single subcutaneous injection of 7-NI suspended in the peanut oil produces a significant and comparable time-related inhibition of NOS activity in both the cerebellum and striatum. Maximum inhibition was observed 30 min after injection of 7-NI with doses of 25 and 50 mg/kg, reducing NOS activity by roughly 50% and 80%, respectively (Przedborski et al., 1996).

Our study shows that 30-min pretreatment with 7-NI significantly antagonized N_2O- and CP-induced increases in time spent in the light compartment. 7-NI also significantly reduced CP-induced increased transitions at one dose of CP but not higher, possibly because the CP-induced transitions were less at higher doses. On the other hand, 7-NI did not influence the N_2O-induced increase in light/dark transitions. This unilateral influence is reminiscent of BZ inverse agonists increasing time spent in the light compartment but not affecting the number of transitions (Crawley and Goodwin, 1980; Kilfoil et al., 1989). One contributing factor to this asymmetry of drug effect is the stimulation of locomotor stimulation by N_2O (Dorris and Truong, 1993; Hynes and Berkowitz, 1979), which possibly masks the expected reduction in number of transitions. Locomotor stimulation might also be caused by the 7-NI itself, as NOS inhibitors may be capable of inhibiting the enzyme monoamine oxidase (Desvignes et al., 1999), thus elevating dopamine, which has been demonstrated to be involved in N_2O-induced locomotor stimulation (Dorris and Truong, 1993; Hynes and Berkowitz, 1979).

In summary, the results of this study demonstrate that BZ receptor blockade, $GABA_A$ receptor blockade and inhibition

of nNOS enzyme activity all generally reduce the behavioral effects of N_2O and CP in the mouse light/dark exploration test. It is suggested that BZ and $GABA_A$ receptors and NO, specifically NO produced by nNOS, are all involved in mediating the behavioral effects of N_2O and CP.

It must be acknowledged, though, that the actual role of NO in anxiety is controversial and remains to be elucidated. While our laboratory has consistently demonstrated that inhibition of NO production appears to impair behavioral effects of N_2O and CP in models of experimental anxiety, other investigators working with NOS inhibitors alone have reported equivocal findings as to whether NO is involved in the genesis or alleviation of "anxious" behavior. Acute treatment of rats with L-NOARG (30–120 mg/kg) was found to reduce open-arm activity in the elevated plus-maze (Lino de Oliveira et al., 1997). Central microinjections of L-NOARG (50 nmol) or L-NAME (100 nmol) into the dorsal periaqueductal grey region of rats increased open-arm activity in the elevated plus-maze (Guimaraes et al., 1994); however, increasing the doses of these same drugs twofold significantly decreased open-arm activity (Guimaraes et al., 1994). Other studies have reported that treatment of rats with L-NAME increased open-arm activity in the elevated plus-maze in the dose range 1–10 mg/kg in one study (Volke et al., 1995) and 10–60 mg/kg in another (Faria et al., 1997). Subchronic 7-NI treatment increased open-arm activity in the rat elevated plus-maze (Dunn et al., 1998). 7-NI also produced an anxiolytic-like profile in the mouse light/dark compartment test and in the elevated plus-maze test (Volke et al., 1997), but the doses required were higher (80–120 mg/kg) than in rats. In addition, one study found that 25–50 mg/kg 7-NI did not elicit any behavioral abnormalities (Przedborski et al., 1996). The resolution of the precise role of NO in anxiety must await development of more selective NOS inhibitors or knockout/knockdown procedures for assessing the role of NOS in various brain functions.

Acknowledgments

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