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# NMDA receptor channel blocker-like discriminative stimulus effects of nitrous oxide gas

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FR – fixed ratio

FFR – first fixed ratio

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## ABSTRACT

Nitrous oxide ($N_2O$) gas is a widely used anesthetic adjunct in dentistry and medicine that is also commonly abused. Studies have shown that $N_2O$ alters the function of the NMDA, $GABA_A$, opioid and serotonin receptors among others. However the receptors systems underlying the abuse-related CNS effects of $N_2O$ are unclear. The goal of this study was to explore the receptor systems responsible for producing the discriminative stimulus effects of $N_2O$. B6SJLF1/J male mice previously trained to discriminate 10 min of exposure to 60% $N_2O$+40% oxygen versus 100% oxygen in a two-lever food reinforced operant task served as subjects. Both the high affinity NMDA receptor channel blocker (+)-MK-801 and the low affinity blocker memantine partially mimicked the stimulus effects of $N_2O$. Neither the competitive NMDA antagonist, CGS-19755 nor the NMDA glycine-site antagonist, L701-324 produced $N_2O$-like stimulus effects. A range of $GABA_A$ agonists and positive modulators including midazolam, pentobarbital, muscimol and gaboxadol all failed to produce $N_2O$-like stimulus effects. Mu, kappa and delta opioid agonists as well as 5-$HT_{1B/2C}$ and 5-$HT_{1A}$ agonists also failed to produce $N_2O$-like stimulus effects. Ethanol partially substituted for $N_2O$. Both (+)-MK-801 and ethanol but not midazolam pretreatment also significantly enhanced the discriminative stimulus effects of $N_2O$. The present results support the hypothesis that the discriminative stimulus effects of $N_2O$ 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 $N_2O$, other mechanisms may also be involved.

## INTRODUCTION

Nitrous oxide gas ($N_2O$) is a widely used anesthetic adjunct in dentistry and surgical anesthesia. $N_2O$ is also subject to widespread abuse (Garland et al., 2009) with as many as 88,000 people aged 12 - 17 years old annually initiating nonmedical recreational use of $N_2O$ (Johnston et al., 2014, Office of Applied Studies, 2009). The National Survey on Drug Use and Health estimated in 2005 that 21% of adolescent inhalant abusers first experience using an inhalant was with $N_2O$ (Office of Applied Studies, 2009). At the present time the neurotransmitter system or systems responsible for the subjective intoxication produced by $N_2O$ are not well understood (Zacny et al., 1994, Beckman et al., 2006) which significantly hampers the development of interventions to treat and prevent $N_2O$ abuse.

*In vitro* and *in vivo* experiments have shown that $N_2O$ modulates the activity of a number of neurotransmitter receptors. A significant body of evidence implicates the NMDA receptor complex as an important mediator of $N_2O$'s effects. $N_2O$ inhibits human NR1A and NR2A NMDA receptor subunits in *Xenopus oocytes* (Ogata et al., 2006, Yamakura & Harris, 2000). The locomotor incoordinating effects of $N_2O$ are reduced in *C. 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 non-competitive and voltage-dependent manner by $N_2O$ (Jevtović-Todovorić et al., 1998, Mennerick et al., 1998) and NMDA-evoked striatal dopamine release is reduced by $N_2O$ (Balon et al., 2003). Finally, $N_2O$ alters isoflurane (Petrenko et al., 2010) and sevoflurane (Sato et al., 2005) minimal alveolar concentration in NMDA receptor epsilon-1 subunit gene knock out in mice.

Considerable evidence also implicates $GABA_A$ receptors as a mediator of $N_2O$'s effects. $N_2O$ potentiates $GABA_A$ receptor current in the presence of an agonist, suggesting it may act as a

positive allosteric modulator (Hapfelmeier et al., 2000). $N_2O$ exposure increases current flow in $GABA_A$ $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_2\gamma_{2L}$ receptors expressed in *Xenopus oocytes* (Hapfelmeier et al., 2000, Yamakura & Harris, 2000). $N_2O$ also potentiates the effects of the $GABA_A$ agonist muscimol in cultured hippocampal neurons (Dzoljic & Van Duijn, 1998). In mice the benzodiazepine site antagonist flumazenil and the $GABA_A$ competitive antagonist SR-95531 (Gabazine) reverse the anxiolytic-like effects of $N_2O$ (Czech & Quock, 1993, Czech & Green, 1992, Li & Quock, 2001). Finally, flumazenil reduces ratings of subjective "high" produced by $N_2O$ in human subjects (Zacny et al., 1995).

Opioid receptors have been implicated as being involved in the analgesic and antinociceptive properties of $N_2O$. The kappa opioid antagonist nor-binaltorphimine (nor-BNI) but not the delta opioid antagonist naltrindole attenuates $N_2O$ analgesia (Koyama & Fukuda, 2010). β-chlornaltrexamine, a mixed agonist/antagonist at mu opioid receptors, reverses $N_2O$ antinociceptive responses (Emmanouil et al., 2008). However, in humans naloxone does not alter $N_2O$'s subjective or cognitive impairing effects nor $N_2O$-induced changes in pain perception (Zacny et al., 1999, Zacny et al., 1994). Lastly, $N_2O$ results in release of serotonin in rat spinal cord (Mukaida, Shichino, & Fukuda, 2007) and other data suggest that the anxiolytic and antinociceptive effects of $N_2O$ may involve a serotonergic mechanism (Emmanouil, et al., 2006, Mueller & Quock, 1992).

Taken together these studies suggest that the pharmacological properties of $N_2O$ are complex and different receptor systems may be involved dependent upon the response which is examined. It is presently unclear which, if any, of these receptor systems are responsible for the abuse-related subjective effects of $N_2O$. The present study sought to address that question using 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 min of exposure to 60% $N_2O$ can be trained as a discriminative stimulus in mice (Richardson & Shelton 2014). Our data showed that nitrous oxide shares discriminative stimulus effects with toluene but there is little overlap between the discriminative stimulus effects of $N_2O$ and other abused inhalants and vapor anesthetics. The goals of the present study were to examine the role of NMDA, $GABA_A$, opioid and serotonin receptors in transducing the discriminative stimulus effects of $N_2O$.

## MATERIALS AND METHODS

### Subjects

Twenty-four adult male B6SJLF1/J mice (The Jackson Laboratory, Bar Harbor, Maine) served as subjects. These mice had previously been used in a study designed to determine if it was possible to train a discrimination based on inhaled $N_2O$ (Richardson & Shelton 2014). The mice were maintained at 85% of their free feed body weights by regulating food intake to 2-5 grams of standard rodent chow per day (Harlan, Teklad, Madison, WI, USA) post training. Water was available *ad libitum* except during experimental sessions. All subjects were individually housed in 31.5cm x19.5cm clear polycarbonate cages with corncob bedding (Teklad, Madison, WI, USA) on a 12-h light/dark cycle (lights on 6:00 AM) in a colony room maintained at $77^0$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 $N_2O$/oxygen gas mixtures were conducted in one of four 26 liter acrylic exposure cubicles which encased modified two-lever mouse operant conditioning chambers (model ENV-307AW; MED Associates, St. Albans, VT, USA). Two yellow LED stimulus lights, two response levers and a liquid dipper were located on the front wall of each chamber. A single 5-Watt LED house light was located at the top center of the chamber rear wall. Drug discrimination schedule conditions and data recordings were controlled by a MED Associates interface and MED-PC version 4 software (MED Associates, St. Albans, VT, USA).

The milk solution used as a reinforcer consisted of 25% sugar, 25% nonfat powdered milk and 50% tap water by volume.

$N_2O$ and oxygen exposure mixtures were controlled by a manually-operated gas metering system. Briefly, an Airsep Onyx+ oxygen concentrator (Buffalo, NY, USA) generated 98+% oxygen. Nitrous oxide gas was supplied by a compressed $N_2O$ cylinder and a single stage regulator. The $N_2O$ and $O_2$ flow rates were regulated by individual rotometers and the individual gas streams were combined prior to passing into the inhalant exposure chamber. System components were connected with Tygon tubing (Fisher Scientific, Hampton, NH, USA). Waste gas was expelled into a fume hood.

Drugs

Medical grade $N_2O$ gas cylinders were obtained from National Welders Supply (Richmond, Virginia, USA). Memantine, *cis*-4-[Phosphomethyl]-piperidine-2-carboxylic acid (CGS-19755), muscimol, *trans*-(±)-3,4-Dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride (U50-488H), (±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide (8-OH DPAT) and 1-(3-Chlorophenyl)piperazine hydrochloride (mCPP) were purchased from Tocris Bioscience (St. Louis, MO, USA). Pentobarbital, valproic acid, 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridine-3-ol (gaboxadol), (+)-MK-801 maleate (dizocilpine) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Midazolam HCL was purchased from the VCU hospital pharmacy (Nutley, NJ, USA). Ethanol (95% weight/volume) was obtained from Acros Organics (Fair Lawn, NJ, USA). Morphine sulfate and 7-Chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1*H*)-quinolinone (L-701,324) were obtained from the National Institute on Drug Abuse drug supply program (Bethesda, MD, USA). (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-

diethylbenzamide (SNC-80) was generously provided by Kenner Rice at IRP-NIDA (Bethesda, MD, USA).

The vehicle for SNC-80 was 0.9% saline, pH adjusted to between 6 and 7. 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 U50-488H were administered 30 min prior to testing. mCPP was administered 20 min prior to testing. All other injected drugs were administered 10 min prior to testing. $N_2O$ exposures were begun 10 min 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 min exposure to 60% $N_2O$+40% $O_2$ mixture from 100% $O_2$ in once daily (M-F) milk reinforced 5 min operant sessions (Richardson & Shelton, 2014). In the present study, training sessions continued on Mon, Wed and Thurs. Substitution test sessions were conducted each Tues and Fri. Briefly, the first 10 min of each training session was a timeout in which the animals were placed into the operant chambers and gas delivery was initiated. After 10 min of exposure the house and lever lights were illuminated and a 5-min operant session commenced. During the operant session, completion of a fixed-ratio 12 (FR12) response requirement on the active lever resulted in 3 sec of access to a 0.01 ml milk dipper. Responding on the inactive lever reset the FR requirement on the correct lever. $N_2O$ and

$O_2$ vehicle training sessions were presented in a double alternation sequence across training days. In the present study, 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 preceded by 100% $O_2$ and 60% $N_2O$+40% $O_2$ control test sessions. When the test drug was an injected compound both the $O_2$ and $N_2O$ 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 $O_2$ control.

Data collection and analysis

The dependent measures collected were percentage $N_2O$ lever responding (±SEM), operant response rate (±SEM) and the lever upon which the first fixed ratio was completed (FFR). Mean $N_2O$-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) utilizing 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 $N_2O$–lever selection resulting from combining $N_2O$

with MK-801, ethanol and midazolam were by two-way repeated measures ANOVA's. To accommodate missing data due to the failure of some subjects at high dose combinations to emit sufficient responses to generate a lever-selection value, each curve combining a single dose of pretreatment drug with increasing $N_2O$ concentrations was separately compared to the control curve combining $N_2O$ with vehicle. Only those dose combinations in which all subjects emitted at least one complete fixed ratio value were included in the analysis. Subsequent analyses of significant interactions were by Sidak post-hoc tests. Statistical analysis examining response rate alterations resulting from combining $N_2O$ with MK-801, ethanol and midazolam were by two-way repeated measures ANOVA's comparing all $N_2O$ + drug pretreatment conditions. Subsequent analyses of significant interactions were by Sidak post-hoc tests. A significance level of $P< 0.05$ was set for all analyses. All ANOVA's and post-hoc tests were performed using Prism version 6.0 for Macintosh. In addition, when possible confidence limits, potencies and half maximal effective concentrations or doses ($EC_{50}$ or $ED_{50}$) of percentage $N_2O$-lever responding and suppression of operant response rates were calculated using values on the linear portion of each mean dose-effect curve using a Microsoft Excel spreadsheet based on published methods (Bliss, 1967, Tallarida & Murray, 1987).



## RESULTS

Nitrous oxide (n=24) produced concentration-dependent full substitution for the 60% training concentration with an $EC_{50}$ of 33% (CL: 29 – 37%) (Fig 1, upper panel). Control tests of 100% $O_2$ and 60% $N_2O$ + 40% $O_2$ produced a mean of 2% (±1) and 97% (±1) $N_2O$ lever-selection, respectively. Full substitution was produced by both 60% and 80% $N_2O$. There was a main effect of nitrous oxide concentration on operant responding [$F_{(2.5, 56.5)}$=15.0, $P<0.01$] but

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only 80% $N_2O$ (Fig 1, lower panel, filled symbol) significantly ($p < 0.05$) attenuated operant responding below $O_2$ control response rates

The high affinity NMDA receptor channel blocker (+)-MK-801 (n=8) produced dose-dependent partial substitution for 60% $N_2O$ (Fig 2, upper panel, circles) with an $ED_{50}$ of 0.39 mg/kg (CL: 0.20 – 0.77 mg/kg). Maximum mean $N_2O$-lever selection of 55% (±16) was produced by a dose of 0.75 mg/kg (+)-MK-801. (+)-MK-801 (Fig 2, lower panel, circles) attenuated operant responding with an $ED_{50}$ 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 (filled circles). The low affinity NMDA receptor channel blocker memantine (n=7) produced a maximum of 50% (±10) $N_2O$-lever responding at a dose of 56 mg/kg (Fig 2, upper panel, squares). Memantine (Fig 2, lower panel) also dose-dependently [$F_{(2.3, 14)}$=24.16, $P$<0.01] attenuated operant responding with an $ED_{50}$ 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 (filled squares). The competitive NMDA antagonist CGS-19755 (n=8) failed to substitute for 60% $N_2O$ (Fig 2, upper panel, triangles). CGS-19755 (Fig 2, lower panel, triangles) attenuated operant responding [$F_{(2.5, 17.5)}$=40.8, $P$<0.01] with an $ED_{50}$ of 12.0 mg/kg (CL: 8.1 – 17.9 mg/kg). Post-hoc analysis indicated responding was suppressed at doses of 10 and 17 mg/kg ($p < 0.05$, filled triangles). The NMDA receptor glycine site antagonist L-701,324 (n=7) also failed to substitute for $N_2O$ producing no greater than 1% $N_2O$-lever selection at any dose (Fig 2, upper panel, diamonds). L-701,324 (Fig 2, lower panel, diamonds) 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 substitution concentration-effect curves (upper panel) and response rate effects (lower panel) produced by increasing concentrations of $N_2O$ following pretreatment with vehicle, 0.03 or 0.17 mg/kg (+)-MK-801 (n=7). The $EC_{50}$ of $N_2O$+vehicle (Fig 3, circles) was 32% (CL: 25 – 41%). Pretreatment with 0.03 mg/kg (+)-MK-801 (squares) resulted in a $N_2O$ $EC_{50}$ of 26% (CL: 17 – 39%). Pretreatment with 0.17 mg/kg (+)-MK-801 (triangles) produced a more pronounced 1.9 fold leftward shift in the $N_2O$ lever-selection curve, further reducing the $EC_{50}$ of $N_2O$ to 17% (CL: 13 – 23%). There was no significant main effect of 0.03 mg/kg (+)-MK-801 treatment [$F_{(1,6)}$=2.2, P=0.19] nor an interaction between 0.03 mg/kg (+)-MK-801 treatment and $N_2O$ exposure concentration [$F_{(4,24)}$=1.2, P=0.33] on percentage $N_2O$-lever selection. However, there was a main effect of 0.17 mg/kg (+)-MK-801 treatment [$F_{(1,6)}$=7.9, P=0.03] as well as an interaction between the 0.17 mg/kg (+)-MK-801 treatment dose and $N_2O$ exposure concentration [$F_{(3,18)}$=4.9, P=0.01] on percentage $N_2O$-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% $N_2O$ over that produced by $N_2O$ alone (upper panel, filled symbol). There was also both a main effect [$F_{(2,12)}$=13.6 , P<0.01] of (+)-MK-801 pretreatment dose as well as an interaction [$F_{(8,48)}$=6.5, P<0.01] of (+)-MK-801 pretreatment dose and $N_2O$ concentration on operant response rates. Post-hoc tests revealed that 40% + 0.17 mg/kg (+)-MK-801 (t = 4.9) and 60% $N_2O$ + 0.17 mg/kg (+)-MK-801 (t = 9.8) resulted in greater ($p < 0.05$) operant response rate suppression than 40% or 60% $N_2O$ alone (lower panel, filled symbols).

Table 1 shows the results of substitution testing with $GABA_A$ receptor agonists and positive modulators. Gaboxadol (n=8) , a $GABA_A$ receptor agonist selective for delta subunit containing extrasynaptic receptors resulted in a maximum of 4% (±3) $N_2O$-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

$ED_{50}$ of 6.4 mg/kg (CL: 2.6 - 15.7 mg/kg). The synaptic $GABA_A$ receptor agonist muscimol (n=8) produced a maximum of 22% (±22) $N_2O$-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 $ED_{50}$ 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) $N_2O$-lever responding. Valproic acid (n=8) dose-dependently suppressed operant responding [$F_{(2, 14)}$=27.6, $P$<0.01] with an $ED_{50}$ of 430 mg/kg (CL: 384 - 481 mg/kg). The $GABA_A$ receptor benzodiazepine-site positive allosteric modulator midazolam (n=9) produced a maximum of 27% (± 7) $N_2O$-lever responding. Midazolam dose-dependently attenuated operant responding [$F_{(3.2, 25.8)}$=26.4, $P$<0.01] with an $ED_{50}$ of 10.5 mg/kg (CL: 3.2 – 34.8 mg/kg). The $GABA_A$ receptor barbiturate-site positive allosteric modulator pentobarbital (n=8) produced a maximum of 10% (±3) $N_2O$-lever responding. Pentobarbital dose-dependently suppressed operant responding [$F_{(2.9, 20.2)}$=26.2, $P$<0.01] with an $ED_{50}$ of 28.9 mg/kg (CL: 17 – 49 mg/kg).

Figure 4 shows the results of pretreatment with vehicle, 0.3 or 3 mg/kg i.p. midazolam prior to exposure to increasing concentrations of $N_2O$ (n=8). The upper panel depicts $N_2O$-lever selection and the lower panel operant response rates. Vehicle administration prior to $N_2O$ exposure produced a $N_2O$-lever selection $EC_{50}$ of 25% (CL: 14 – 44%). Nitrous oxide combined with 0.3 mg/kg or 3 mg/kg midazolam produced $N_2O$-lever selection $EC_{50's}$ of 35% (CL: 25–47%) and 44% (CL: 35 – 56%), respectively. There was no main effect [$F_{(1,7)}$=1.6, $P=0.25$] or interaction [$F_{(4,28)}$ < 1, $P=0.81$] of 0.3 mg/kg midazolam pretreatment on $N_2O$-lever selection. Analysis of variance was not performed on lever selection data generated when combining 3 mg/kg midazolam + $N_2O$ due to 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 $N_2O$ concentration [$F_{(8,56)}$=4.5, *P<0.01*] on operant rate suppression (Figure 4, lower panel). Post-hoc analysis showed that 3 mg/kg midazolam enhanced ($p < 0.05$) the response-rate suppression produced by concentrations of 20-60% $N_2O$ (filled triangles).

Table 2 shows the results of substitution testing with opioid receptor agonists, ethanol and selected serotonergic agonists. The mu opioid receptor agonist morphine (n=8) produced a maximum of 33% (±33) $N_2O$-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 $ED_{50}$ of 7.9 mg/kg (CL 3.9 – 16.2 mg/kg). The kappa opioid receptor agonist U50-488H (n=8) produced a maximum of 11% (±11) $N_2O$-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 $ED_{50}$ of 3.3 mg/kg (CL: 2.7 – 4.1 mg/kg).. The delta opioid receptor agonist SNC-80 (n=8) produced a maximum of 10% $N_2O$-lever responding. SNC-80 dose-dependently attenuated operant responding [$F_{(2.3, 16.0)}$=13.7, *P*<0.01] with an $ED_{50}$ of 28.6 mg/kg (CL: 16.8 – 48.6 mg/kg). Ethanol (n=9) elicited a maximum of 55% (±13) $N_2O$-lever responding at the highest test dose of 2500 mg/kg. The substitution $ED_{50}$ of ethanol for $N_2O$ 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 $ED_{50}$ of 2109 mg/kg (CL: 1909 – 2332 mg/kg). The 5-$HT_{1B/2C}$ receptor agonist mCPP produced a maximum of 21% (±17) $N_2O$-lever responding at a dose of 10 mg/kg. mCPP (n=8) dose-dependently attenuated operant responding with an $ED_{50}$ 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 [$F_{(2.2, 15.4)}$=25.5, *P*<0.01]. The $5HT_{1A}$ agonist 8-OH DPAT (n=8) produced no greater than 4% $N_2O$-lever selection at any dose. 8-OH DPAT dose-dependently attenuated operant responding with an $ED_{50}$ 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 prior to exposure to increasing concentrations of $N_2O$. The upper panel depicts $N_2O$-lever selection and the lower panel operant response rates. Vehicle pretreatment prior to $N_2O$ exposure (Fig 5, upper panel, circles) resulted in a $N_2O$-lever selection $EC_{50}$ of 31% (CL: 27 – 36%). Pretreatment with a low dose of 500 mg/kg ethanol (Fig 5, upper panel, squares) resulted in a $N_2O$-lever selection $EC_{50}$ of 27% (CL: 23 – 32%). Pretreatment with a higher dose of 1500 mg/kg ethanol (Fig 5, upper panel, triangles) produced a 2.8 fold leftward shift in the $N_2O$ substitution concentration effect curve and a $N_2O$-lever selection $EC_{50}$ of 11% (CL: 7 – 18%). There was no main effect [$F_{(1,7)}$=1.5, $P=0.3$] or interaction [$F_{(4,28)}$ < 1 $P=0.51$] between the 500 mg/kg ethanol pretreatment dose and $N_2O$ concentration on percentage $N_2O$-lever selection. However, there was a main effect [$F_{(1,7)}$=19.8, $P<0.01$] as well as an interaction [$F_{(3,21)}$=3.9, $P=0.02$] between the 1500 mg/kg ethanol pretreatment dose and $N_2O$ concentration on percentage $N_2O$-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% $N_2O$ (upper panel, filled triangle). There 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 $N_2O$ 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) $N_2O$ concentrations (Fig 5, lower panel, filled triangles). 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% $N_2O$. (Fig 5, lower panel, filled square).

## DISCUSSION

The overarching goal of the present study was to explore the receptor mechanisms underlying the discriminative stimulus effects of nitrous oxide. Given the strong *in vitro* evidence that $N_2O$ attenuates NMDA receptor function (Balon et al., 2003, Jevtović-Todovorić et al., 1998, Mennerick et al., 1998, Nagele et al., 2004, Ogata et al., 2006, Petrenko et al., 2010, Sato et al., 2005, Yamakura & Harris, 2000) a number of site-selective NMDA antagonists were tested for their ability to substitute for $N_2O$ (Fig 2). Neither the competitive NMDA antagonist CGS-19755, nor the NMDA receptor glycine site antagonist L-710,324 produced appreciable substitution for $N_2O$. 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 & Balster, 2009) and doses lower than 10 mg/kg have other behavioral effects in rodents (Poleszak et al., 2011, Wlaz & Poleszak, 2011). In contrast, the high affinity NMDA receptor channel blocker (+)-MK-801 produced 55% N2O-lever responding, suggesting a possible channel blocker-like effect of $N_2O$. To systematically replicate this finding we also tested the low affinity NMDA receptor channel blocker memantine which produced a comparable level of 50% $N_2O$-lever responding. The latter result not only confirmed the findings with (+)-MK-801 but also suggest that the relative affinity of NMDA receptor channel blockers is not critical for modulating $N_2O$-like stimulus effects.

While the data with (+)-MK-801 and memantine suggest that N2O may have channel blocker-like stimulus effects, the degree of substitution produced by both drugs was incomplete and could be due to drug effects unrelated to their subjective stimulus properties. For instance, NMDA antagonists disrupt glutamatergic neurotransmission in long term potentiation (Manahan-

Vaughan et al., 2008) which can interrupt memory recall (Florian & Roullet, 2004). A disruption of stimulus control could potentially result in levels of $N_2O$-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 & Zivkovic, 1989, Shelton & Balster, 2004) and other channel blockers (Beardsley et al., 2002, Bowen et al., 1999, Nicholson & Balster, 2003, 2009) 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 $N_2O$.

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, CNS depressants and serotonergics (Koek 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 & Balster, 2004). To address if the partial substitution produced by (+)-MK-801 was due to nonspecific effects, we examined if pretreatment with (+)-MK-801 at doses which produced little or no substitution for $N_2O$ when administered alone would alter the discriminative stimulus properties of $N_2O$ (Figure 3). Our hypothesis was that low doses of (+)-MK-801 would only enhance the stimulus effects of $N_2O$ 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 $N_2O$'s stimulus effects at the higher $N_2O$ test concentrations. The results showing that (+)-MK-801 produced an orderly and significant enhancement of the discriminative

stimulus effects of $N_2O$ support our hypothesis that the discriminative stimulus effects of $N_2O$ has a NMDA channel blocker like component.

Since NMDA antagonists produced incomplete substitution for the training condition seems likely that another mechanism also contributes to the stimulus effects of $N_2O$. To examine a potential GABAergic contribution to the stimulus effects of $N_2O$, five site-selective GABA-positive drugs were tested for their ability to substitute for $N_2O$ (Table 1). Of the potential GABAergic mechanisms which might have played a role in the stimulus effects of $N_2O$, positive allosteric modulation was most strongly implicated in the literature (Hapfelmeier et al., 2000, Quock et al., 1992, Zacny et al., 1995). However, neither the classical benzodiazepine-site positive allosteric modulator midazolam nor the barbiturate pentobarbital produced meaningful levels of substitution for $N_2O$. Further midazolam pretreatment failed to significantly enhance the discriminative stimulus effects of $N_2O$, instead producing a trend toward diminishing the discriminative stimulus effects of $N_2O$ (Fig 4). Likewise, neither the extrasynaptic $GABA_A$ receptor agonist gaboxadol nor the synaptic $GABA_A$ agonist muscimol were $N_2O$-like. Lastly, the relatively nonselective GABA transaminase inhibitor valproic acid produced a low level of partial substitution for $N_2O$, but only at doses which completely suppressed operant responding in 4 of 8 test subjects.

Opioid receptors have been suggested to be involved in the analgesic and antinociceptive effects of $N_2O$ (Emmanouil et al., 2008). However mu, kappa and delta opioid receptors agonists all failed to produce greater than vehicle appropriate responding in $N_2O$-trained mice (Table 2). The poor substitution produced by mu opioid agonist morphine is consistent with data showing that the opioid antagonist naloxone does not attenuate the subjective effects of 30% $N_2O$ in humans (Zacny et al., 1994, 1999). Likewise, the failure of the delta opioid agonist SNC-

80 to substitute for $N_2O$ is consistent with reports that the delta opioid receptor agonist naltrindole does not attenuate $N_2O$ analgesia (Koyama & Fukuda, 2010). Our data showing that the selective kappa opioid agonist U50-488H does not substitute for $N_2O$ is, however, in conflict with a previous study in which $N_2O$ generalized to the purported kappa opioid agonist ethylketocyclazocine in guinea pigs trained to discriminate ethylketocyclazocine from vehicle (Hynes & Hymson, 1984). More recent data has suggested that ethylketocyclazocine is a mixed mu/kappa opioid agonist and some of the discriminative stimulus effects of ethylketocyclazocine may result from mu opioid receptor actions (Wessinger et al., 2011) but this does not entirely reconcile the prior finding with the present data. It is possible the differences between studies were the result of species or methods. Additional work will be necessary to resolve this conflict but based on the present data it does not appear that $N_2O$ has opioid-like discriminative stimulus properties under our training conditions.

A number of studies suggest a relationship between the behavioral effect of $N_2O$ and ethanol. For instance, $N_2O$ reduces 10% ethanol consumption in alcohol preferring and heavy drinking strains of rats (Kosobud et al., 2006). Although alcohol drinking prior to $N_2O$ exposure does not appear to augment the subjective effects of $N_2O$ (Walker & Zacny, 2001), $N_2O$ is chosen more frequently by moderate alcohol drinkers than light drinkers (Zacny et al., 2008). Most recently, our lab reported that ethanol produces partial substitution in mice trained to discriminate $N_2O$ from vehicle (Richardson & Shelton, 2014). In the present study we systematically replicated and expanded our prior results by demonstrating that not only will ethanol partially substitute for $N_2O$ but that, like NMDA channel blockers, ethanol pretreatment will robustly and significantly facilitate the discriminative stimulus effects of $N_2O$ (Figure 5).

The discriminative stimulus effects of ethanol have been repeatedly demonstrated to be based upon a combination of $GABA_A$ positive modulation, NMDA antagonism and $5\text{-}_{HT1B/2C}$ 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 $N_2O$ we examined both the $5HT_{1B/2C}$ agonist mCPP, which has ethanol-like effects in drug discrimination, as well as the $5HT_{1A}$ agonist 8-OH DPAT. Neither mCPP nor 8-OH-DPAT produced $N_2O$-like stimulus effects (Table 2). Overall these finding suggest that the ethanol-like discriminative stimulus effects of $N_2O$ are probably mediated exclusively through a common NMDA channel blocker-like cue component (Grant & Colombo, 1993, Shelton & Grant, 2002, Vivian et al., 2002).

In summary the present results probed the most probable receptor mechanisms underlying the discriminative stimulus effects of $N_2O$. Of these mechanisms, the only class of drugs which engendered meaningful levels of $N_2O$-appropriate responding were NMDA receptor channel blockers. However, even channel blockers failed to elicit full substitution suggesting that other mechanisms are also involved in transducing the stimulus effects of $N_2O$. Several additional candidate mechanisms have been suggested by the literature including $5HT_3$ antagonism (Suzuki et al., 2002, Yamakura & Harris, 2000), neuronal nicotinic acetylcholine (nACh) inhibition (Suzuki et al., 2003, Yamakura & Harris, 2000), interactions with neuronal nitric oxide synthase (nNOS) enzymes [review see (Emmanouil & 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 $N_2O$.

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## AUTHORSHIP CONTRIBUTIONS

*Participated in research design:* Richardson and Shelton

*Conducted experiments:* Richardson

*Performed data analysis:* Richardson and Shelton

*Wrote or contributed to the writing of the manuscript:* Richardson and Shelton

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## FOOTNOTES

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## FIGURE LEGENDS

Figure 1. Mean percentage $N_2O$ lever responding (±SEM) shown in the upper panel and operant response rates shown in the lower panel for 24 mice trained to discriminate 10 min of exposure to 60% $N_2O$+40% oxygen from 100% oxygen. Points above $O_2$ and $N_2O$ reflect the 100% oxygen and 60% $N_2O$+40% oxygen control conditions. Filled symbols in the lower panel indicate significant ($P< 0.05$) suppression of response rates relative to the oxygen control condition.

Figure 2. Mean percentage $N_2O$ lever responding (±SEM) shown in the upper panel and operant response rates (±SEM) shown in the lower panel produced by (+)-MK-801 (n=8) [circles], memantine (n=7) [squares], CGS-19755 (n=8) [triangles], and L-701,324 (n=7) [diamonds] in mice trained to discriminate $N_2O$ from oxygen. Points above $O_2$ and $N_2O$ reflect the 100% oxygen and 60% $N_2O$+40% oxygen control conditions. Numbers in brackets indicates 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 significant ($P< 0.05$) suppression of response rates relative to the oxygen control condition.

Figure 3. Mean percentage $N_2O$ lever responding (±SEM) shown in upper panel and operant response rates (±SEM) shown in the lower panel after pretreatment with vehicle [circles], 0.03 mg/kg [squares] or 0.17 mg/kg (+)-MK-801 [triangles] prior to 10 min of exposure to increasing concentrations of $N_2O$ (n=7). Points above $O_2$ and $N_2O$ reflect the 100% oxygen and 60% $N_2O$+40% oxygen control conditions. Filled symbols in the upper and lower panels indicate significant ($P< 0.05$) differences from the corresponding $N_2O$ + vehicle control values.

Figure 4. Mean percentage $N_2O$ lever responding (±SEM) shown in upper panel and operant response rates (±SEM) shown in the lower panel after pretreatment with vehicle [circles], 0.3 mg/kg [squares] or 3.0 mg/kg midazolam [triangles] prior to 10 min of exposure to increasing concentrations of $N_2O$ (n=8). Numbers in brackets indicates 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 significant ($P< 0.05$) suppression of response rates from the corresponding $N_2O$ + vehicle control values.

Figure 5. Mean percentage $N_2O$ lever responding (±SEM) shown in upper panel and operant response rates (±SEM) shown in the lower panel after pretreatment with vehicle [circles], 500 mg/kg [squares] or 1500 mg/kg ethanol [triangles] prior to 10 min of exposure to increasing concentrations of $N_2O$ (n=8). Points above $O_2$ and $N_2O$ reflect the 100% oxygen and 60% $N_2O$+40% oxygen control conditions. Filled symbols in the upper and lower panels indicate significant ($P< 0.05$) differences from the corresponding $N_2O$ + vehicle control values.

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Table 1. Percentage $N_2O$ lever responding (±SEM) and responses per second (±SEM) produced by gaboxadol, muscimol, valproic acid, midazolam and pentobarbital.

| Test drug | Drug dose (mg/kg) | Percentage $N_2O$ lever responding (±SEM) | Response rate in responses per second (±SEM) |
|---|---|---|---|
| Gaboxadol (n=8) | $O_2$ + vehicle | 0.0 (0.0) | 1.4 (0.1) |
| | $N_2O$ + vehicle | 99.4 (0.3) | 1.2 (0.1) |
| | 0.3 | 1.6 (1.5) | 1.5 (0.1) |
| | 1 | 3.9 (2.6) | 1.6 (0.1) |
| | 3 | 1.6 (1.2) | 1.4 (0.1) |
| | 10 | - | 0.0 (0.0) * |
| Muscimol (n=8) | $O_2$ + vehicle | 0.6 (0.6) | 1.6 (0.1) |
| | $N_2O$ + vehicle | 96.4 (2.2) | 1.2 (0.2) |
| | 0.3 | 1.0 (0.9) | 1.6 (0.1) |
| | 1 | 1.3 (1.1) | 1.4 (0.1) |
| | 1.7 | 21.8 (21.8) [4/8] | 0.6 (0.3) * |
| | 3 | - | 0.0 (0.0) * |
| Valproic acid (n=8) | $O_2$ + vehicle | 1.5 (1.5) | 1.5 (0.1) |
| | $N_2O$ + vehicle | 95.8 (2.3) | 1.2 (0.2) |
| | 100 | 1.0 (1.0) | 1.6 (0.1) |
| | 300 | 2.3 (2.3) | 1.3 (0.1) |
| | 560 | 33.0 (14.8) [4/8] | 0.4 (0.2) * |
| Midazolam (n=9) | $O_2$ + vehicle | 2.1 (1.7) | 1.4 (0.1) |
| | $N_2O$ + vehicle | 98.4 (0.7) | 1.3 (0.1) |
| | 1 | 4.9 (1.6) | 1.2 (0.1) |
| | 3 | 4.4 (3.5) | 1.3 (0.1) |
| | 10 | 11.3 (4.8) [8/9] | 0.6 (0.1) * |
| | 17 | 19.7 (8.7) | 0.5 (0.1) * |
| | 30 | 16.2 (9.1) | 0.6 (0.1) * |
| | 56 | 27.3 (7.2) | 0.5 (0.1) * |
| Pentobarbital (n=8) | $O_2$ + vehicle | 1.8 (1.5) | 1.5 (0.1) |
| | $N_2O$ + vehicle | 95.8 (2.0) | 1.1 (0.2) |
| | 3 | 8.3 (6.5) | 1.4 (0.2) |
| | 10 | 1.0 (1.0) | 1.6 (0.1) |
| | 17 | 1.8 (1.2) | 1.5 (0.1) |
| | 30 | 9.5 (3.4) | 1.0 (0.1) * |
| | 50 | - | 0.2 (0.1) * |

Values in [brackets] indicates number of subjects earning at least one reinforcer (first value) and subjects tested (second value). * indicate significant ($p < 0.05$) difference in response rates compared to the oxygen + vehicle control condition.

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Table 2. Percentage $N_2O$ lever responding (±SEM) and response rate (±SEM) produced by morphine, U-50488H, SNC-80, ethanol, mCPP and 8-OH-DPAT.

| Test drug | Drug dose (mg/kg) | Percentage $N_2O$ lever responding (±SEM) | Response rate in responses per second (±SEM) |
|---|---|---|---|
| Morphine (n=8) | $O_2$ + vehicle | 2.4 (1.6) | 1.8 (0.1) |
| | $N_2O$ + vehicle | 96.1 (2.5) | 1.6 (0.1) |
| | 1 | 2.4 (1.6) | 1.7 (0.1) |
| | 3 | 15.1 (12.2) | 1.3 (0.2) |
| | 10 | 3.3 (2.2) | 0.9 (0.2) * |
| | 30 | 33.3 (33.3) [3/8] | 0.1 (0.0) * |
| U-50488H (n=8) | $O_2$ + vehicle | 2.4 (1.5) | 1.5 (0.1) |
| | $N_2O$ + vehicle | 95.8 (2.4) | 1.3 (0.1) |
| | 1 | 2.4 (1.6) | 1.7 (0.1) |
| | 3.2 | 1.6 (1.6) [5/8] | 0.9 (0.3) * |
| | 7 | 10.7 (10.7) [3/8] | 0.1 (0.1) * |
| SNC-80 (n=8) | $O_2$ + vehicle | 1.9 (1.3) | 1.7 (0.1) |
| | $N_2O$ + vehicle | 93.0 (0.8) | 1.5 (0.2) |
| | 1 | 1.4 (0.8) | 1.7 (0.1) |
| | 10 | 10.3 (2.9) | 1.2 (0.1) * |
| | 17 | 7.9 (3.0) | 1.1 (0.2) * |
| | 30 | 8.7 (1.8) [7/8] | 0.8 (0.2) * |
| | 56 | 6.8 (1.8) [5/8] | 0.5 (0.2) * |
| Ethanol (n=9) | $O_2$ + vehicle | 2.3 (1.5) | 1.6 (0.1) |
| | $N_2O$ + vehicle | 96.6 (1.5) | 1.3 (0.2) |
| | 1000 | 9.6 (6.6) | 1.4 (0.1) * |
| | 1500 | 11.6 (5.5) | 1.3 (0.1) * |
| | 2000 | 44.9 (15.3) | 0.8 (0.1) * |
| | 2500 | 55.0 (13.2) | 0.4 (0.1) * |
| mCPP (n=8) | $O_2$ + vehicle | 2.6 (1.7) | 1.7 (0.1) |
| | $N_2O$ + vehicle | 96.9 (1.7) | 1.6 (0.1) |
| | 0.1 | 1.9 (1.1) | 1.8 (0.1) |
| | 1 | 2.0 (1.3) | 1.6 (0.1) |
| | 5.6 | 3.4 (1.7) | 0.9 (0.2) * |
| | 10 | 20.6 (17.0) [5/8] | 0.4 (0.2) * |
| 8-OH DPAT (n=8) | $O_2$ + vehicle | 1.6 (1.6) | 1.6 (0.1) |
| | $N_2O$ + vehicle | 98.3 (1.5) | 1.5 (0.1) |
| | 0.1 | 1.6 (1.6) | 1.4 (0.1) |
| | 0.3 | 1.4 (1.1) | 1.1 (0.1) * |
| | 1 | 1.1 (1.1) [7/8] | 0.5 (0.1) * |
| | 1.56 | 4.3 (2.4) [6/8] | 0.4 (0.1) * |

Values in [brackets] indicates number of subjects earning at least one reinforcer (first value) and subjects tested (second value). * indicate significant ($p < 0.05$) difference in response rates compared to the oxygen + vehicle control condition.

Figure 1

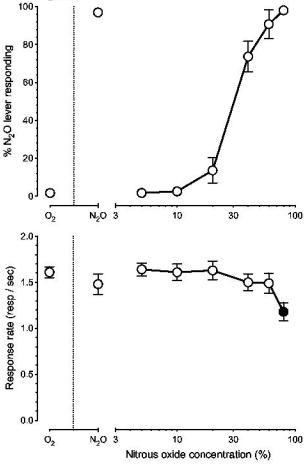

% $N_2O$ lever responding
100
80
60
40
20
0
$O_2$
$N_2O$
3
10
30
100
Response rate (resp / sec)
2.0
1.5
1.0
0.5
0.0
$O_2$
$N_2O$
3
10
30
100
Nitrous oxide concentration (%)

Figure 2

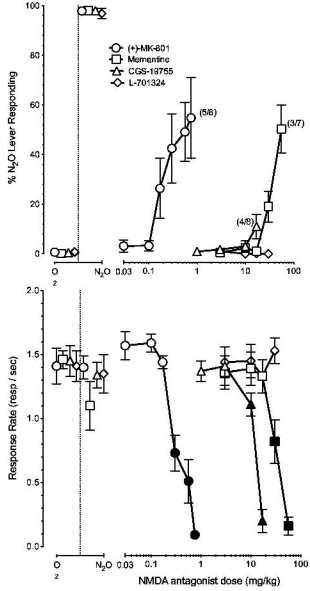

% $N_2O$ Lever Responding
(+)-MK-801
Memantine
CGS-19755
L-701324
(5/6)
(3/7)
(4/6)
O
2
$N_2O$
0.03
0.1
1
10
100
Response Rate (resp / sec)
NMDA antagonist dose (mg/kg)

Figure 2

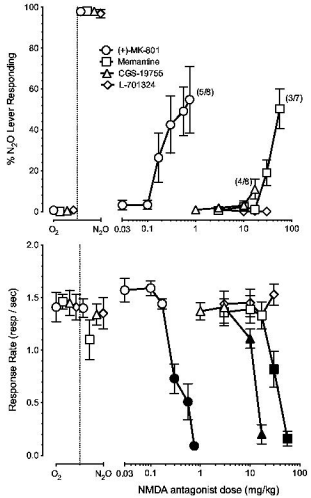

% N2O Lever Responding
(+)-MK-801
Memantine
CGS-19755
L-701324
(5/8)
(3/7)
(4/8)
O2
N2O
0.03
0.1
1
10
100
Response Rate (resp / sec)
NMDA antagonist dose (mg/kg)

Figure 4

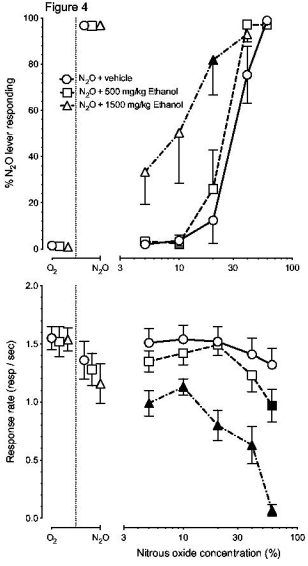
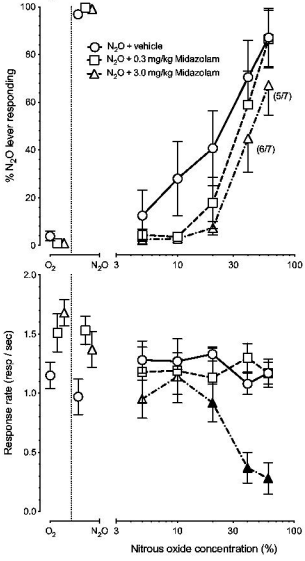

% $N_2O$ lever responding
$N_2O$ + vehicle
$N_2O$ + 500 mg/kg Ethanol
$N_2O$ + 1500 mg/kg Ethanol
$O_2$
$N_2O$
Response rate (resp./ sec)
Nitrous oxide concentration (%)

Figure 5


% $N_2O$ lever responding
$N_2O$ + vehicle
$N_2O$ + 0.3 mg/kg Midazolam
$N_2O$ + 3.0 mg/kg Midazolam
(5/7)
(6/7)
$O_2$
$N_2O$
3
10
30
100
Response rate (resp / sec)
Nitrous oxide concentration (%)