N-Methyl-d-Aspartate Receptor Channel Blocker–Like Discriminative Stimulus Effects of Nitrous Oxide Gas

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ABSTRACT
Nitrous oxide (N\textsubscript{2}O) gas is a widely used anesthetic adjunct in dentistry and surgery that is also commonly abused. Studies have shown that N\textsubscript{2}O alters the function of the N-methyl-d-aspartate (NMDA), GABA\textsubscript{A}, opioid, and serotonin receptors among others. However, the receptors systems underlying the abuse-related central nervous system effects of N\textsubscript{2}O are unclear. The present study explores the receptor systems responsible for producing the discriminative stimulus effects of N\textsubscript{2}O. B6SJLF1/J male mice trained to discriminate 10 minutes of exposure to 60% N\textsubscript{2}O + 40% oxygen versus 100% oxygen served as subjects. Both the high-affinity NMDA receptor channel blocker (−)-MK-801 maleate [([SS,10R]−(−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] and the low-affinity blocker memantine partially mimicked the stimulus effects of N\textsubscript{2}O. Neither the competitive NMDA antagonist, CGS-19755 ([cis-4-[phosphomethyl]-piperidine-2-carboxylic acid], nor the NMDA glycine-site antagonist, L701-324 [7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolinoine], produced N\textsubscript{2}O-like stimulus effects. A range of GABA\textsubscript{A} agonists and positive modulators, including midazolam, pentobarbital, muscimol, and gaboxadol (4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-ol), all failed to produce N\textsubscript{2}O-like stimulus effects. The µ-, κ-, and δ-opioid agonists, as well as 5-hydroxytryptamine (serotonin) 1B/2C (5-HT\textsubscript{1B/2C}) and 5-HT\textsubscript{1A} agonists, also failed to produce N\textsubscript{2}O-like stimulus effects. Ethanol partially substituted for N\textsubscript{2}O. Both (+)-MK-801 and ethanol but not midazolam pretreatment also significantly enhanced the discriminative stimulus effects of N\textsubscript{2}O. Our results support the hypothesis that the discriminative stimulus effects of N\textsubscript{2}O are at least partially mediated by NMDA antagonist effects similar to those produced by channel blockers. However, as none of the drugs tested fully mimicked the stimulus effects of N\textsubscript{2}O, other mechanisms may also be involved.

Introduction
Nitrous oxide gas (N\textsubscript{2}O), a widely used anesthetic adjunct in dentistry and surgical anesthesia, is subject to widespread abuse (Garland et al., 2009), with as many as 88,000 people aged 12–17 years annually initiating nonmedical recreational use (http://oas.samhsa.gov/2k9/inhalantTrends/inhalantTrends.htm). The National Survey on Drug Use and Health estimated in 2005 that 21% of adolescent abusers first experience using an inhalant was with N\textsubscript{2}O (http://oas.samhsa.gov/2k9/inhalantTrends/inhalantTrends.htm). At the present time, the neurotransmitter system or systems responsible for the subjective intoxication produced by N\textsubscript{2}O are not well understood (Zacny et al., 1994; Beckman et al., 2006), which significantly hampers the development of interventions to treat and prevent N\textsubscript{2}O abuse.

In vitro and in vivo experiments have shown that N\textsubscript{2}O modulates the activity of a number of neurotransmitter receptors. A significant body of evidence implicates the N-methyl-d-aspartate (NMDA) receptor complex as an important mediator of N\textsubscript{2}O effects. N\textsubscript{2}O inhibits human NR1A and NR2A NMDA receptor subunits in Xenopus oocytes (Yamakura and Harris, 2000; Ogata et al., 2006). The locomotor incoordinating effects of N\textsubscript{2}O are reduced in Caeorhabditis elegans, with a nmr-1 gene loss-of-function mutation encoding a NMDA-type glutamate receptor (Nagele et al., 2004). NMDA receptors in rat hippocampal neurons are inhibited in a noncompetitive and voltage-dependent manner by N\textsubscript{2}O (Jevtović-Todorić et al., 1998; Mennerick et al., 1998), and NMDA-evoked striatal dopamine release is reduced by N\textsubscript{2}O (Balon et al., 2003). Finally, N\textsubscript{2}O alters isoflurane (Petrenko et al., 2010) and sevoflurane (Sato et al., 2005) minimal alveolar concentration in NMDA receptor epsilon-1 subunit gene knockout in mice.

Considerable evidence also implicates GABA\textsubscript{A} receptors as a mediator of N\textsubscript{2}O’s effects. N\textsubscript{2}O potentiates GABA\textsubscript{A} receptor current in the presence of an agonist, suggesting it may act as a positive allosteric modulator (Hapfelmeier et al., 2000). N\textsubscript{2}O exposure increases the current flow in GABA\textsubscript{A} α\textsubscript{2}β\textsubscript{2}γ\textsubscript{2L} Receptors expressed in Xenopus oocytes (Hapfelmeier et al., 2000; Yamakura and Harris, 2000). N\textsubscript{2}O also potentiates

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ABBREVIATIONS: ANOVA, analysis of variance; gaboxadol, 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-ol; CGS-19755, cis-4-[phosphomethyl]-piperidine-2-carboxylic acid; FR, fixed ratio; 5-HT, 5-hydroxytryptamine, serotonin; L-701,324, 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolinoine; mCPP, 1-(3-chlorophenyl)piperazine hydrochloride; (−)-MK-801 maleate, (SS,10R)-(−)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDA, N-methyl-d-aspartate; N\textsubscript{2}O, nitrous oxide; 8-OH DPAT, (−)-8-hydroxy-2-dipropylaminotetralin hydrobromide; SNC-80, (−)-4-[isoR]-α-(35S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethybenzamide; SR-95531, 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide; USO-488H, trans-(−)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride.
the effects of the GABA<sub>A</sub> agonist muscimol in cultured hippocampal neurons (Dzoljic and Van Duijn, 1998). In mice, the benzodiazepine site antagonist flumazenil and the GABA<sub>A</sub> competitive antagonist, benzamamide hydrobromide, reverse the anxiolytic-like effects of N<sub>2</sub>O (Czech and Green, 1992; Czech and Quock, 1993; Li and Quock, 2001). Finally, flumazenil reduces ratings of subjective “high” produced by N<sub>2</sub>O in human subjects (Zaeny et al., 1995).

Opioid receptors have been implicated as being involved in the analgesic and antinociceptive properties of N<sub>2</sub>O. The κ-opioid antagonist nor-binaltorphimine but not the δ-opioid antagonist naltrindole attenuates N<sub>2</sub>O analgesia (Koyama and Fukuda, 2010). A mixed agonist/antagonist at μ-opioid receptors, β-chronalortrexamine reverses N<sub>2</sub>O antinociceptive responses (Emmanouil et al., 2008). However, in humans naloxone does not alter N<sub>2</sub>O’s subjective or cognitive impairing effects nor N<sub>2</sub>O-induced changes in pain perception (Zaeny et al., 1994, 1999). Lastly, N<sub>2</sub>O results in release of serotonin in the rat spinal cord (Mukaida et al., 2007), and other data suggest that the anxiolytic and antinociceptive effects of N<sub>2</sub>O may involve a serotonergic mechanism (Mueller and Quock, 1992; Emmanouil et al., 2006).

Taken together these studies suggest that the pharmacologic properties of N<sub>2</sub>O are complex and that different receptor systems may be involved depending on the response that is being examined. It is presently unclear which, if any, of these receptor systems are responsible for the abuse-related subjective effects of N<sub>2</sub>O. We sought to address that question by use of the drug-discrimination procedure in mice. Drug discrimination models human subjective intoxication and is an extremely powerful behavioral research tool for examining the receptor mechanisms underlying abuse-related behavioral effects of drugs (Colpaert, 1999). We have previously demonstrated that 10 minutes of exposure to 60% N<sub>2</sub>O can be trained as a discriminative stimulus in mice (Richardson and Shelton, 2014). Our data showed that N<sub>2</sub>O shares discriminative stimulus effects with toluene, but there is little overlap between the discriminative stimulus effects of N<sub>2</sub>O and other abused inhalants and vapor anesthetics. In the present study, we examined the role of NMDA, GABA<sub>A</sub>, opioid, and serotonin receptors in transducing the discriminative stimulus effects of N<sub>2</sub>O.

Materials and Methods

Subjects. Twenty-four adult male B6SJLF1/J mice (The Jackson Laboratory, Bar Harbor, ME) served as subjects. These mice had previously been used in a study designed to determine whether it was possible to train a discrimination based on inhaled N<sub>2</sub>O (Richardson and Shelton 2014). The mice were maintained at 85% of their free-feed body weights by regulating food intake to 2–5 g of standard rodent chow per day (Harlan, Teklad, Madison, WI) after training. Water was available ad libitum except during experimental sessions. All subjects were individually housed in 31.5 cm × 19.5 cm clear polycarbonate cages with corn cob bedding (Teklad, Madison, WI) on a 12-hour light/dark cycle (lights on 6:00 AM) in a colony room maintained at 77°F with 44% humidity. Studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and conducted in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Apparatus. Exposures to oxygen and N<sub>2</sub>O/oxygen gas mixtures were conducted in one of four 26-l acrylic exposure cubicles that encased modified two-lever mouse operant conditioning chambers (model ENV-307AW; MED Associates, St. Albans, VT). Two yellow light-emitting diode stimulus lights, two response levers, and a liquid dipper were located on the front wall of each chamber. A single 5-watt light-emitting diode house light was located at the top center of the chamber rear wall. The drug-discrimination schedule conditions and data recordings were controlled by a MED Associates interface and MED-PC version 4 software (MED Associates). The milk solution used as a reinforcer consisted of 25% sugar, 25% nonfat powdered milk, and 50% tap water by volume.

N<sub>2</sub>O and oxygen exposure mixtures were controlled by a manually operated gas metering system. Briefly, an Airsep Oxyx<sup>®</sup> oxygen concentrator (Buffalo, NY) generated ≥98% oxygen. N<sub>2</sub>O gas was supplied by a compressed N<sub>2</sub>O cylinder and a single-stage regulator. The N<sub>2</sub>O and O<sub>2</sub> flow rates were regulated by individual rotameters, and the individual gas streams were combined before passing into the inhalant exposure chamber. System components were connected with Tygon tubing (Fisher Scientific, Hampton, NH). Waste gas was expelled into a fume hood.

Drugs. Medical grade N<sub>2</sub>O gas cylinders were obtained from National Welders Supply (Richmond, VA). Memantine, CGS-19755 (cis-4-phosphomethyl-piperidine-2-carboxylic acid), muscimol, U50,488H [trans-(±),3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride], 8-OH DPAT [(±)-8-hydroxy-2-dipropylaminothinolin hydrobromide], and mCPP [(1-3-chlorophenyl)piperazine hydrochloride] were purchased from Tocris Bioscience (St. Louis, MO). Pentobarbital, valprocic acid, gaboxadol (4,5,6,7-tetrahydrosioxazolo[4,5-c]pyridine-3-ol), and (3R)-MK-801 maleate [(S,S),10fR-5-10 dihydrol-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate] were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam HCl was purchased from the VCU hospital pharmacy. Ethanol (95% weight/volume) was obtained from Acros Organics (Fair Lawn, NJ). Morphine sulfate and L-701,324 [(-)-4-hydroxy-3-(3-phenoxypheryl)-2H-quinolinolone] were obtained from the National Institute on Drug Abuse drug supply program (Bethesda, MD). The SNC-80 [(–)-4-[[3a(R)]-5-(1-pyrrolidinyl)cyclohexyl][a]-quinolinoline] was generously provided by Kenner Rice at the Intramural Research Program of the National Institute on Drug Abuse (Bethesda, MD).

The vehicle for SNC-80 was 0.9% saline, pH adjusted to between 6.0 and 7.0. L-701,324 was solubilized in 10% Cremophor in sterile water. All other injected compounds were prepared in 0.9% sterile saline. All drugs except ethanol were prepared to achieve a constant injection volume of 10 ml/kg. To prevent tissue damage, ethanol doses higher than 1000 mg/kg were produced by increasing injection volumes of a 100-mg/ml ethanol solution. Morphine sulfate was administered subcutaneously. All other injected compounds were administered intraperitoneally. SNC-80, memantine, muscimol, gaboxadol, CGS 19755, L-701,324, and USF-488H were administered 30 minutes before testing. The mCPP was administered 20 minutes before testing. All other injected drugs were administered 10 minutes before testing. N<sub>2</sub>O exposures were begun 10 minutes before the start of and continued for the duration of the operant test session. All drug doses are expressed as their salt weight.

Training, Acquisition, and Substitution Test Procedure. Subjects were previously trained to discriminate a 10-minute exposure to 60% N<sub>2</sub>O + 40% O<sub>2</sub> mixture from 100% O<sub>2</sub> in once daily (Monday–Friday) milk-reinforced 5-minute operant sessions (Richardson and Shelton, 2014). In our present study, training sessions continued on Monday, Wednesday, and Thursday. Substitution test sessions were conducted each Tuesday and Friday. Briefly, the first 10 minutes of each training session was a time-out in which the animals were placed into the operant chambers and gas delivery was initiated. After 10 minutes of exposure, the house and lever lights were illuminated, and a 5-minute operant session commenced.

During the operant session, completion of a fixed-ratio 12 (FR12) response requirement on the active lever resulted in 3 seconds of access to a 0.01-ml milk dipper. Responding on the inactive lever reset the FR requirement for the correct lever. N<sub>2</sub>O and O<sub>2</sub> vehicle training sessions were presented in a double alternation sequence across training days. Subjects were eligible to test if they maintained
accurate stimulus control on training sessions between tests. Specifically, the subject must have emitted their first complete FR12 on the correct lever and a minimum 80% of total lever presses on the correct lever in all of the training sessions since the last test session. If an animal failed to maintain this level of performance, the double alternation training schedule was continued until the subject met the daily accuracy criteria for three consecutive days.

On test days, both levers were active, and completion of the FR12 requirement on either lever was reinforced. Generally, substitution concentration–effect or dose–effect curves were examined in ascending dose order and were preceded by 100% O₂ and 60% N₂O + 40% O₂ control test sessions. When the test drug was an injected compound, both the O₂ and N₂O control test exposures were preceded by vehicle injections. When possible, doses were increased until maximal substitution was apparent or a test condition resulted in a greater than 50% mean suppression of responding compared with the O₂ control.

Data Collection and Analysis. The dependent measures collected were the percentage of N₂O lever responding (± S.E.M.), the operant response rate (± S.E.M.), and the lever upon which the first fixed ratio was completed. Mean N₂O-lever appropriate responding of less than 20% was defined as no substitution, 21%–79% as partial substitution, and 80%–100% as full substitution. Suppression of operant responding produced by each drug was examined by one-way repeated measures analysis of variance (ANOVA) using Geisser-Greenhouse corrections for sphericity. Significant main effects were followed by Dunnett post hoc tests comparing each dose to its vehicle control. Statistical analysis examining percentage N₂O-lever selection

**Fig. 1.** Mean percentage N₂O lever responding (± S.E.M.) shown in the upper panel and operant response rates shown in the lower panel for 24 mice trained to discriminate 10 minutes of exposure to 60% N₂O + 40% oxygen from 100% oxygen. Points above O₂ and N₂O reflect the 100% oxygen and 60% N₂O + 40% oxygen control conditions. Filled symbols in the lower panel indicate statistically significant (P, 0.05) suppression of response rates relative to the oxygen control condition.

**Fig. 2.** Mean percentage N₂O lever responding (± S.E.M.) shown in the upper panel and operant response rates (± S.E.M.) shown in the lower panel produced by (+)-MK-801 (n = 8) [ ], memantine (n = 7) [ ], CGS-19755 (n = 8) [ ], and L-701,324 (n = 7) [ ] in mice trained to discriminate N₂O from oxygen. Points above O₂ and N₂O reflect the 100% oxygen and 60% N₂O + 40% oxygen control conditions. Numbers in brackets indicate the number of subjects that earned at least one reinforcer (first value) and the total number of subjects tested at that dose (second value). Filled symbols in the lower panel indicate statistically significant (P < 0.05) suppression of response rates relative to the oxygen control condition.
NMDA Channel Blocker–Like Stimulus Effects of Nitrous Oxide

**Results**

N₂O (n = 24) produced concentration-dependent full substitution for the 60% training concentration with an EC₅₀ of 33% (CL: 29%–37%) (Fig. 1, upper panel). Control tests of 100% O₂ and 60% N₂O + 40% O₂ produced a mean of 2% (±1) and 97% (±1) N₂O lever-selection, respectively. Full substitution was produced by both 60% and 80% N₂O. There was a main effect of N₂O concentration on operant responding [F(2.5, 56.5) = 15.0, P < 0.01] but only 80% N₂O (Fig. 1, lower panel, filled symbol) significantly (P < 0.05) attenuated operant responding below the O₂ control response rates.

The high-affinity NMDA receptor channel blocker (+)-MK-801 (n = 8) produced dose-dependent partial substitution for 60% N₂O (Fig. 2, upper panel, ◊) with an ED₅₀ of 0.39 mg/kg (CL: 0.20–0.77 mg/kg). Maximum mean N₂O-lever selection of 55% (±16) was produced by a dose of 0.75 mg/kg (+)-MK-801. In addition, (+)-MK-801 (Fig. 2, lower panel, ◊) attenuated operant responding with an ED₅₀ of 0.39 mg/kg (CL: 0.30–0.50 mg/kg). There was a main effect of (+)-MK-801 dose on operant responding [F(2.4, 16.4) = 30.6, P < 0.01] with suppression of responding (P < 0.05) at doses of 0.30–0.75 mg/kg (●).

The low-affinity NMDA receptor channel blocker memantine (n = 7) produced a maximum of 50% (±10) N₂O-lever responding at a dose of 56 mg/kg (Fig. 2, upper panel, □). Memantine (Fig. 2, lower panel) also dose dependently [F(2.3, 14) = 24.16, P < 0.01] attenuated operant responding with an ED₅₀ of 29.2 mg/kg (CL: 24.9–34.3 mg/kg). Operant responding relative to vehicle was significantly reduced (P < 0.05) by memantine doses of 30 and 56 mg/kg (■).

The competitive NMDA antagonist CGS-19755 (n = 8) failed to substitute for 60% N₂O (Fig. 2, upper panel, △). CGS-19755 (Fig. 2, lower panel, △) attenuated operant responding [F(2.5, 17.5) = 40.8, P < 0.01] with an ED₅₀ of 12.0 mg/kg (CL: 8.1–17.9 mg/kg). Post hoc analysis indicated that responding was suppressed at doses of 10 and 17 mg/kg (P < 0.05, △). The NMDA receptor glycine site antagonist L-701,324 (n = 7) also failed to substitute for N₂O, producing no greater than 1% N₂O-lever selection at any dose (Fig. 2, upper panel, ◊). L-701,324 (Fig. 2, lower panel, ◊) failed to attenuate operant responding [F(2.5, 17.5) < 1, P = 0.44] up to the maximum dose tested of 30 mg/kg.

Figure 3 shows the substitution concentration–effect curves (upper panel) and response rate effects (lower panel) produced by increasing concentrations of N₂O after pretreatment with vehicle, or 0.03 or 0.17 mg/kg (+)-MK-801 (n = 7). The EC₅₀ of N₂O + vehicle (Fig. 3, ○) was 32% (CL: 25%–41%). Pretreatment with 0.03 mg/kg (+)-MK-801 (□) resulted in a N₂O EC₅₀ of 26% (CL: 17%–39%). Pretreatment with 0.17 mg/kg (+)-MK-801 (△) produced 33% (CL: 29%–37%). Pretreatment with 0.17 mg/kg (+)-MK-801 (□) produced a maximum of 50% (CL: 24.9–34.3 mg/kg). Operant responding relative to vehicle was significantly reduced (P < 0.05) with an ED₅₀ of 12.0 mg/kg (+)-MK-801 (□). Point above O₂ and N₂O reflect the 100% oxygen and 60% N₂O + 40% oxygen control conditions. Filled symbols in the upper and lower panels indicate statistically significant (P < 0.05) differences from the corresponding N₂O + vehicle control values.

![Fig. 3](image-url)
(+)-MK-801 (Δ) produced a more pronounced 1.9-fold leftward shift in the N\textsubscript{2}O lever-selection curve, further reducing the EC\textsubscript{50} of N\textsubscript{2}O to 17\% (CL: 13\%–23\%). There was no statistically significant main effect of 0.03 mg/kg (+)-MK-801 treatment [F(1,6) = 2.2, P = 0.19], nor was there an interaction between 0.03 mg/kg (+)-MK-801 treatment and N\textsubscript{2}O exposure concentration [F(4,24) = 1.2, P = 0.33] on the percentage of N\textsubscript{2}O lever selection. There was a main effect of 0.17 mg/kg (+)-MK-801 treatment [F(1,4) = 7.9, P = 0.03] as well as an interaction between the 0.17 mg/kg (+)-MK-801 treatment dose and N\textsubscript{2}O exposure concentration [F(3,18) = 4.9, P = 0.01] on the percentage of N\textsubscript{2}O lever selection. Post hoc tests revealed that pretreatment with 0.17 mg/kg (+)-MK-801 enhanced (t = 4.6, P < 0.05) the discriminative stimulus effects of 20\% N\textsubscript{2}O over that produced by N\textsubscript{2}O alone (upper panel, filled symbol). There was also both a main effect [F(3,12) = 13.6, P < 0.01] of (+)-MK-801 pretreatment dose as well as an interaction [F(8,48) = 6.5, P < 0.01] of the (+)-MK-801 pretreatment dose and N\textsubscript{2}O concentration on operant response rates. Post hoc tests revealed that 40\% + 0.17 mg/kg (+)-MK-801 (t = 4.9) and 60\% N\textsubscript{2}O + 0.17 mg/kg (+)-MK-801 (t = 9.8) resulted in a greater (P < 0.05) operant response rate suppression than 40\% or 60\% N\textsubscript{2}O alone (lower panel, filled symbols).

Table 1 shows the results of substitution testing with GABA\textsubscript{A} receptor agonists and positive modulators. Gaboxadol (n = 8), a GABA\textsubscript{A} receptor agonist selective for δ-subunit–containing extrasynaptic receptors resulted in a maximum of 4\% ±3 N\textsubscript{2}O-lever responding at a dose of 1.0 mg/kg. Gaboxadol attenuated operant responding [F(2,4,17.1) = 105, P < 0.01] with an ED\textsubscript{50} of 6.4 mg/kg (CL: 2.6–15.7 mg/kg). The synaptic GABA\textsubscript{A} receptor agonist muscimol (n = 8) produced a maximum of 22\% ±22 N\textsubscript{2}O-lever responding at a dose of 1.7 mg/kg. Muscimol dose-dependently attenuated operant response rates [F(1,5,10.4) = 32.4, P < 0.01] with an ED\textsubscript{50} of 1.2 mg/kg (CL: 0.9–1.6 mg/kg).

The anticonvulsant valproic acid, which inhibits GABA transaminase, produced a maximum of 33\% ±15 N\textsubscript{2}O-lever responding. Valproic acid (n = 8) dose-dependently suppressed operant responding [F(2,14) = 27.6, P < 0.01] with an ED\textsubscript{50} of 430 mg/kg (CL: 384–481 mg/kg). The GABA\textsubscript{A} receptor benzodiazepine-site positive allosteric modulator midazolam (n = 9) produced a maximum of 27\% ±7 N\textsubscript{2}O-lever responding. Midazolam dose-dependently attenuated operant responding [F(3,2,25.8) = 26.4, P < 0.01] with an ED\textsubscript{50} of 10.5 mg/kg (CL: 3.2–34.8 mg/kg). The GABA\textsubscript{A} receptor barbiturate-site positive allosteric modulator pentobarbital (n = 8) produced a maximum of 10\% ±3 N\textsubscript{2}O-lever responding. Pentobarbital dose-dependently suppressed operant responding [F(2,9,20.2) = 26.2, P < 0.01] with an ED\textsubscript{50} of 28.9 mg/kg (CL: 17–49 mg/kg).

Figure 4 shows the results of pretreatment with vehicle, or 0.3 or 3 mg/kg i.p. midazolam before exposure to increasing concentrations of N\textsubscript{2}O (n = 8). The upper panel depicts N\textsubscript{2}O-lever selection and the lower panel the operant response rates. Vehicle administration before N\textsubscript{2}O exposure produced

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**TABLE 1**

<table>
<thead>
<tr>
<th>Test Drug</th>
<th>Drug Dose</th>
<th>Percentage of N\textsubscript{2}O Lever Responding (± S.E.M.)</th>
<th>Response Rate in Responses per Second (± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaboxadol (n = 8)</td>
<td>O\textsubscript{2} + vehicle</td>
<td>0.0 (0.0)</td>
<td>1.4 (0.1)</td>
</tr>
<tr>
<td>N\textsubscript{2}O + vehicle</td>
<td>99.4 (0.3)</td>
<td>1.2 (0.1)</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>3.9 (2.3)</td>
<td>1.1 (0.5)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.6 (1.2)</td>
<td>1.0 (0.4)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>1.0 (0.0)*</td>
<td></td>
</tr>
<tr>
<td>Muscimol (n = 8)</td>
<td>O\textsubscript{2} + vehicle</td>
<td>0.6 (0.6)</td>
<td>1.6 (0.1)</td>
</tr>
<tr>
<td>N\textsubscript{2}O + vehicle</td>
<td>96.4 (2.2)</td>
<td>1.2 (0.2)</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>1.0 (0.9)</td>
<td>1.1 (0.8)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.3 (1.1)</td>
<td>1.0 (0.1)</td>
<td></td>
</tr>
<tr>
<td>1.7</td>
<td>21.8 (21.8)</td>
<td>0.6 (0.3)*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>0.0 (0.0)*</td>
<td></td>
</tr>
<tr>
<td>Valproic acid (n = 8)</td>
<td>O\textsubscript{2} + vehicle</td>
<td>1.5 (1.5)</td>
<td>1.5 (0.1)</td>
</tr>
<tr>
<td>N\textsubscript{2}O + vehicle</td>
<td>95.8 (2.3)</td>
<td>1.2 (0.2)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.0 (1.0)</td>
<td>1.0 (0.1)</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>2.3 (2.3)</td>
<td>1.3 (0.1)</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>33.0 (14.8)</td>
<td>0.4 (0.2)*</td>
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</tr>
<tr>
<td>Midazolam (n = 9)</td>
<td>O\textsubscript{2} + vehicle</td>
<td>2.1 (1.7)</td>
<td>1.4 (0.1)</td>
</tr>
<tr>
<td>N\textsubscript{2}O + vehicle</td>
<td>98.4 (0.7)</td>
<td>1.3 (0.1)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.9 (1.6)</td>
<td>1.2 (0.1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.4 (3.5)</td>
<td>1.3 (0.1)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>11.3 (4.8)</td>
<td>0.6 (0.1)*</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>19.7 (8.7)</td>
<td>0.5 (0.1)*</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>16.2 (9.1)</td>
<td>0.5 (0.1)*</td>
<td></td>
</tr>
<tr>
<td>Pentobarbital (n = 8)</td>
<td>O\textsubscript{2} + vehicle</td>
<td>1.8 (1.5)</td>
<td>1.5 (0.1)</td>
</tr>
<tr>
<td>N\textsubscript{2}O + vehicle</td>
<td>95.8 (2.0)</td>
<td>1.1 (0.2)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.3 (6.5)</td>
<td>1.4 (0.2)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.0 (1.0)</td>
<td>1.6 (0.1)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1.8 (1.2)</td>
<td>1.5 (0.1)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>9.5 (3.4)</td>
<td>1.0 (0.1)*</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>—</td>
<td>0.2 (0.1)*</td>
<td></td>
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</table>

*Statistically significant (P < 0.05) difference in response rates compared with the oxygen + vehicle control condition.
a N2O-lever selection EC50 of 25% (CL: 14%–44%). N2O combined with 0.3 mg/kg or 3 mg/kg midazolam produced N2O-lever selection EC50’s of 35% (CL: 25%–47%) and 44% (CL: 35%–56%), respectively. There was no main effect for F(1,7) = 1.6, P = 0.25 or interaction for F(4,28) < 1, P = 0.81 of 0.3 mg/kg midazolam pretreatment on N2O-lever selection.

Analysis of variance was not performed on lever selection data generated when combining 3 mg/kg midazolam + N2O because of missing data resulting from complete suppression of responding in some subjects in the higher dose-combination conditions. There was both a main effect of midazolam pretreatment dose [F(2,14) = 6.6, P < 0.01] as well as an interaction of midazolam pretreatment dose and N2O concentration [F(8,56) = 4.5, P < 0.01] on operant rate suppression (Fig. 4, lower panel). Post hoc analysis showed that 3 mg/kg of midazolam enhanced (P < 0.05) the response-rate suppression produced by concentrations of 20%–60% N2O (▲).

Table 2 shows the results of substitution testing with opioid receptor agonists, ethanol, and selected serotonergic agonists. The µ-opioid receptor agonist morphine (n = 8) produced a maximum of 33% (±33) N2O-lever responding at the highest test dose of 30 mg/kg. Morphine dose-dependently attenuated operant responding [F(2.5,17.9) = 39.13, P < 0.01] with an ED50 of 7.9 mg/kg (CL: 3.9–16.2 mg/kg). The κ-opioid receptor agonist U50-488H (n = 8) produced a maximum of 11% (±11) N2O-lever responding at a dose of 7 mg/kg. U50-488H dose-dependently reduced operant response rates [F(1,4,9,7) = 32.4, P < 0.01] with an ED50 of 3.3 mg/kg (CL: 2.7–4.1 mg/kg). The δ opioid receptor agonist SNC-80 (n = 8) produced a maximum of 10% N2O-lever responding. SNC-80 dose-dependently attenuated operant responding [F(2.3,16.0) = 13.7, P < 0.01] with an ED50 of 28.6 mg/kg (CL: 16.8–48.6 mg/kg).

Ethanol (n = 9) elicited a maximum of 55% (±13) N2O-lever responding at the highest test dose of 2500 mg/kg. The substitution ED50 of ethanol for N2O was 2238 mg/kg (CL: 1397–3587 mg/kg). Ethanol dose-dependently attenuated operant response rates [F(1,8,14) = 34.7, P< 0.01] with an ED50 of 2109 mg/kg (CL: 1909–2332 mg/kg).

The 5-hydroxytryptamine (serotonin) 1B/2C (5-HT1B/2C) receptor agonist mCPP produced a maximum of 21% (±17) N2O-lever responding at a dose of 10 mg/kg. Also, mCPP (n = 8) dose-dependently attenuated operant responding with an ED50 of 3.7 mg/kg (CL 2.2–6.5 mg/kg) and suppressed operant responding at doses of 5.6 and 10 mg/kg [F(2,1,5.4) = 25.5, P < 0.01]. The 5-HT1A agonist 8-OH DPAT (n = 8) produced no greater than 4% N2O-lever selection at any dose; 8-OH DPAT dose-dependently attenuated operant responding with an ED50 of 0.5 mg/kg (CL: 0.38–0.71 mg/kg) and suppressed operant responding [F(2,3,15.8) = 31.4, P < 0.01] at doses of 0.3–1.56 mg/kg.

Figure 5 shows the effect of pretreatment with either 500 or 1500 mg/kg i.p. ethanol before exposure to increasing concentrations of N2O. The upper panel depicts N2O-lever selection and the lower panel the operant response rates. Vehicle pretreatment before N2O exposure (Fig. 5, upper panel, ○) resulted in a N2O-lever selection EC50 of 31% (CL: 27%–36%). Pretreatment with a low dose of 500 mg/kg ethanol (Fig. 5, upper panel, □) resulted in a N2O-lever selection EC50 of 27% (CL: 23%–32%). Pretreatment with a higher dose of 1500 mg/kg ethanol (Fig. 5, upper panel, △) produced a 2.8-fold leftward shift in the N2O substitution concentration effect curve and a N2O-lever selection EC50 of 11% (CL: 7%–18%).

There was no main effect for F(1,7) = 1.5, P = 0.3 or interaction for F(4,28) < 1, P = 0.51 between the 500 mg/kg ethanol pretreatment dose and N2O concentration on the percentage N2O-lever selection. However, there was a main effect for F(1,7) = 19.8, P < 0.01 as well as an interaction for F(3,21) = 3.9, P = 0.02 between the 1500 mg/kg ethanol pretreatment dose and N2O concentration on the percentage of N2O-lever selection. Post hoc analysis revealed that pretreatment with 1500 mg/kg ethanol enhanced (t = 5.6, P < 0.05) the discriminative stimulus effects of 20% N2O (Fig. 5, upper panel, △).
was a main effect of ethanol pretreatment dose \(F(2,14) = 21.1, P < 0.01\) as well as an interaction \(F(8,56) = 4.1, P < 0.01\) of ethanol pretreatment dose and N2O concentration on rates of operant responding (Fig. 5, lower panel). Post hoc analysis revealed that 1500 mg/kg ethanol suppressed \((P, 0.05)\) operant responding at the 5% \((t = 3.9)\), 10% \((t = 2.7)\), 20%, \((t = 6.4)\), and 60% \((t = 9.4)\) N2O concentrations (Fig. 5, lower panel, ▲). Pretreatment with a lower dose of 500 mg/kg ethanol only enhanced \((t, 2.4, P, 0.05)\) the operant response rate suppressing effects of 60% N2O. (Fig. 5, lower panel, ●).

### Discussion

The overarching goal of the present study was to explore the receptor mechanisms underlying the discriminative stimulus effects of N2O. Given the strong in vitro evidence that N2O attenuates NMDA receptor function (Jevtović-Todorović et al., 1998; Mennerick et al., 1998; Yamakura and Harris, 2000; Balon et al., 2003; Nagele et al., 2004; Sato et al., 2005; Ogata et al., 2010), a number of site-selective NMDA antagonists were tested for their ability to substitute for N2O (Fig. 2). Neither the competitive NMDA antagonist CGS-19755 nor the NMDA receptor glycine site antagonist L-710,324 produced appreciable substitution for N2O. However, doses up to 30 mg/kg of L-701,324 also failed to suppress operant responding. It is therefore possible that an adequate dose range of L-701,324 was not tested, but this seems unlikely given a report showing that 10 mg/kg of L-701,324 has behavioral effects in other rodent discrimination procedures (Nicholson and Balster, 2009) and doses lower than 10 mg/kg have other behavioral effects in rodents (Poleszak et al., 2011; Wlaz and Poleszak, 2011). In contrast, the high-affinity NMDA receptor channel blocker \((-\text{MK}-801)\) produced 55% N2O-lever responding, suggesting a possible channel blocker-like effect of N2O.

To systematically replicate this finding, we also tested the low-affinity NMDA receptor channel blocker memantine, which produced a comparable level of 50% N2O-lever responding, suggesting a possible channel blocker-like effect of N2O.
glutamatergic neurotransmission in long-term potentiation (Manahan-Vaughan et al., 2008), which can interrupt memory recall (Florian and Roullet, 2004). A disruption of stimulus control could potentially result in levels of N2O-lever selection in the range of 50%, which is that which would be expected if the animals were responding randomly on both levers. However, (+)-MK-801 (Sanger and Zivkovic, 1989; Shelton and Balster, 2004) and other channel blockers (Bowen et al., 1999; Beardsley et al., 2002; Nicholson and Shelton and Balster, 2004) are easily trained in drug-discrimination procedures. Further, if a simple disruption of performance were responsible for the present data, one might have also expected partial substitution with the competitive NMDA antagonist CGS-19755 rather than a complete failure of GCS-19755 to substitute for N2O.

A more plausible alternative explanation for the partial substitution produced by (+)-MK-801 and memantine is insufficient specificity of the drug-discrimination assay. This hypothesis is based on data showing that under some conditions NMDA channel blockers will generate intermediate levels of substitution in rodents trained to discriminate stimulants, central nervous system depressants, and serotonergics (Koeck et al., 1995). The converse is also true in that benzodiazepines have been demonstrated to produce partial substitution in mice trained to discriminate (+)-MK-801 from vehicle (Shelton and Balster, 2004). To address whether the partial substitution produced by (+)-MK-801 was due to nonspecific effects, we examined whether pretreatment with (+)-MK-801 at doses that produced little or no substitution for N2O when administered alone would alter the discriminative stimulus properties of N2O (Fig. 3). Our hypothesis was that low doses of (+)-MK-801 would only enhance the stimulus effects of N2O if they were acting through a similar mechanism. Indeed, if (+)-MK-801 simply disordered behavior, it might be expected to produce a net antagonism of N2O’s stimulus effects at the higher N2O test concentrations. The results showing that (+)-MK-801 produced an orderly and significant enhancement of the discriminative stimulus effects of N2O support our hypothesis that the discriminative stimulus effects of N2O has a NMDA channel blocker-like component.

Because NMDA antagonists produced incomplete substitution for the training condition, it seems likely that another mechanism also contributes to the stimulus effects of N2O. To examine a potential GABAergic contribution to the stimulus effects of N2O, five site-selective GABA-positive drugs were tested for their ability to substitute for N2O (Table 1). Of the potential GABAergic mechanisms that might have played a role in the stimulus effects of N2O, positive allosteric modulation was most strongly implicated in the literature (Quock et al., 1992; Zacny et al., 1995; Hapfelmeier et al., 2000). However, neither the classic benzodiazepine-site positive allosteric modulator midazolam nor the barbiturate pentobarbital produced meaningful levels of substitution for N2O. Further, midazolam pretreatment failed to significantly enhance the discriminative stimulus effects of N2O, instead producing a trend toward diminishing the discriminative stimulus effects of N2O (Fig. 4). Likewise, neither the extrasynaptic GABA A receptor agonist gaboxadol nor the synaptic GABA A agonist muscimol were N2O-like. Lastly, the relatively nonselective GABA transamine inhibitor valproic acid produced a low level of partial substitution for N2O, but only at doses that completely suppressed operant responding in four of eight test subjects.

Opioid receptors have been suggested to be involved in the analgesic and antinoceceptive effects of N2O (Emmanouil et al., 2008). However, µ-, κ-, and δ-opioid receptors agonists all failed to produce greater than vehicle appropriate responding in N2O-trained mice (Table 2). The poor substitution produced by µ-opioid agonist morphine is consistent with data showing that the opioid antagonist naloxone does not attenuate the subjective effects of 30% N2O in humans (Zacny et al., 1994, 1999). Likewise, the failure of the δ-opioid agonist SNC-80 to substitute for N2O is consistent with reports that the δ-opioid receptor agonist naltrindole does not attenuate N2O analgesia (Koyama and Fukuda, 2010). Our data showing that the selective κ-opioid agonist U50-488H does not substitute for N2O...
is, however, in conflict with a previous study in which N₂O generalizes to the purported κ-opioid agonist ethylketocyclazocine in guinea pigs trained to discriminate ethylketocyclazocine from vehicle (Hynes and Hymson, 1984). More recent data have suggested that ethylketocyclazocine is a mixed μ-/κ-opioid agonist and that some of the discriminative stimulus effects of ethylketocyclazocine may result from μ-opioid receptor actions (Wessinger et al., 2011); however, this does not entirely reconcile the prior finding with our present data. It is possible that the differences between studies were the result of species or methods. Additional work will be necessary to resolve this conflict, but based on our present data it does not appear that N₂O has opioid-like discriminative stimulus properties under our training conditions.

A number of studies suggest a relationship between the behavioral effect of N₂O and ethanol. For instance, N₂O reduces 10% ethanol consumption in alcohol-prefering and heavy-drinking strains of rats (Kosobud et al., 2006). Although alcohol drinking before N₂O exposure does not appear to augment the subjective effects of N₂O (Walker and Zacny, 2001), N₂O is chosen more frequently by moderate alcohol drinkers than light drinkers (Zacny et al., 2008). Most recently, our laboratory reported that ethanol produces partial substitution in mice trained to discriminate N₂O from vehicle (Richardson and Shelton, 2014). In our present study, we systematically replicated and expanded our prior results by demonstrating not only that ethanol partially substitutes for N₂O but also that, like NMDA channel blockers, ethanol predominantly substitutes for the discriminative stimulus effects of N₂O (Fig. 5).

The discriminative stimulus effects of ethanol have been repeatedly demonstrated to be based upon a combination of GABAergic positive modulation, NMDA antagonism, and 5-HT₁B/₂C agonism (Grant et al., 1997), any one of which alone is sufficient to elicit ethanol-like stimulus effects. As we had already determined the degree of NMDA and GABAergic involvement in the stimulus effects of N₂O, we examined both the 5-HT₁B/₂C agonist mCPP, which has ethanol-like effects in drug discrimination, as well as the 5-HT₁A agonist 8-OH DPAT. Neither mCPP nor 8-OH-DPAT produced N₂O-like stimulus effects (Table 2). Overall, these findings suggest that the ethanol-like discriminative stimulus effects of N₂O are probably mediated exclusively through a common NMDA channel blocker—cue component (Grant and Colombo, 1993; Shelton and Grant, 2002; Vivian et al., 2002).

In summary, our results probe the most probable receptor mechanisms underlying the discriminative stimulus effects of N₂O. Of these mechanisms, the only class of drugs that engaged meaningful levels of N₂O-appropriate responding were NMDA receptor channel blockers. However, even the channel blockers failed to elicit full substitution, suggesting that other mechanisms are also involved in transducing the stimulus effects of N₂O. Several additional candidate mechanisms have been suggested by the literature, including 5-HT₃ antagonism (Yamakura and Harris, 2000; Suzuki et al., 2002), neuronal nicotinic acetylcholine inhibition (Yamakura and Harris, 2000; Suzuki et al., 2003), interactions with neuronal nitric oxide synthase enzymes (for a review, see Emmanouil and Quock, 2007), or TREK-1 potassium channel activation (Gruss et al., 2004). Additional research examining these mechanisms will be required to fully characterize the discriminative stimulus properties of N₂O.

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Authorship Contributions

Participated in research design: Richardson, Shelton.
Conducted experiments: Richardson.
Performed data analysis: Richardson, Shelton.
Wrote or contributed to the writing of the manuscript: Richardson, Shelton.

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