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Characterization of the discriminative stimulus effects of nitrous oxide

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Characterization of the discriminative stimulus effects of nitrous oxide

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy at Virginia Commonwealth University

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List of abbreviations and acronyms

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARCI	Addiction Research Center Inventory
AV	atrioventricular
CGS-19755	*cis*-4-[Phosphomethyl]-piperidine-2-carboxylic acid
CL	confidence limits
CNS	central nervous system
EC_{50}	half maximal (50%) effective concentration
ED_{50}	half maximal (50%) effective dose
i.p.	intraperitoneal
IC_{50}	half maximal (50%) inhibitory concentration
L	liters
L-701,324	7-Chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1*H*)-quinolinone
L-NAME	NG-Nitro-L-arginine methyl ester hydrochloride
LPM	liters per minute
LSD	Lysergic acid diethylamide
mCPP	1-(3-Chlorophenyl)piperazine hydrochloride
mins	minutes
MBG	morphine-benzedrine group
N_2O	nitrous oxide
NMDA	N-methyl-D-aspartate
NSDUH	National Survey on Drug Use and Health
O_2	oxygen
POMS	Profile of moods states

s.c.   subcutaneous

SA   sinoatrial

SNC-80   (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-
            N,N-diethylbenzamide

TCE   1,1,1-trichloroethane

U50-488H   trans-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]
            benzeneacetamide hydrochloride

Abstract

CHARACTERIZATION OF THE DISCRIMINATIVE STIMULUS EFFECTS OF NITROUS OXIDE

By Kellianne Jean Muse Richardson, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Major Director: Keith L. Shelton, Ph.D., Assistant Professor, Department of Pharmacology & Toxicology

Nitrous oxide (N_2O) is a widely used anesthetic adjunct in dentistry and medicine that is also commonly abused. N_2O alters the function of several receptors *in vitro* and *ex vivo*, however, the receptors systems underlying its abuse-related intoxicating effects are poorly understood. The goals of this dissertation were to (1) establish N_2O as a discriminative stimulus, (2) characterize the temporal properties of the discriminative stimulus, (3) determine the degree of similarity between N_2O and other inhalants and (4) explore the neurochemical effects responsible for the stimulus properties of N_2O. Twenty-four mice were trained to discriminate 10 minutes exposure to 60% N_2O+40% O_2 from 100% O_2 in daily 5 minute food-reinforced operant sessions. Mice acquired the discrimination in a mean of 38 sessions. N_2O produced concentration-dependent full substitution for itself. Full substitution required 7 minutes of N_2O exposure but the offset of stimulus effects following cessation of N_2O exposure were more rapid. Varying degrees of partial substitution for N_2O were engendered by abused vapors and vapor anesthetics. The aromatic hydrocarbon toluene produced the most robust substitution for N_2O. One or more toluene concentrations produced full substitution for N_2O in 7 of 8 subjects, suggesting that these two abused inhalants share common neurochemical mechanisms. The NMDA receptor open

channel blockers (+)-MK-801, ketamine and memantine produced dose-dependent partial substitution for N_2O. A competitive NMDA antagonist and NMDA glycine site antagonist did not substitute for N_2O. Pretreatment with (+)-MK-801 as well as ethanol produced dose-dependent leftward shifts in the N_2O concentration effect curve further suggesting some overlap in their mechanisms of action. $GABA_A$ agonists and positive allosteric modulators, opioid agonists, serotonergic agonists, nicotine, a nNOS inhibitor and the psychomotor stimulant amphetamine all failed to appreciably substitute for N_2O and/or failed to alter the N_2O concentration effect curve when administered prior to N_2O exposure. No drug tested produced greater than 80% mean N_2O-lever selection leaving open the possibility of other neurochemical contributors to the stimulus effects of N_2O.

Introduction

Epidemiology of inhalant abuse

Inhalants are a large class of volatile and gaseous chemicals such as toluene, 1,1,1-trichloroethane and nitrous oxide (N_2O) that are grouped only by their route of administration. Most inhalants are components of common household products such as computer dusters, correction fluid, gasoline and lacquer thinner. Worldwide inhalant abuse is a serious social and medical issue (Substance Abuse, 2010, Garriott & Petty, 1980, Kumar et al., 2008, Li et al., 2011, Padilla et al., 1979, Potocka-Banas et al., 2011, Smart, 1988, Szapocznik et al., 1977, Vaille, 1988). Demographics that are particularly at risk for abuse of inhalants include adolescents, medical staff, recreational drug users as well as those living below the poverty line in the USA and abroad.

It is believed that inhalants are a drug of choice for adolescents because they are commonly found around the home, legal, inexpensive and easily concealable (World Health Organization, 1999). Prevalence of inhalant abuse amongst adolescents rises and falls cyclically in the Unites States. For example, from 1967 through 1970 roughly 13% of incarcerated adolescents in New York state admitted inhalant abuse, whereas in 1977 the percentage was considerably lower (Hein, Cohen, & Litt, 1979). The percentage of twelfth grade students using inhalants rose steadily from 4% in 1981 to 7% by 1987 (Johnston et al., 2012). Since the introduction of 8th and 10th grade students to the Monitoring the Future Survey in 1991, inhalant abuse has been consistently estimated as more prevalent amongst 8th and 10th grade students than 12th grade students every year through 2013 (Johnston et al., 2014). The number per 1,000

person years metric used to estimate prevalence of inhalant abuse in 1991 is difficult to convert into an exact percentage of the population but estimates of youth 12 to 17 years old whom initiated inhalant usage almost doubled from 10.7 in 1991 to 21.8 in 1995 (Substance Abuse, 1997).

In both 2006 and 2009 the rate of inhalant abuse amongst high school students in the United States was ranked third after alcohol and marijuana and continuously more prevalent than cocaine or heroin abuse (Johnston et al., 2007, 2009). In the most recent dataset, inhalants continues to rank second to marijuana as most used illicit drug in 8th, 10th and 12th grade youths (Johnston et al., 2012). Interestingly as was the case with several other drugs, within all three grade levels inhalant abuse has slowly declined in the last decade from its peak in 2005 to the present. In 2005 the estimated percentage of past year users in 8th 10th and 12th grade were 10%, 6% and 5%, respectively; the most recent data from 2013 estimated the percentage of past year users in 8th 10th and 12th grade were 5%, 4% and 3%, respectively (Johnston et al., 2014).

Several factors increase the likelihood that an adolescent will abuse volatile substances. A study conducted in 12 to 18 year old school children in Bogotá, Colombia showed that the strongest factors in past year abusers were having friends who misused volatile substances and having experienced being drunk (Lopez-Quintero & Neumark, 2011). Being male, 14–16 years old, having poor academic achievement record and being from a neither wealthy nor poor family were also positively correlated with volatile solvent misuse. Having friends that misused volatile substances and low perception of the dangers of inhalants were most strongly associated with non-users with who were self-admittedly "likely" or "very likely" to misuse volatile substances in the next year. Public school attendance, lack of exposure to drug use prevention programs and poor academic achievement in the last year were also positively

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correlated in these non-using students with a self-identified risk of abusing volatile substances in the next year (Lopez-Quintero & Neumark, 2011).

As is the case with other drugs of abuse there is also a correlation between inhalant abuse and mental illness. For instance, the likelihood of an adult using an illicit drug more than doubles from 11.6% to 26.5% if they are mentally ill (Substance Abuse, 2010b). Likewise the instance of inhalant use in youths aged 12-17 more than doubles from 3.4% to 8.0% if they were diagnosed as having a major depressive episode in the last year (Substance Abuse, 2010b).

While inhalants in aggregate are a serious public health concern, the number of scientific studies devoted to individual inhalants varies widely. One inhalant which is widely available and frequently abused, yet has received relatively little attention, is N_2O. Very few studies have quantified the subcategory of adolescents abusing N_2O within the larger context of adolescents abusing inhalants (Garland, Howard, & Perron, 2009) however in 2005 Monitoring the Future estimated that 21% of 12 to 17 year old adolescents initiated inhalant abuse with N_2O (Substance Abuse, 2006). Further the National Survey on Drug Use and Health (NSDUH) estimates 21.3% of adolescents that initiated inhalant abuse began with N_2O (Office of Applied Studies, 2009).

Nitrous oxide gas is commonly used in dentistry and medicine as an analgesic and anxiolytic. Nitrous oxide is available to the general public through diversion of the gas from a variety of sources. These include medical N_2O cylinders, cylinders used as an oxidizing agent for both vehicle performance enhancement and hobby rocketry and most frequently whipped cream dispenser charging cylinders. Nitrous oxide abusers are a unique subset of the inhalant abusing population compared to those who abuse volatile solvents. Unlike most inhalants which are primarily abused by younger adolescents, N_2O is most commonly abused by older

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adolescents aged 16-17 (Substance Abuse, 2008). From 2002 to 2006 the NSDUH estimated N_2O was the drug choice for 4.7% of past-year inhalant initiates age 12 but was the drug choice for 59.3% of past-year inhalant initiates age 17.

In addition to a high prevalence of use in adolescents, health care professional such as dentists, anesthesiologists and nurse anesthetists also have an elevated risk of N_2O abuse compared to the general public (Bell et al., 1999, Seidberg & Sullivan, 2004). Given the easy access to medical-grade N_2O the drug choice in these demographics is unsurprising. It has been hypothesized that dentists may initiate misuse of N_2O as self-medication for stress relief which progresses to more frequent abuse (Seidberg & Sullivan, 2004). When randomly selected members of the American Association of Nurse Anesthetists across the United States were polled to determine the prevalence of drug abuse, N_2O was ranked second only to benzodiazepines as a preferred drug for misuse by certified nurse anesthetists (Bell, McDonough, Ellison, & Fitzhugh, 1999). For the subset of admitted poly-drug abusers, daily use of N_2O accounted for 252 separate instances of abuse per month as compared with only 152 instances of benzodiazepine misuse.

Medical consequences of N₂O abuse

The intentional misuse of inhalants can lead to medical complications such as encephalopathy (Ross, 1982), dissolution of myelin sheath surrounding neurons (Filley, Halliday, & Kleinschmidt-DeMasters, 2004), kidney stones, leukemia, cardiac arrhythmias (Garriott & Petty, 1980) and damage to the tissues of the lungs and liver (Devathasan et al., 1984). The specific dangers of N₂O abuse include B-12 deficiency myelopathy (Hathout & El-Saden, 2011), necrosis of tissue from frostbite (Hwang, Himel, & Edlich, 1996) and cardiorespiratory failure (Potocka-Banas et al., 2011). In rare instances death attributed to sudden sniffing syndrome has been reported (DiMaio & Garriott, 1978, Fagan & Forrest, 1977). In fact poison control reports indicate N₂O ranks 4th out of 25 categories in percentage fatality which estimates the number of cases resulting in death per 1000 poison control calls (Marsolek, White, & Litovitz, 2010).

The mechanism behind a number of N₂O-induced pathologies are fairly well understood. Nitrous oxide use interrupts vitamin B-12 metabolism (Alt et al., 2011, Chiang et al., 2013, Diamond et al., 2004, Hathout & El-Saden, 2011, Sethi et al., 2006). Vitamin B-12 is a cofactor necessary for red blood cell formation and maintenance of the nervous system. Nitrous oxide irreversibly oxidizes cobalt in Vitamin B-12 from Co^{1+} to Co^{2+}. The oxidized cobalt is needed for methylmalonyl CoA but the reduced form of cobalt is needed for methionine synthase. Decreased methionine synthase activity impairs ongoing regeneration of tetrahydrofolate which in turn delays DNA synthesis. Impaired methionine synthase activity also increases homocystine levels. This indirect effect on methionine synthase underlies the dermal and hematological consequences of N₂O abuse. Megaloblastic anemia secondary to

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repeated N_2O abuse has also been reported, probably as a result of a delay in DNA synthesis leading to impaired nuclear maturation (Barbosa et al., 2000, Trivette et al., 2013). Furthermore, individuals with megaloblastic anemia can develop hyperpigmentation of the skin secondary to vitamin B-12 deficiency but repeated N_2O abuse alone can lead to hyperpigmentation in a relatively short period of time (Chiang et al., 2013, Gilliam & Cox, 1973).

Repeated abuse of N_2O also has deleterious effects on the brain and spinal cord (Alt et al., 2011, Diamond et al., 2004, Hathout & El-Saden, 2011, Tatum et al., 2010). The etiology of myelopathies secondary to N_2O abuse also relates to dysfunction of vitamin B-12 related enzymes and possibly B-12's role in regulating growth factors and cytokines [for review see (Hathout & El-Saden, 2011)]. However, even N_2O abusers with normal serum vitamin B-12 levels have presented at the clinic with pathologies such as a Guillain-Barre-like syndrome (Tatum et al., 2010). Guillain-Barre syndrome occurs when the immune system attacks the peripheral nervous system leading to nerve inflammation, demyelination that slows nerve signaling and eventual paralysis. Early symptoms of Guillain-Barre syndrome are numbness beginning in the extremities, poor balance, muscle weakness and pain. Nitrous oxide effects at Methylmalonyl CoA (an intermediate in the Krebs cycle) may impede energy metabolism to cause some of these symptoms (Maze & Fujinaga, 2000).

The cardiac effects of N_2O are less clear. Death by cardiorespiratory failure after hypoxia from N_2O abuse (Potocka-Banas et al., 2011) and unexplained death after N_2O exposure (Mody, 1975) have been reported. Nitrous oxide does not alter heart rate or blood pressure (Zacny et al., 1994) or lead to heart attack (Sanders et al., 2012). However, several case studies show N_2O administration during surgery disrupts normal sinus rhythm (Roizen, Plummer, & Lichtor, 1987). Deviations from normal sinus rhythm were speculated to be due to direct effects

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of N_2O on either the sinoatrial (SA) node, intra-arterial conduction or the atrioventricular (AV) node or possibly other increases in vagal stimulation and (less likely) decreases in sympathetic tone. An increased percentage of patients given N_2O in addition to epinephrine under anesthesia show an irregular echocardiogram (Lampe et al., 1990). Specifically, AV wave dissociation or progressive delay of the P wave into the QRS complex occurred in 61% patients given N_2O but 41% of patients not administered N_2O (Lampe et al., 1990). The mechanism of this complication is also unknown.

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Current status of inhalant abuse research in the United States

Case studies reported by individual groups and geographical/socioeconomic patterns of abusers underscore the prevalence of inhalant abuse in the United States. Abuse rates in excess of those reported for many other classes of abuse drugs are well-documented by the combined efforts of several U.S. government offices including the Office of Applied Studies (OAS), Substance Abuse and Mental Health Services Administration (SAMHSA) and U.S. Department of Health and Human Services (HHS). Additionally, the University of Michigan Institute for Social Research receives funding to publish an annual Monitoring the Future report on emerging patterns of drug abuse amongst adolescent populations examining the prevalence of inhalant abuse amongst adolescents. However, primary research investigating the mechanisms of action of inhalants and the long term effects of inhalant use are underfunded. A search of the publically available Research Portfolio Online Reporting Tools (RePORT) reveals roughly $2.9 million of NIDA funding will directly benefit the study of inhalants including $999,807 to aid prevention studies, $569,766 towards ongoing epidemiological studies and $1,372,542 for primary research. This is approximately 0.35% of NIDA's 2013 fiscal year research budget which is staggeringly low when compared to the problem. In order to grasp the full impact of inhalant abuse on society and understand their abuse-related effects, more tangible support in the form of increased targeted research funding to encourage additional investigations in the area is desperately needed. The studies described here are designed to address that need as it relates to nitrous oxide. Understanding the abuse-related stimulus properties of nitrous oxide is critical. It will provide a much needed basic understanding of the behaviorally relevant pharmacological effects

of nitrous oxide. This knowledge is a prerequisite for developing effective treatment strategies to curb nitrous oxide abuse.

Physiochemical properties and pharmacokinetics of N₂O

N₂O in nature

Nitrous oxide is a small, inorganic, linear molecule with a molar mass of 44 grams per mole. Nitrous oxide is colorless and has been described as either entirely odorless or slightly sweet smelling (Erowid entry 'Nitrous oxide', 2014). Nitrous oxide synthesis can occur naturally as a byproduct of nitrifying and denitrifying reactions in earthworms, archaea and some anoxic bacteria. Commercially produced N_2O has many applications: it is an oxidant used in internal combustion engines and hobbyist/professional rocketry; it is used in food products as an aerosol propellant; it is a reliable low cost analgesic and anesthetic in medical and dental practices. Some of the physical properties of N_2O lend it to being classification as a greenhouse gas. In some circumstances reactions downstream of N_2O and oxygen lead to production of atmospheric nitric oxide, a free radical reported to deplete the ozone layer. In the body, N_2O crosses the blood brain barrier and interacts with one or more neuroreceptor systems to exert its psychoactive properties.

N₂O absorption, distribution, metabolism and elimination

The only route of entry of N_2O into the body is via inhalation (Cowley & Lambertsen, 1979). The gas rapidly enters the bloodstream through alveolar absorption. Nitrous oxide circulates freely in the blood without requiring a carrier molecule. Due to its low blood gas partition coefficient, once in the bloodstream it distributes between the blood and organ tissues (Becker & Rosenberg, 2008). As a result of its amphipathic properties it can rapidly cross lipid bilayers such as the blood brain barrier. There is no evidence of N_2O conversion to nitrogen

after entering the brain and nitrogen perfusion has not elicited a recorded receptor response (Yamakura & Harris, 2000) therefore it is believed N_2O itself is active in the CNS and exerts its effects via interactions with one or more neurotransmitter receptor systems. Excretion occurs almost exclusively via the lungs (Sawyer, Eger, & Bahlman, 1977) with over 99% cleared by exhalation and 0.004% metabolized by flora native to the intestines (Hong, Trudell, O'Neil, & Cohen, 1980).

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Receptor systems implicated in the biological effects of N₂O

<u>NMDA receptors</u>

The excitatory amino acid glutamate has three ionotropic receptors in the CNS; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA). The NMDA subtype of glutamate receptors is named due to its responsiveness to exogenous administration of the agonist NMDA. NMDA receptors are heterotetrameric ligand-gated ion channels. There are three known NMDA receptor subunits: NR1 and NR3 which binds glycine and NR2 which binds L-glutamate. NMDA receptor heterotetramers require the presence of one NR1 subunit (Pérez-Otaño et al., 2001). Activation of the NMDA receptor requires coincidence of presynaptic glutamate release and postsynaptic depolarization which results in the removal of a voltage-dependent Mg^{2+} blockade from the channel. The binding of both L-glutamate and the co-agonist glycine confers maximum activation.

NMDA receptors can be antagonized by drugs acting at several distinct sites. Antagonists can act through the native glutamate binding site, the native glycine binding site, the polyamine binding site or through blockade of the receptor ion channel. Kashiwagi et al. demonstrated that the interactions with channel blockers are determined by amino acid residues constituting M2 pore-forming regions and M1, M3 and M4 channel spanning regions of both NR1 and NR2 subunits (Kashiwagi et al., 2002). Point mutations of some residues effects IC_{50} values of the open channel blocker MK-801 but not memantine in the NR1 and NR2A variants (Kashiwagi et al., 2002) suggesting amino acid residues within the channel have differential effects on channel blockade depending on the structure of the antagonist. A number of *in vitro* findings indicate that N₂O acts as a NMDA antagonist. Nitrous oxides antagonizes NMDA

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receptor function in a reversible, non-competitive and modestly voltage dependent manner (Jevtović-Todovorić et al., 1998, Mennerick et al., 1998). The nature of the interaction between N_2O and NMDA receptors has been primarily characterized using patch clamp techniques on cells native to brain regions densely populated with NMDA receptors (Balon et al., 2003, Jevtović-Todovorić et al., 1998, Mennerick et al., 1998, Ranft et al., 2007) as well as in heterologous expression systems (Ogata et al., 2006, Petrenko et al., 2010, Sato et al., 2005).

Studies in amygdalar slice preparations suggest both a pre- and post-synaptic component to N_2O's actions on NMDA receptor-mediated excitatory postsynaptic currents (NMDAR-EPSCs) (Ranft et al., 2007). NMDA receptor-mediated EPSCs are decreased by application of N_2O, an effect which is consistent with a post-synaptic action. Application of the channel blocker (+)-MK-801 (dizocilpine) decreased the amplitude of the NMDAR-EPSCs. Co-application of N_2O prolonged the magnitude of the blockade produced by MK-801 which the authors speculated was due to a decrease in the probability of glutamate release (Ranft et al., 2007); this effect is consistent with a pre-synaptic action. When NMDA is applied to rat hippocampal cultured neurons inward current increases. The addition of N_2O produces a rightward and downward shift in the dose-response curve (Jevtović-Todovorić et al., 1998). Furthermore, whole cell recording of rat hippocampal neurons showed inhibition by N_2O when co-applied with NMDA (Mennerick et al., 1998).

Studies of monosynaptic communication by NMDA receptors allow the investigation of receptor function without confounding polysynaptic network interference (Mennerick et al., 1998). Excitatory synaptic transmission in microcultures of rat hippocampal cultures show that NMDA receptor-mediated excitatory autaptic currents are attenuated by $49 \pm 6\%$ when 80% N_2O is bubbled into the extracellular solution. Based on NMDA current change, the N_2O blockade of

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NMDA receptors is believed to be less voltage dependent than ketamine or Mg^{2+} (Mayer, Westbrook, & Guthrie, 1984) but easily reversible.

There are numerous variations in assembled NMDA receptors *in vivo*. Agonist and antagonist binding affinities may vary according to subunit composition. *Xenopus oocytes* expressing human NR1A and NR2A NMDA receptor subunits show inhibition by $31\pm2\%$ when 0.58 atm N_2O is bubbled into the extracellular solution (Yamakura and Harris, 2000). There are 4 established types of NR2 subunits. NR2 subunits modify, among other properties, channel conductance and current kinetics (Sobolevsky, 2007). Heterologous expression systems could potentially examine which subunit composition or NR2 subtypes are more sensitive to N_2O but these studies have yet to be conducted.

NMDA heterotetramers may assemble with a NR3 subunit. Though less studied than NR1-NR2 variants, NR1-NR3 subunit are activated by glycine alone (Cavara, Orth, & Hollmann, 2009). There are indications that functional and physiologically relevant NMDA receptors require NR3 coassembly with NR1/NR2 subunits. NR3A mRNA expression is low in adult rodent brain and NR3B expression remains constant in the adult brainstem and spinal cord motor neurons during development (Yamakura et al., 2005). Coexpression of NR3 subunits decreased calcium permeability (Matsuda et al., 2003) but not the extent of magnesium blockade (Yamakura et al., 2005). However, there is little evidence that NR3 subunits are involved in the physiological effects of N_2O given data demonstrating that NMDA receptor inhibition by 0.6 atm N_2O was similar in NR1/NR2B comprised receptors and NR1/NR2B/NR3B comprised receptors (Yamakura et al., 2005).

Only two *in vivo* studies have directly examined interactions between N_2O and NMDA receptors. Like the volatile anesthetic halothane (Crowder, Shebester, & Schedl, 1996), N_2O

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effects coordinated movement in *C. elegans* worms (Nagele, Metz, & Crowder, 2004). Quality but not quantity of locomotion is altered by N_2O exposure in wild-type *C. elegans*. Specifically, reversal of their direction and time spent moving backwards are half that of air-exposed worms. However, *C. elegans* with a loss-of-function mutation in NMR-1, which encoded a NMDA-type glutamate receptor, are unaffected by 70% N_2O. *C. elegans* with transformation rescue in *nmr-1* gene mutation had restored sensitivity to 70% N_2O such that quality of locomotion was again altered in a manner similar to that in wild-type worms. To confirm that these findings were due to NMDA-like receptors, a non-NMDA mechanism was investigated by examining a loss of function mutation in a gene most closely associated with the AMPA subtype of glutamate receptors. These *glr-1* mutants were as sensitive as wild-type worms to N_2O mediated decreases in reversals showing that N_2O antagonizes NMDA receptors and not AMPA receptors to impair locomotion in *C. elegans*. In the second *in vivo* demonstration of N_2O's effects at NMDA receptors, rats were implanted with electrodes and voltammetric measurements were monitored using a polarograph. NMDA receptors located on dopaminergic substantia nigra pars compacta cells were sensitive to N_2O exposure (Balon et al., 2003). Specifically, administration of 500 pM NMDA to the substantia nigra pars compacta increased dopamine release in the striatum of freely moving rats. Nitrous oxide exposure reduced the percentage striatal dopamine release elicited by 500 pM NMDA (Balon et al., 2003). Taken together these studies strongly suggest that N_2O antagonizes NMDA receptor function.

GABA_A receptors

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian CNS (Chebib & Johnston, 1999, Jacob, Moss, & Jurd, 2008, Kumar & Kuppast, 2012). There are three subclasses of GABA receptors: $GABA_A$, the closely related $GABA_C$, as

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well as G-protein coupled $GABA_B$ receptors. Upon activation, $GABA_A$ receptors act to hyperpolarize neurons and inhibit neuronal firing by gating chloride ion influx. Of the three subclasses, only the $GABA_A$ subtype of receptors are currently implicated in the actions of N_2O.

Nitrous oxide does not potentiate $GABA_A$ receptor current in the absence of an agonist suggesting it is a positive modulator (Hapfelmeier et al., 2000). Distinct positive modulatory sites have been identified which bind benzodiazepines, barbiturates and GABA-positive neurosteroids. Investigations of N_2O's ability to increase GABAergic current have been conducted in mammalian cells with subunit combinations found to have widespread distribution in the rodent brain (Pirker et al., 2000). In *Xenopus oocytes* expressing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ recombinant receptors, N_2O exposure potentiates GABAergic current resulting from application of 10 µM GABA by a modest 20% (Yamakura & Harris, 2000). In another patch clamp study, N_2O (100% or 29.2 mM) increased chloride ion current flow through $\alpha_1\beta_2\gamma_{2L}$ recombinant mammalian $GABA_A$ receptors by 69% and decreased rise in current time by 45% (Hapfelmeier et al., 2000). The same 100% N_2O concentration in $\alpha_1\beta_2$ recombinant mammalian $GABA_A$ receptors increased peak current by 88% and decreased rise time by 30% (Hapfelmeier et al., 2000). Application of 29.2 mM N_2O increased current in whole cell patches by 154% over that of 5 µM GABA alone in $\alpha_1\beta_2\gamma_{2L}$ recombinant receptors in human embryonic kidney cells (Hapfelmeier et al., 2001). Nitrous oxide also potentiated responses to the exogenously applied direct $GABA_A$ agonist muscimol in *ex vivo* cultured hippocampal neurons (Dzoljic & Van Duijn, 1998). Specifically, 80% N_2O enhanced GABA induced chloride ion movement by 20% (Dzoljic & Van Duijn, 1998). These *in vitro* and *ex vivo* data showing an interaction of N_2O with the $GABA_A$ receptor suggest that potentiation of $GABA_A$ receptor function may also underlie the behavioral effects of N_2O.

Additional indirect evidence of an interaction of N_2O with $GABA_A$ receptors comes from data examining the interaction of N_2O and ethanol. A number of the behavioral effects of ethanol are believed to be $GABA_A$-receptor mediated. The behavioral effects of N_2O have some overlap with those of ethanol. For instance, both ethanol and N_2O are anxiolytics (Lapin, 1993) and the discriminative stimulus effects of ethanol are partially mediated through $GABA_A$ positive modulatory effects (Shannon et al., 2004, Shelton & Balster, 1994, Shelton & Grant, 2002). Nitrous oxide reduced 10% ethanol consumption in alcohol preferring and heavy drinking strains of rats (Kosobud, Kebabian, & Rebec, 2006). Nitrous oxide is chosen more frequently by moderate alcohol drinkers than light drinkers (Zacny, Walker, & Derus, 2008) however alcohol drinking prior to N_2O choice does not appear to augment the subjective effects of N_2O (Walker & Zacny, 2001).

The possibility that the behavioral effects of N_2O are at least in part due to interactions with the $GABA_A$ receptor is also suggested by studies examining the anxiolytic properties of N_2O. It is well accepted that benzodiazepines produce anxiolysis through positive modulatory effects at $GABA_A$ receptors. It was demonstrated that the benzodiazepine site antagonist flumazenil reversed 30% N_2O and 40% N_2O-mediated reductions in anxiogenic phenotypes in the conditioned defensive burying assay (Czech & Quock, 1993). In this study subjects that received 2mA shock conditioning followed by N_2O exposure showed reductions in both duration of burying and bedding height (Czech & Quock, 1993). Flumazenil also dose dependently attenuated the anxiolytic effect of 50% N_2O in the hole-board assay (Czech & Green, 1992). Furthermore, the $GABA_A$ competitive antagonist SR-95531 (Gabazine) significantly attenuated N_2O mediated increases in time spent in the light compartment as well as number of transitions in the light/dark box assay (Li & Quock, 2001). Finally, a double-blind randomized study

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quantified human subjective effects during exposure to 30% N_2O and after pretreatment with flumazenil. Visual analogue scale ratings of subjective "high" produced by N_2O were significantly reduced by flumazenil pretreatment. Ratings of "drunk", "elated" and "drug liking" were also diminished, though not significantly (Zacny et al., 1995). In summary N_2O potentiates $GABA_A$ receptor function. It is therefore possible that the $GABA_A$ positive modulatory effects of N_2O may also be responsible for the subjective intoxication produced by N_2O.

Opioid receptors

There are three subtypes of opioid receptors: mu (Lord, Waterfield, Hughes, & Kosterlitz, 1977), delta (Wolozin & Pasternak, 1981) and kappa (Martin, Eades, Thompson, Huppler, & Gilbert, 1976) [For review see (Pasternak, 2005, Pasternak & Pan, 2013)]. Both mu and kappa opioid receptors have been implicated in the analgesic and antinociceptive properties of N_2O. For example, exposure to 75% N_2O reduced the number of writhes subsequent to intraperitoneal injection of 0.7% glacial acetic acid. The kappa opioid antagonist nor-binaltorphimine (nor-BNI) but not the delta opioid antagonist naltrindole suppressed N_2O analgesia (Koyama & Fukuda, 2010). β-Chlornaltrexamine, a mixed agonist/antagonist at mu opioid receptors, reversed antinociceptive responses to 70% N_2O in subjects that received 0.6% glacial acetic acid (Emmanouil et al., 2008).

However, evidence also exists which argue against an interaction of N_2O with mu opioid receptors. Specifically, the subjective effects of 30% N_2O are not attenuated by naloxone (Zacny et al., 1999, Zacny et al., 1994) nor did naloxone antagonize N_2O-induced deficits in the digit substitution test and pain perception (Zacny et al., 1999). While 30% N_2O is a relevant clinical concentration in dentistry it is relatively low, suggesting that the effects of N_2O on opioid

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receptors may require exposure to higher concentrations. Unfortunately, these studies were

unable to test concentrations above 30% as they are reported as being aversive in some patients

(Block et al., 1988, Dohrn et al., 1992, Walker & Zacny, 2003).

The reports of N_2O's aversive effects may be due to an interaction with kappa opioid

receptors. Since emotional states cannot be attributed to animals, avoidance and aversion

phenotypes are used as models of dysphoric effects in humans. Activity at the dynorphin/kappa

opioid receptor system has been shown to produce aversive effects in animals. For example, the

kappa agonist U50-488 dose dependently increased the development of odorant paired swim

stress aversion in mice (Land et al., 2008). In a second conditioned aversion paradigm,

conditioned aversion to a footshock-paired compartment was prevented by pretreatment with the

kappa opioid antagonist norBNI and could not be elicited in prodynorphin knockout mice (Land

et al., 2008). Lastly, there is also direct evidence of overlapping stimulus properties of N_2O and

kappa opioid agonists in drug discrimination. Specifically, N_2O substituted in animals trained to

discriminate the kappa opioid agonist ethylketocyclazocine but not in mice trained to

discriminate the mu opioid agonist morphine (Hynes & Hymson, 1984). Considering the

pharmacologically specificity of drug discrimination it is plausible that kappa opioid agonism

may partially mediate the subjective effects of N_2O.

Other potential targets

There are a scattering of additional *in vitro* and *ex vivo* studies in the literature suggesting

that nitrous oxide interacts with a number of other receptor systems. However, these reports

have generally not be systematically replicated nor expanded upon in follow-up experiments.

Individual studies have shown that nicotinic acetylcholine receptors (Suzuki, Ueta, Sugimoto,

Uchida, & Mashimo, 2003, Yamakura & Harris, 2000), serotonin receptors (Suzuki et al., 2002, Yamakura & Harris, 2000), glycine receptors (Yamakura & Harris, 2000), Dopamine D-2 receptors (Koyanagi et al., 2008) as well as GIRK (Yamakura, Lewohl, & Harris, 2001) and TREK-1 (Gruss et al., 2004) potassium channels may all be modulated by N_2O. There is also evidence that N_2O interacts with neuronal nitric oxide synthase (nNOS), a family of enzymes regulating nitric oxide (NO) production [review see (Emmanouil & Quock, 2007)]. While nNOS expression is relevant to anxiety-related biological activity (Chakraborti, Gulati, & Ray, 2008) there is little evidence to suggest these interactions primarily mediate the activity of other known drugs of abuse (Green, Gatto, & Grant, 1997) or will apply to the discriminative stimulus properties of N_2O.

Drug discrimination: A model of the subjective intoxicating effects of drugs in humans

Adaptation for studying drugs of abuse

The subjective intoxication produced by a drug can only be verbally reported by humans. Subjective effects of drugs vary by individual (Preston & Bigelow, 1991) but are made more easily quantifiable with standardized questionnaires like the Addiction Research Center Inventory (ARCI), Profile of Moods States (POMS), drug class questionnaires and adjective rating scales (Karch, 2006). The drug discrimination procedure is an extremely powerful research tool which models human subjective intoxicating effects of drugs in humans or animals. Humans, non-human primates, guinea pigs, gerbils, pigeons, and rodents have all served as subjects in drug discrimination studies (Colpaert, 1999).

When an animal serves as a subject discrimination training typically occurs using an operant procedure. The subject is trained to perform one behavior following drug administration in order to receive reinforcement whereas they must perform an alternative behavior following vehicle administration to receive reinforcement. Either positive or negative reinforcement is effective in training drugs as discriminative stimuli (Järbe & Ohlin, 1979, Järbe & Rollenhagen, 1978). Once subjects reliably perform the discrimination behavior, the degree to which other drugs elicit discriminative stimulus effects similar to the training drug can be examined. The discriminative stimulus effects of the training drug are the result of the interoceptive cue of that drug. This interoceptive cue results from the neurochemical effects produced by the training drug. Pharmacological properties of drugs with CNS activity such as the site of action, receptor specificity, receptor subtype, site selective binding and efficacy can all be explored using drug discrimination (Colpaert, 1999).

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The specific parameters of drug discrimination training and testing vary according to the variables of importance and the species used in the study. For instance in early studies, guinea pigs were trained to run into one arm in a T-maze to receive a reinforcer following pentobarbital injection and run into the opposite arm to receive a reinforcer following saline injection (Overton, 1964). Similar T-maze procedures have been conducted with other training drugs including nicotine (Schechter & Rosecrans, 1972a). Pigeons have also been used as subjects. In one early study pigeons were trained to peck one key following Δ-9-THC and another key following saline (Henriksson, Johansson, & Järbe, 1975).

During the 1960s through the 1980s a limited number of studies examined the drug discrimination task in humans (Chait, Uhlenhuth, & Johanson, 1984, Wolbach, Isbell, & Miner, 1962). Laboratories that conducted the procedure in human subjects allowed for comparison of animal discrimination data with human discrimination data (Schechter & Rosecrans, 1972b, Wolbach, Miner, & Isbell, 1962) as well as human discrimination data with human subjective effects measures (Chait et al., 1988, Lee et al., 1989, Preston et al., 1989, Shannon & Holtzman, 1977, Young et al., 1984). A few studies even specifically compared and contrasted data generated in humans with data generated in nonhuman subjects from the same drugs (Chait, Uhlenhuth, & Johanson, 1988, Lee, Stafford, & Hoebel, 1989). Overall these studies clearly demonstrated that there is an excellent correlation between human subjective and discriminative stimulus effects and the discriminative stimulus effects of drugs in non-human subjects.

Although early procedures used tasks such as the T-maze, the research community rapidly adopted the use of operant conditioning chambers for conducting drug discrimination studies in non-human primates, rodents and pigeons. The most common variant is the two-lever discrimination procedure. In a typical two lever operant procedure subjects learn that responding

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on one lever is reinforced following administration of a training drug whereas responding on the alternative lever is only reinforced following administration of vehicle. The lever resulting in reinforcement is predictable only by attending to the interoceptive cue of the drug or lack thereof. Initially responding approximates chance levels but improves with repeated daily training sessions. Eventually the majority of responding within a training session will occur on the injection-appropriate lever. After acquiring the behavior to pre-defined criteria, subjects that maintain satisfactory levels of discriminatory control move to a training/testing session alternation across days. Schedules of reinforcement may or may not be the same during a test as during training. For instance, testing may be conducted under conditions in which responding on either lever is reinforced (Shannon & Holtzman, 1977) or the test sessions may be conducted under extinction (Holtzman, 1988, Järbe & Ohlin, 1979, Leberer & Fowler, 1977, Stolerman & Olufsen, 2001). There is some indication that conducting testing during extinction may generate more graded dose-effect curves but in practice both methods yield information about subjective similarity of the test drug injection compared to that of the training drug.

In addition to data on the subjective similarity of a test drug injection to that of the training drug, the drug discrimination procedure also provides information on the temporal rate of operant lever-pressing behavior. Response rate can be sensitive to administration of drug (Harris & Balster, 1968, Heffner, Drawbaugh, & Zigmond, 1974). Disruption of operant performance can be due to either excessive stimulation resulting in the emergence of competing behaviors such as stereotypy or due to CNS depression (Solinas, Panlilio, Justinova, Yasar, & Goldberg, 2006).

Generally in drug discrimination assays several doses of the training drug or a probe drug are used to generate a generalization curve. Very low doses of the training drug elicit

responding on the vehicle appropriate lever. Increasing doses of the training drug result in increases in group mean drug-lever selection. Generally, the training dose and doses higher than the training dose elicit responding only on the training drug-appropriate lever.

The most powerful feature of the drug discrimination procedure is the cross-substitution or generalization test. The neurochemistry underlying the interoceptive cue of the training drug dictates the behavior of a subject during a substitution test (Schechter & Rosecrans, 1972a). Novel test compounds may engender either vehicle or drug-lever responding. Test compounds that elicit responding on the training drug-appropriate lever often share similar neurochemical effects with the training drug. Test compounds that engender only vehicle-appropriate responding either do not cross the blood brain barrier or, more likely do not act through the same neurochemical mechanism as the training drug (Colpaert, 1999).

Properties of discriminative stimuli

The discriminative stimulus properties of drugs have been shown in a number of experiments to be CNS mediated. For example, it was demonstrated that the discriminative stimulus of nicotine could be antagonized by the CNS-penetrant nicotinic antagonist mecamylamine but not by the nicotinic antagonist hexamethonium, which does not cross the blood brain barrier (Rosecrans & Chance, 1977). The discriminative stimulus effects of drugs are also receptor specific. To illustrate, in an early study it was shown that physostigmine, a reversible acetylcholinesterase inhibitor would not substitute in animals trained to discriminate pentobarbital, a GABAergic positive modulator, from saline (Overton, 1966). Generally a test drug will only substitute for a training drug if they bind the same receptor. For example,

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Lysergic acid diethylamide (LSD) is a hallucinogen which interacts with many serotonergic receptor subtypes, chiefly, 5HT1$_A$ and 5HT$_{2A}$ (Burris, Breeding, & Sanders-Bush, 1991, Burris & Sanders-Bush, 1992, Glennon, Rosecrans, & Young, 1983, Watts et al., 1995, Winter, 2009). Psilocybin and mescaline are both hallucinogens that act as partial agonists for several serotonergic receptors (Geyer & Vollenweider, 2008). Psilocybin and mescaline but not the monoamine releaser D-amphetamine produced a LSD-like cue in rats trained to discriminate LSD from saline (Schechter & Rosecrans, 1972b). The substitution of psilocybin and mescaline are attributed to the pharmacological effects they share with LSD. Their similarity is also corroborated by the fact that psilocybin and mescaline also produce cross-tolerance with LSD (Wolbach, Isbell, et al., 1962).

In some cases drug discrimination has selectivity such that it can distinguish between drugs acting upon different sites on the same receptor complex. For instance, NMDA receptors have several binding sites through which compounds can decrease receptor function (Nankai, 1998, Scatton, 1993). NMDA antagonists may competitively bind glutamate or glycine binding sites as well as uncompetitively block the channel or modulate polyamine sites (Gibson et al., 2002). NMDA antagonists produce psychomimetic effects in humans depending on how the compound binds the receptor. For instance, polyamines like spermine have biological activity (Igarashi & Kashiwagi, 2010, Pegg, 2009) such as interacting with RNA and DNA, roles in cell proliferation and differentiation and inhibition of neuronal nitric oxide synthase (nNOS) but may also have behavioral effects such as antagonism of NMDA receptors (Carter et al., 1988, Patat et al., 1994). However, the polyamines spermine, spermidine and arcaine were not similar to the high affinity open-channel NMDA antagonist phencyclidine (Nicholson & Balster, 1998). Similarly, eliprodil, another drug that non-competitively binds a polyamine site the NMDA

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receptor did not substitute in subjects trained to discriminate PCP from saline (Balster, Nicholson, & Sanger, 1994).

Drug discrimination may also be capable of differentiating between drugs that have the same site of action but differing efficacy levels.   For example, zolpidem is a full agonist at α_1-containing $GABA_A$ receptors. Partial and full agonists binding allosteric sites on $GABA_A$ receptors were evaluated for their ability to substitute in subjects trained to discriminate 3.0 mg/kg zolpidem from saline. The $GABA_A$ $\alpha 1$ partial agonist SL651498 only partially substituted for zolpidem however the full agonist CL 218872 completely substituted for zolpidem (Mirza, Rodgers, & Mathiasen, 2006).

Analysis of drug discrimination data

The degree of similarity of a test drug to that of the training drug is generally empirically quantified. Two of the most common methods of quantification are the examination of group mean drug-lever selection data and the examination of first fixed ratio (FFR) completed data. Mean group percentage drug-lever selection is perhaps the common method in published studies. An advantage of this method is that it is data inclusive. Group mean data summarizes the behavior of all subjects during a test session and provides a graded metric from what is often quantal individual data. Specifically, within individual subjects, drug-lever selection is often an all or none phenomena. All responding is often allocated on the vehicle-appropriate lever until a dose is reached at which all responding switches to the drug-appropriate lever. The dose in which this switch takes place often differs between subjects. Aggregating this individual subject data produces a graded mean dose response curve facilitating calculations such as ED_{50} and EC_{50} values and potencies. A disadvantage of group mean data is that is an artificial construct that

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does not generally represent the responding of any of the individual subjects. An alternative metric of drug-appropriate responding tabulates the lever on which each subject completes the first fixed ratio value, generally referred to as the first fixed ratio (FFR). Whereas percent drug lever responding averages the graded responses of individual subjects, the FFR pools quantal yes/no data from individual subjects before the presentation of a reinforcer. Although FFR data, if pooled to produce a group mean is also an artificial construct, it may have some advantage in that it is free from the influences of reinforcer presentation upon subsequent behavioral allocation when both responses are reinforced during testing.

Interpretation of substitution data

The degree to which a subject identifies a novel drug stimulus as a training drug is often segregated into three descriptive categories: full substitution, partial substitution and no substitution. The numerical ranges which are defined as full, partial or no substitution are entirely arbitrary. In some laboratories 60% to 79 % and 80% to 100% indicates partial and full substitution, respectively (Wiebelhaus, Vunck, Meltzer, & Porter, 2012). In the present proposal for consistency with other publication from our laboratory I have defined responding of less than 20% as no substitution, 21%-79% as partial substitution and 80%-100% as full substitution (Shelton, 2007, 2009, 2010, Shelton & Nicholson, 2010, 2012, Shelton & Slavova-Hernandez, 2009).

There is little argument within the drug discrimination field regarding the interpretation of full substitution and no substitution. However, when a novel compound is administered and the maximal drug-appropriate responding across the entire range of doses tested only reaches the level of partial substitution, interpretation of the data can become more challenging. Several

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possible data sets can yield similar mean group levels of partial substitution. For instance, one common occurrence is that a test drug produces full substitution in a subset of subjects but fails to substitute in the remaining subjects. Another possibility is that partial substitution is produced in all subjects. Unfortunately, most publications do not provide sufficient data to allow for extended interpretation of partial substitution results.

In some studies it is possible to explain partial substitution mechanistically based on drug affinity, efficacy or intrinsic activity (Solinas et al., 2006). For example, high affinity NMDA channel blockers fully substitute for the high affinity NMDA antagonist PCP but moderate affinity channel blockers sometimes produce partial substitution for PCP (Nicholson & Balster, 2003). The most objective explanation of partial substitution, therefore, is the existence of a limited similarity in the neurochemistry underlying the stimulus properties of the training drug and the test drug.

Studies of compound cues and drug mixtures

Given the repeated demonstrations that N_2O interacts with multiple ligand gated ion channels and G protein-coupled receptors (i.e. opioids, GIRK) more than one interaction may mediate the discriminative stimulus effects of N_2O. Considering the sensitivity of the task to the neuropharmacology of drugs, studies of compound stimuli and drug mixtures modeling artificial compound stimuli may aid in the interpretation of cross-substitution data if N_2O has multiple cue components. Two types of compound cues have been demonstrated in drug mixture discrimination studies: redundant cues and conditional cues. In a redundant cue either component of a discriminative stimulus based on a drug mixture will fully substitute for the discriminative stimulus effects of that drug mixture. In a conditional cue both components of a drug mixture must be presented together to fully substitute for the discriminative stimulus effects of a training drug mixture. Administration of a singular component of the mixture will not elicit substitution for the mixture training cue. As an example, in a classic study performed by Stolerman, a group of rats was trained to discriminate a mixture of 0.4 mg/kg nicotine combined with 0.2 mg/kg midazolam versus saline (Stolerman, Rauch, & Norris, 1987). Nicotine and midazolam have distinct mechanisms of action and little to no overlap in stimulus effects under circumstances where either drug is trained versus vehicle. However, when trained together, both nicotine administered alone and midazolam administered alone produced partial substitution for the "AND-mixture". These findings have been both directly and systematically replicated in studies of nicotine + midazolam mixtures (Garcha & Stolerman, 1989) as well as amphetamine + pentobarbitone mixtures (Mariathasan, Garcha, & Stolerman, 1991). Further, these mixture cues appear to react predictably to antagonist pretreatment. For example, complete antagonism of the

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nicotine and pentobarbital mixture stimulus was only possible by administering a mixture of flumazenil (Ro 15-1788) and mecamylamine (Stolerman et al., 1987). These data demonstrate that each component of a drug mixture is capable of eliciting at least partial substitution for that mixture, demonstrating that the individual components of a drug mixture cue are perceived separately rather than as a new and unique stimulus complex.

These studies provide two major considerations for the interpretation of data if N_2O has a compound cue. The first ramification of these studies is the possibility that mimicking any individual component of nitrous oxides stimulus may not engender full substitution. Again, both nicotine administered alone and midazolam administered alone produced at best partial substitution for a nicotine+midazolam mixture (Stolerman, Rauch, & Norris, 1987). It might therefore be expected that if the stimulus effects of N_2O are mediated by NMDA antagonism and GABA$_A$ positive modulation that neither class of drugs alone would produce full substitution for nitrous oxide. A second ramification of these studies is that it may not be possible to pharmacologically antagonize the stimulus effects of nitrous oxide with any one drug. Instead it may require that antagonists for all parts of nitrous oxides stimulus effects be given together to fully block its cue.

Substitution results inferred based on combinations of two distinct drugs are informative. However, a more appropriate comparison may be individual drugs which have actions at multiple receptors. Two examples of drugs with compound cues are ethanol as well as the vapor anesthetic isoflurane. Ethanol interacts with several receptor systems *in vitro* and *in vivo* including GABA$_A$ (Helms, Rogers, & Grant, 2009), NMDA (Kotlinska & Liljequist, 1997) and 5HT$_{1B/2C}$ (Andrade et al., 2011). When tested in ethanol trained animals, benzodiazepines (Grant et al., 2000, Shelton & Grant, 2002), barbiturates (York, 1978), NMDA channel blockers

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(Kotlinska & Liljequist, 1997, Shelton & Grant, 2002) and, depending on the dose of ethanol trained, serotonergic agonists fully substitute for ethanol (Grant, Colombo, & Gatto, 1997). These data somewhat contradict the prior discrete mixture data in showing that a single component of a compound training cue is sufficient to elicit complete substitution for that cue.

The volatile vapor isoflurane may also have a multiple component discriminative stimulus. Isoflurane has been reported to potentiate $GABA_A$, glycine, kainate and $5HT_3$ receptor currents and reduce $\alpha 4\beta 2$ and $\alpha 4\beta 4$ containing nicotinic acetylcholine, NMDA and AMPA receptor current in *Xenopus oocytes* (Yamakura & Harris, 2000). The discriminative stimulus effects of isoflurane appear to be mediated by both $GABA_A$ receptor positive modulation and NMDA receptor antagonism (Shelton & Nicholson, 2010). In mice trained to discriminate 6,000 ppm isoflurane vapor from air the benzodiazepines midazolam and zaleplon, the barbiturate pentobarbital as well as the competitive NMDA antagonist CGS 19755 all produced at least partial and in some cases full substitution for isoflurane (Shelton & Nicholson, 2010). As was the case with ethanol, the presence of multiple stimulus contributors did not appear to diminish the ability of any of these individual drugs to engender significant substitution.

The phenomena of overshadowing may also have implications in subjects trained to discriminate N_2O from vehicle. Overshadowing can occur when the stimulus properties of one cue component of a compound stimulus are much more robust than other components (Jarbe & Johansson, 1976, Johansson & Jarbe, 1976). In these cases the stronger stimulus component may mask the ability of drugs which mimic the weaker cue component(s) to engender substitution. For instance, four groups of rats were trained to discriminate the anticholinergic drug ditran alone from vehicle, ditran plus the acetylcholinesterase inhibitor neostigmine from vehicle, and either ditran plus a low or high dose of physostigmine from vehicle. Neostigmine indirectly

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stimulate both nicotinic and muscarinic receptors by reversibly inhibiting cholinesterase. Ditran plus a low or high physostigmine dose did not substitute for ditran alone. It was believed that the presence of physostigmine prevented recognition of the ditran portion of the mixture. In a later study physostigmine plus ditran did not produce physostigmine-like stimulus effects (Johansson & Jarbe, 1976). Therefore it is possible that even if N_2O has multiple stimulus components, if a minor component is significantly weaker than the primary component this may produce a false negative.

Cross substitution of inhalants

One of the goals of the present series of studies is to examine the similarity between the stimulus effects of N_2O and other abused inhalants. The discriminative stimulus properties of several abused inhalants have been characterized in our laboratory. 1,1,1-trichlorethane (TCE) has been trained as a discriminative stimulus in mice (Shelton, 2009, 2010, Shelton & Nicholson, 2012). The mu opioid agonist morphine, several NMDA antagonists and nicotine all fail to substitute for TCE (Shelton, 2010). In contrast, midazolam, diazepam and pentobarbital produce dose-dependent increases in TCE-appropriate responding (Shelton & Nicholson, 2012). However, the benzodiazepine antagonist flumazenil did not antagonize TCE's discriminative stimulus therefore it is unlikely that TCE acts at the benzodiazepine recognition site. The abused solvent toluene and N_2O have numerous overlapping molecular targets [for review see (Bowen et al., 2006)]. Targets of toluene include NMDA, $GABA_A$, glycine, $5HT_3$, neuronal nicotinic acetylcholine, dopaminergic and muscarinic receptors as well as sodium, calcium and potassium channels. Several studies have characterized toluene's discriminative stimulus (Knisely et al., 1990, Rees et al., 1987, Shelton, 2007, Shelton & Nicholson, 2013, Shelton &

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Slavova-Hernandez, 2009). Benzodiazepines (Knisely, Rees, & Balster, 1990, Shelton &

Nicholson, 2013) and barbiturates (Rees, Knisely, Jordan, & Balster, 1987) produce robust

substitution in toluene trained subjects. However, numerous other classes of drugs which have

been implicated in molecular actions of toluene fail to substitute in toluene-trained mice (Shelton

& Nicholson, 2013). These studies suggest that even where *in vitro* and *ex vivo* data

demonstrated multiple receptor involvement in the CNS effects of a drug, the stimulus effects of

that drug need not be as promiscuous.

In sum the drug discrimination task is exceptionally useful for investigating the

mechanisms of action underlying the abuse-related behavioral effects of drugs and exploring

their pharmacological sites of action. This paradigm has been applied in the study of many drugs

of abuse some of which, like N_2O may affect more than a single CNS target. I therefore believe

that it will be a useful procedure for examining the abuse-related effects of N_2O.

Experimental Hypotheses

Given the above background I formulated several hypotheses in regard to the receptor mechanism underlying the discriminative stimulus effects of N_2O. First, N_2O reduces agonist mediated NMDA current in amygdalar slices (Ranft et al., 2007), hippocampal preparations (Jevtović-Todovorić et al., 1998, Mennerick et al., 1998) and in substantia nigra cells (Balon et al, 2003) as well as in heterologous expression systems (Ogata et al., 2006, Petrenko et al., 2010, Sato et al., 2005). Due to the strong evidence of interactions at NMDA receptors I believe NMDA antagonism is the primary mediator of the discriminative stimulus effects of N_2O.

NMDA receptors have several binding sites through which compounds can decrease receptor function (Nankai, 1998, Scatton, 1993). The binding sites include the glutamate binding site which can be antagonized directly as well as the binding site for the co-agonist glycine. In addition NMDA receptor function can be uncompetitively antagonized by drugs which binding within the channel or at the polyamine site (Gibson, Harris, Rogers, & Littleton, 2002). Given data showing animals can distinguish between NMDA antagonists acting upon the different sites it may be possible to more accurately pinpoint the site of action of N_2O using specific pharmacological tools, but at this point the available data do not readily lend themselves to a specific hypothesized interaction domain.

In addition to antagonizing NMDA receptors, in isolated systems as well as heterologous expression systems, N_2O potentiates agonist mediated $GABA_A$ currents (Dzoljic & Van Duijn, 1998, Hapfelmeier et al., 2000, Hapfelmeier et al., 2001). Further, the subjective effects of N_2O are attenuated by administration of a benzodiazepine site antagonist, flumazenil (Zacny et al., 1995). Therefore I hypothesize that $GABA_A$ receptor positive allosteric modulation may also

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play a role in the abuse-related discriminative stimulus effects of N_2O. $GABA_A$ receptor activity can be increased by drugs which bind at the agonist-binding site, drugs which indirectly increasing GABA levels as well drugs which bind at one of the positive allosteric sites. Positive allosteric modulatory sites have been identified for the binding of barbiturates, benzodiazepines and GABA positive neurosteroids. Rodent drug discrimination assays appear to be capable of differentiating between drugs acting at some but not all of these sites. For instance, direct agonists such as muscimol can be differentiated from positive allosteric modulators (Jones & Balster, 1998). Specifically, the direct extrasynaptic $GABA_A$ receptor agonist gaboxadol produces full substitution for muscimol, however, the positive allosteric modulator diazepam produced ~50% substitution for muscimol (Jones & Balster, 1998). However, there is little difference in the degree of substitution produced by barbiturates and benzodiazepines in cross substitution testing. When pentobarbital is trained as a discriminative stimulus midazolam produced full substitution for pentobarbital (Grech & Balster, 1994). Furthermore, both midazolam and pentobarbital produced about the same degree of substitution in isoflurane-trained mice whereas muscimol failed to substitute for isoflurane entirely (Shelton & Nicholson, 2010). Given that $GABA_A$ receptor positive allosteric modulators are distinguishable from direct $GABA_A$ agonists in the drug discrimination task I would expect that some degree of $GABA_A$ receptor site specificity of N_2O can be determined but it will likely not be as clear as data generated with NMDA antagonists.

My final aim was to determine the degree of similarity between N_2O and other abused inhalants. Based on the existing data showing that both toluene and 1,1,1-trichloroethane have GABAergic effects in *ex vivo* and in *in vitro* assays (Beckley & Woodward, 2011, Beckstead et al., 2000, Filley, Halliday, & Kleinschmidt-DeMasters, 2004) as well as $GABA_A$ positive

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modulator-like effect in drug discrimination (Rees et al., 1987), I predict that there may be some overlap in the discriminative stimulus effects of N_2O and these inhalants. A prior drug discrimination study from our laboratory has shown that the discriminative stimulus effects of isoflurane are elicited by both NMDA antagonists as well as $GABA_A$ positive modulators (Shelton & Nicholson, 2010). As I hypothesize that the stimulus effects of N_2O are also the result of positive $GABA_A$ modulation and NMDA antagonism, I predict that the stimulus effects of N_2O will be more similar to isoflurane than either toluene or 1,1,1-trichloroethane. However, this hypothesis is somewhat more tentative given other data from our laboratory showing that N_2O does not produce isoflurane-like stimulus effects in mice trained to discriminate isoflurane from air (Shelton & Nicholson, 2010).

Materials

<u>Subjects</u>

Forty adult male B6SJLF1/J mice (The Jackson Laboratory, Bar Harbor, Maine) served as subjects. These F1 hybrid mice, derived from C57BL6/J female and SJL/J male parents, have been used extensively in prior inhalant drug discrimination studies conducted in our laboratory (Shelton, 2007, 2009, 2010, Shelton & Nicholson, 2010, 2012, Shelton & Slavova-Hernandez, 2009). Nine of forty mice were not naïve; having been previously trained to discriminate 40% N_2O from 100% oxygen under the supervision of another student in the laboratory (unpublished). All subjects were individually housed on a 12-h light/dark cycle (lights on 6:00 AM). To promote operant responding the mice were maintained at 85% free feed body weights by restricting 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. The first determination of 85% free feed body weight occurred approximately three weeks after arrival from the vendor and re-determination occurred after 1-2 week biannual *ad libitium* feeding periods. For example, a mouse weighing 32 grams week-3 of the study was initially maintained at a food restricted weight of 27 grams. .When not in the laboratory the mice were housed on an Enviro-Gard^{TM} B ventilator cage rack (model 59016; Lab Products Inc., Seaford, DE, USA) in a colony room maintained at 77^0F with 44% humidity.

<u>Apparatus</u>

Operant sessions were conducted in two-lever mouse operant conditioning chambers equipped with 0.01-ml liquid dippers (model ENV-307AW; MED Associates, St. Albans, VT, USA). One yellow LED lever light was above each of two response levers which were located

on the front chamber wall. 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 reinforcer consisted of 25% sugar (Great Value Foods; Walmart, Richmond, VA, USA), 25% nonfat powdered milk (Great Value Foods; Walmart, Richmond, VA, USA), and 50% tap water by volume.

Initially exposures to oxygen and N_2O /oxygen gas mixtures were conducted within a converted 9.9-L Secador mini vacuum desiccator cabinet (Bel-Art Products, Pequannock, NJ, USA) that served as an exposure chamber. Following exposure, discrimination training was conducted in standard mouse operant conditioning chambers housed inside 63.5 cm x 41.9 cm x 39.4 cm sound attenuating cubicles (Med Associates, St Albans, VT, USA). The appropriate mixture of N_2O and oxygen was controlled by a manually-operated metering system. An Airsep Onyx+ oxygen concentrator (Buffalo, NY, USA) generated 98+% oxygen directed by Tygon tubing (Fisher Scientific, Hampton, NH, USA) through a rotometer to regulate oxygen flow rate. Nitrous oxide gas (National Welders Supply, Richmond, VA, USA) flowed from a medical compressed N_2O cylinder through a single stage regulator. The N_2O flow rate was regulated by a second rotometer. Downstream from the rotometers oxygen and N_2O were combined at a Y fitting prior to passing through a hose barb into the 9.9-L inhalant exposure chamber. Waste gas was expelled through a second length of Tygon tubing into a fume hood.

After several months of training and testing it was concluded that the above system was inadequate due to the fast offset/limited duration of action of N_2O following the cessation of exposure (see Results). Therefore, the remainder of the study was conducted using a system which combined the inhalant exposure and operant test equipment a single apparatus (Appendix

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1). The revised apparatus consisted of four 26.0-L acrylic exposure cubicles which encased modified two-lever mouse operant conditioning chambers. To accommodate substitution tests of vapors each exposure chamber was also fitted with an internal 80mm 24-Volt DC fan mounted in an acrylic frame with a perforated metal filter paper attachment grill. The fan motors were connected to MED-PC 28v output allowing automated control of vapor volatilization. Vapor exposures were accomplished by injection of a fixed volume of volatile liquid onto filter paper using a glass gas-tight syringe. The ideal gas law as derived for vapors at standard laboratory temperature and pressure was used to determine the appropriate volume of volatile liquid introduced into the exposure chamber (Shelton, 2007). Nitrous oxide/oxygen flow was accomplished using the delivery system previously described (Appendix 2).

Drugs

Medical N_2O cylinders were obtained from National Welders Supply (Richmond, Virginia, USA). Oxygen was produced by an Airsep Onyx+ oxygen concentrator (Buffalo, NY, 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), NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME), (±)-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, gaboxadol (THIP), (+)-MK-801 (dizocilpine), nicotine bitartrate, toluene, TCE and 2-butanol were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Isoflurane and ketamine were purchased from Webster/Patterson Veterinary Supply (Devens, MA, USA). Midazolam was a gift of Roche Pharmaceuticals (Nutley, NJ, USA). Methoxyflurane was obtained from Pitman-Moore (Mundelein, IL, USA).

Sarcosine and ethanol (95% weight/volume) were obtained from Acros Organics (Fair Lawn, NJ, USA). Morphine sulfate, D-amphetamine 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-[(αR)-α-((2S,5R)-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).

All drugs except ethanol were prepared to achieve a constant injection volume of 10 ml/kg. Ethanol doses up to 1 g/kg were prepared to achieve an injection volume of 10 ml/kg. To prevent tissue damage, ethanol doses higher than 1 g/kg were accomplished by administering higher volumes of 100 mg/ml ethanol. Nicotine doses were based on the weight of the base and pH adjusted to between 6 and 7 with 0.1 N NaOH. L-701,324 was dissolved in 10% cremophor in sterile water. The vehicle for SNC-80 was 0.9% saline with one or two drops of hydrochloric acid pH adjusted to between 6 and 7. All other injected compounds were dissolved in 0.9% saline.

Morphine sulfate and nicotine bitartrate were administered subcutaneously (s.c.). All other injected compounds were administered intraperitoneally (i.p.). Exposure to isoflurane, methoxyflurane, toluene, TCE and 2-butanol were accomplished by fixed volume injection onto filter paper with volatilization and circulation aided by the MED-PC controlled fans. 10% cremophor dissolved in sterile water served as the vehicle control for L-701,324. 0.9% saline solution was used for injections during all other control tests.

SNC-80, sarcosine, memantine, muscimol, gaboxadol (THIP), CGS 19755, L-701,324, L-NAME and U50-488H were administered with a 30 minute pre-treatment time. mCPP was administered with a 20 minute pre-treatment time. All other injected drugs were administered 10

minutes before the operant test session. Pre-treatment conditions and doses/concentrations used for substitution tests are summarized in Table 1. Except when indicated, N_2O exposures were begun 10 minutes before the start of the operant session and continued for the duration of the operant test session. Exposures to volatile vapors, oxygen and N_2O-oxygen mixtures were begun 10 minutes before the session and continued for the duration of the 5 minute test session.

Table 1. Summary of pretreatment conditions, doses tested and corresponding sources used for substitution tests in subjects trained to discriminate 60% N_2O + 40% O_2 from 100% O_2.

Drug	Vehicle	Route of administration	Pretreatment time	Doses/concentrations tested
N_2O [13,14]	oxygen	inhaled	10 mins	5, 10, 20, 40, 60, 80%
L-701,324 [13,14]	10% cremophor/ sterile water	i.p.	30 mins	3, 10, 17, 30 mg/kg
CGS 19755 [13,14]	0.9% saline	i.p.	30 mins	1, 3, 10, 17 mg/kg
(+)-MK-801 [11,13,14]	0.9% saline	i.p.	10 mins	0.03, 0.17, 0.3, 0.56, 0.75 mg/kg
Ketamine [6]	0.9% saline	i.p.	10 mins	3.0, 10.0, 15.6, 30.0 mg/kg
Memantine [5]	0.9% saline	i.p.	30 mins	3, 10, 17, 30, 56 mg/kg
Sarcosine [4]	0.9% saline	i.p.	30 mins	100, 300, 600, 1200 mg/kg
Pentobarbital [13,16]	0.9% saline	i.p.	10 mins	3, 10, 17, 30, 50 mg/kg
Midazolam [11]	0.9% saline	i.p.	10 mins	1, 3, 10, 17, 30, 56 mg/kg
Valproic acid [13,16]	0.9% saline	i.p.	30 mins	100, 300, 560 mg/kg
THIP [14]	0.9% saline	i.p.	30 mins	0.3, 1.0, 3.0, 10.0 mg/kg
Muscimol [13]	0.9% saline	i.p.	30 mins	0.3, 1.0, 1.7, 3.0 mg/kg
Isoflurane [9,13]	air	inhaled	10 mins	1000, 2000, 4000, 6000 ppm
Methoxyflurane [12]	air	inhaled	10 mins	500, 1000, 2000, 4000 ppm
Toluene [2,9,10,13,15]	air	inhaled	10 mins	500, 1000, 2000, 4000, 8000 ppm
TCE [10,11,13,14]	air	inhaled	10 mins	1000, 4000, 8000, 12000 ppm
2-butanol [10]	air	inhaled	10 mins	10, 30, 100 ppm
Ethanol [14]	0.9% saline	i.p.	10 mins	1.0, 1.5, 2.0, 2.5 g/kg
mCPP [14]	0.9% saline	i.p.	20 mins	0.1, 1.0, 5.6, 10.0 mg/kg
8-OH DPAT [1]	0.9% saline	i.p.	10 mins	0.1, 0.3, 1.0, 1.56 mg/kg
D-amphetamine [2,17]	0.9% saline	i.p.	10 mins	0.1, 0.3, 1.0, 1.56 mg/kg
Morphine [11]	0.9% saline	s.c.	10 mins	1.0, 1.7, 3.0, 10.0, 30.0 mg/kg
SNC-80 [3,8]	0.9% saline	i.p.	30 mins	3, 10, 17, 30, 56 mg/kg
U50-488H [17]	0.9% saline	i.p.	30 mins	1.0, 3.2, 7.0, 10.0 mg/kg
nicotine [11]	0.9% saline	s.c.	10 mins	0.1, 0.3, 1.0, 1.7, 2.5 mg/kg
L-NAME [7]	0.9% saline	i.p.	30 mins	1, 10, 30 mg/kg

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Methods

<u>Training procedure</u>

Upon arrival mice were individually housed in 31.5cm x19.5cm clear polycarbonate cages with corncob bedding (Teklad, Madison, WI, USA). Mice were habituated to the home cage and maintained on free feed (Teklad Lab Diet, Madison, WI, USA) for seven days. During the last two days of habituation the mice were weighed, handled and tails were marked with a color coded numeric identifier in permanent marker. Beginning the following week mice were trained once daily. Adjustment to the desired final fixed ratio requirement, timeout length and session length occurred in four stages.

<u>1. Initial training</u>

Initial operant training began with one 14-hour overnight session conducted during the dark cycle. At the beginning of the session the house light and light above each lever were illuminated. The first 6 hours of the program provided non-contingent intermittent access to a 0.01 ml dipper cup of sweetened milk (25% sugar, 25% nonfat powdered milk and 50% tap water by volume) to engender head entries into the dipper aperture. During this 6 hour period the dipper cup was available for the first 10 seconds of every 60 second period. For the subsequent 8 hours, presentation of milk only occurred after completion of a response on either lever under a fixed ratio 1 (FR-1) schedule of reinforcement. Each lever press resulted in 10 seconds of milk access and a 5 second timeout in which no responses were recorded. Following the overnight session mice were trained once daily Monday to Friday for the remainder of the study.

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2. Decreasing session length and one active lever

The first session following the initial 14 hour training procedure was a 4 hour session under a FR 1 schedule of reinforcement where completion of the FR requirement on either lever produced reinforcement. Each lever press resulted in 3 seconds of access to 0.01 ml sweetened milk. Over the next four days the session length was shortened to 2 hours, 1 hour, 40 minutes then 30 minutes.

3. Increasing the fixed ratio and decreasing the session length

Change in active lever assignment, FR or session length were accomplished by changing only one variable each training day. Initially the active lever was alternated daily at FR 1. On the third day the FR requirement was increased to FR 2. On the fourth day the alternate lever was reinforced on a FR 2 schedule. Once mice were reliably alternating responding between levers the session length was shortened to 20 minutes.

For the subjects trained in the 9.9-L apparatus the operant session length was gradually decreased from 20 minutes to 5 minutes across successive days. Once the session length was decreased to 5 minutes the 100% oxygen and 60% $N_2O+40\%$ O_2 pairing began. Subjects were trained to discriminate a 10 minute exposure to 60% $N_2O+40\%$ O_2 mixture from 100% O_2. Subjects were assigned one lever as correct after oxygen exposure and the alternate lever as correct after 60% $N_2O+40\%$ O_2 exposure. Lever assignments were counterbalanced so that an equal number of subjects had right and left levers designated as N_2O appropriate. Drug and vehicle were presented in a double alternation sequence across days (O_2, O_2, N_2O, N_2O).

For the subjects trained in the 26.0-L apparatus three parameters were adjusted over the course of initial training. The operant session length was gradually decreased from 30 minutes to

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15 minutes and then a timeout of increasing duration, up to the desired timeout of 10 min, was introduced prior to the start of the operant session. During the timeout all lights were off and responses were not recorded. Once the animals reliably responded only after the completion of the timeout, the session length was decreased to five minutes and discrimination training between 60% N_2O+40% O_2 and 100% O_2 began. Over successive training days the FR requirement was gradually increased to the final target value of FR 12.

Discrimination acquisition criteria and N_2O concentration-effect curve

The double alternation of training conditions continued Monday through Friday until mice acquired the discrimination according to the designated acquisition criteria. In at least 8 of 10 consecutive training days the subject must have emitted the first fixed ratio on the correct lever. Additionally, in each of these 8 sessions a minimum of 80% of total lever presses must have been emitted on the correct lever. After meeting both criteria, subjects were eligible to test if they maintained accurate stimulus control on training sessions between tests. Specifically the subject must have emitted the first fixed ratio on the correct lever and minimum 80% of total lever presses on the correct lever in all of the training sessions between each Tuesday and Friday 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.

<u>Data collection</u>

The dependent measures collected were percentage nitrous oxide lever responding (±SEM), operant response rate (±SEM) and the lever upon which the first full fixed ratio was completed. Mean percentage nitrous oxide lever responding was the number of responses on N_2O-appropriate lever ÷ (responses on N_2O-appropriate lever + responses on vehicle-appropriate lever). Session data were recorded in 30-second bins. For generalization curves generated using the 9.9-L exposure apparatus which necessitated operant testing under room air, only the 1st minute data were analyzed. For generalization curves generated in the 26.0-L apparatus which housed operant test equipment inside an inhalant exposure chamber full 5 minute test session data were analyzed.

<u>Pharmacokinetic testing procedure: Onset of discriminative stimulus effect</u>

To determine the onset of discriminative stimulus effects of N_2O, mice were exposed to 60% N_2O for progressively shorter periods of time prior to the start of the 5 minute operant test session. The timeout period prior to the onset of the operant session remained 10 minutes but introduction of N_2O to oxygen flow into the operant chamber began later into the timeout. Group mean percentage drug lever responding and operant response rates were calculated for 1, 3, 7 and 10 minute pre-exposure durations.

<u>Pharmacokinetic testing procedure: Effect of exposure duration and duration of stimulus effects</u>

To approximate whether the training exposure duration of 10 minutes was sufficient to produce steady-state tissue concentrations of N_2O, the mice were exposed to a concentration of

N$_2$O which produced partial substitution for both the normal 10 minute pretest exposure duration as well as for a longer 20 minute pretest exposure duration. To compare the exposure conditions, an ANOVA with repeated measures (concentration X time) was used. This was conducted for both percentage drug-lever responding data and response rate data during the 60% N$_2$O/10 minute control session and for both 10 minute and 20 minute 30% N$_2$O exposures.

To estimate the offset of discriminative stimulus effects, mice were exposed to 100% O$_2$ or 60% N$_2$O+40% O$_2$ for 10 minutes. Progressively longer timeout periods prior to the discrimination session were then instituted. Group mean percentage N$_2$O lever responding and operant response rates were calculated for 1, 3 and 5 minute test delays.

Cross substitution test procedure

Substitution tests were conducted each Tuesday and Friday provided that the mice continued to exhibit accurate stimulus control during Monday, Wednesday and Thursday training sessions. On test days, both levers were active. Completion of the FR on either lever resulted in reinforcer presentation. When the test drug was an injected compound both the O$_2$ and N$_2$O control test exposures were preceded by vehicle injections.

Data analysis

Both the percentage N$_2$O-lever responding and operant response rates generated for overall responding were analyzed using an analysis of variance (ANOVA) with repeated measures. In all cases an effect was considered significant if $P< 0.05$. Dunnet's post hoc comparison tests were conducted where appropriate. Responding of less than 20% is considered no substitution, 21%-79% is considered partial substitution for N$_2$O and 80%-100% is considered full substitution for N$_2$O. Where possible confidence limits, potencies and half

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maximal effective concentration or dose values (EC_{50} or ED_{50}) of percentage N_2O-lever

responding and operant response rates were calculated using a Microsoft Excel spreadsheet

based on published methods (Bliss, 1967).

Results

<u>Aim 1: Training N$_2$O as a discriminative stimulus and characterizing the pharmacokinetics of its discriminative stimulus effects</u>

Of the 16 mice which began training, 13 acquired the discrimination between 60% N$_2$O+40% O$_2$ and 100% O$_2$ vehicle when operant training was conducted in standard operant chambers following cessation of gas exposure. Figure 1 plots the number of training sessions required to reach the acquisition criteria for each individual subject as a step graph. The shortest number of days to acquire was 88 and the most extended was 180 with a mean of 137 ($\pm$9) training sessions for 13 of 16 mice to meet criteria. One of the remaining subjects was moved to the new system as a consequence of failing to acquire the discrimination after an excess of 195 training sessions. Training under room air was ceased after 45 sessions for two remaining subjects newly added to the study.

When exposed in 9.9-L exposure system and tested in room air, N$_2$O produced concentration-dependent substitution for the 60% training concentration [$F_{(12,72)}$=1.61, P<0.05] (Figure 2, upper panel). Only data from the first minute of the 5 minute test session are shown. Control tests that followed 10 minutes exposure to oxygen or the N$_2$O-oxygen mixture produced a mean of 15% ($\pm$8) and 86% ($\pm$8) N$_2$O respectively. Concentrations of 20% to 60% N$_2$O produced partial substitution. Full substitution for the 60% N$_2$O+40% O$_2$ training condition occurred at a test concentration of 66% N$_2$O. Nitrous oxide exposure did not alter operant response rates (Figure 2, lower panel). Response rates across all N$_2$O test concentrations did not significantly differ from oxygen control response rate [$F_{(12,72)}$=3.22, P=0.42]. Using these training and test procedures acquisition was so lengthy and eligibility to test infrequent that the

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training apparatus was modified to allow exposure to the N_2O or oxygen training conditions to be continued during the operant training sessions.

The same sixteen mice were subsequently retrained in a 26.0-L continuous exposure/operant test system under conditions in which 60% N_2O+40% O_2 or 100% O_2 exposure was continued throughout the operant discrimination training session (Table 2). Subsequent re-training under these new conditions required a mean of 45.4 (±8.4) training days to re-establish the discrimination. Further, the three subjects who previously did not meet acquisition criteria were able to acquire the discrimination in a mean 67.3 (± 17.7) additional training days. As subjects acquired more rapidly in continuous exposure/operant test system six naïve subjects were ordered to supplement the remaining subjects and replace two subjects which died.

Figure 3 (upper panel) shows the N_2O concentration effect curve in all subjects retrained in the continuous exposure/test system. Nitrous oxide produced concentration-dependent full substitution for the 60%+40% O_2 training concentration with an EC_{50} of 28% (CL 11% – 67%). Concentrations below 40% N_2O did not produce significant substitution for the training condition. Full substitution occurred at the training concentration as well as the 80% N_2O exposure condition. Nitrous oxide exposure significantly attenuated operant response rates at the highest tested concentration of 80% (Figure 3, lower panel) [$F_{(12,72)}$=5.3, P<0.05].

At this point in the study the subjects were euthanized due to the presence of an unrelated viral disease in the rodent colony which is normally asymptomatic in adult mice. However, this unfortunate circumstance permitted the training of new subjects which did not have a complicated acquisition training history. A total of 24 naïve subjects were trained to discrimination 60% N_2O+40% O_2 from 100% O_2 in the continuous exposure/test apparatus. The

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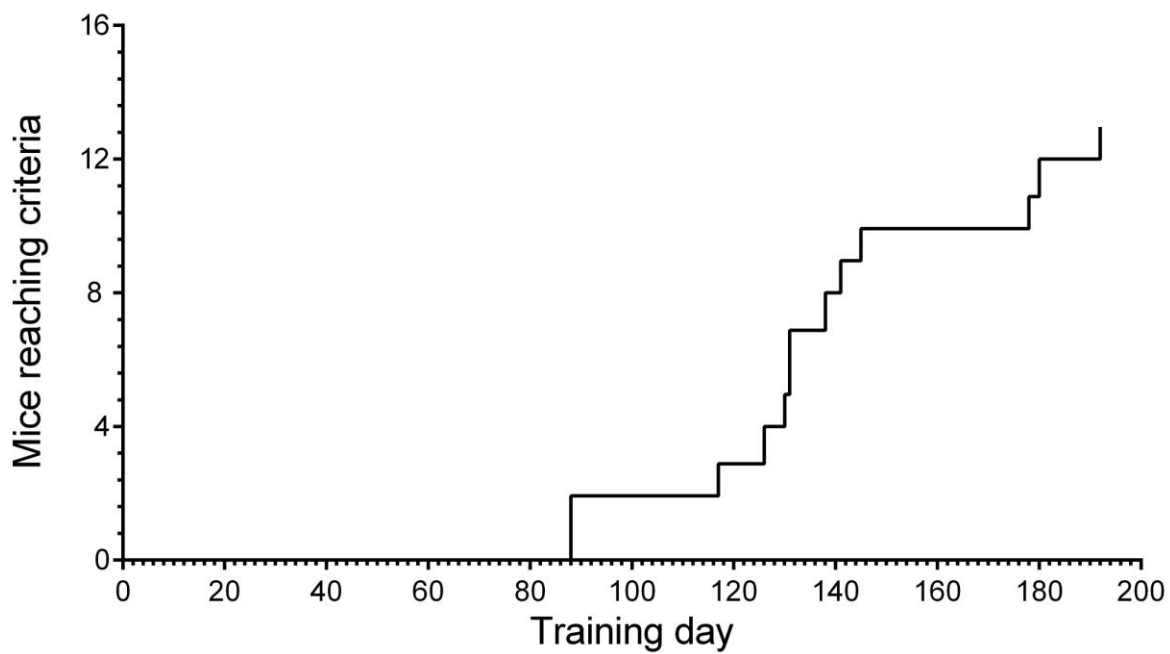
Figure 1. Stepwise acquisition plot showing the day acquisition criteria was met for 13 of 16 mice trained to discriminate 60% nitrous oxide + 40% oxygen from 100% oxygen. Data are from animals in which operant training sessions were conducted post-exposure in room air.
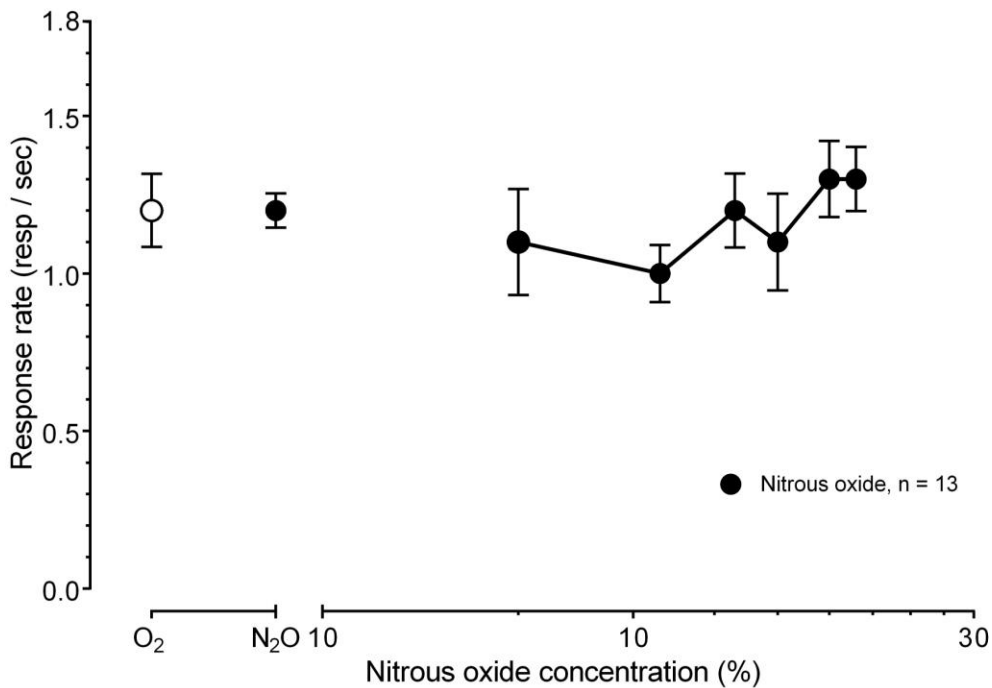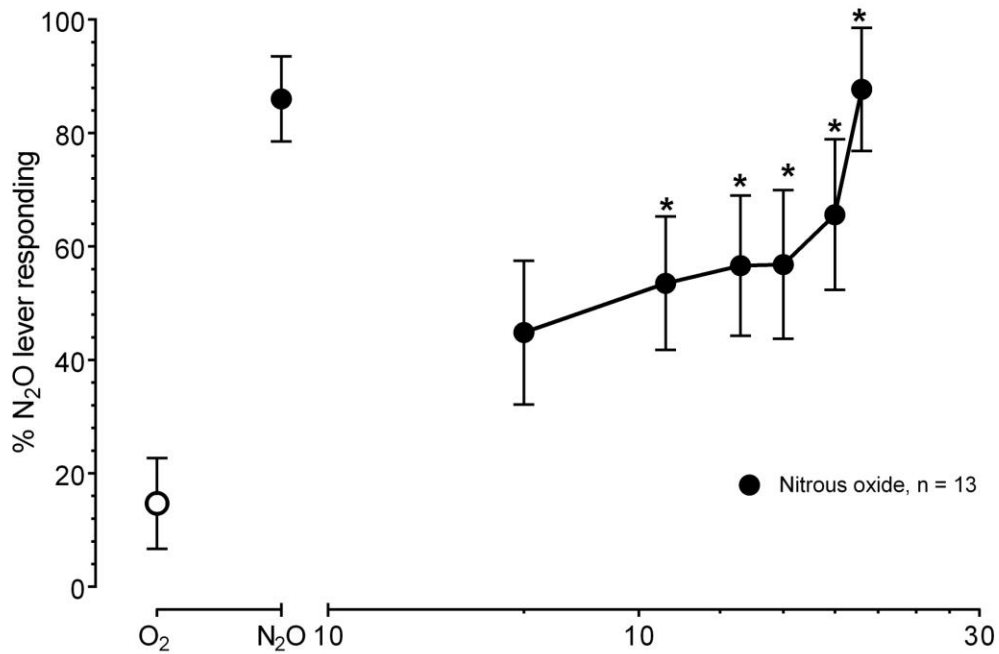
Figure 2. Nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% nitrous oxide + 40% oxygen from 100% oxygen following the cessation of 10 minutes of exposure. Points above O_2 and N_2O reflect the 60% nitrous oxide + 40% oxygen and the 100% oxygen control conditions. * indicate significant ($p < 0.05$) differences from oxygen control.

Table 2. Number of training days for individual subjects to acquire the initial 60% N_2O+40% oxygen versus 100% oxygen discrimination. Column data shown acquisition days when operant training followed cessation of gas exposure as well as days to reacquire the discrimination when gas exposure continued throughout the operant session. # denotes subjects which failed to meet acquisition criteria under initial training conditions.

Subject number	Acquisition Days Training after cessation of exposure	Reacquisition days Training under concurrent exposure
1A	138	88
2A	131	33
3A	180	33
4A	145	36
5A	178	112
6A	131	23
7A	192	33
9A	88	39
10A	141	36
11A	#	33
13A	#	104
14A	#	65
1N	117	Deceased
2N	88	32
3N	126	Deceased
4N	130	34
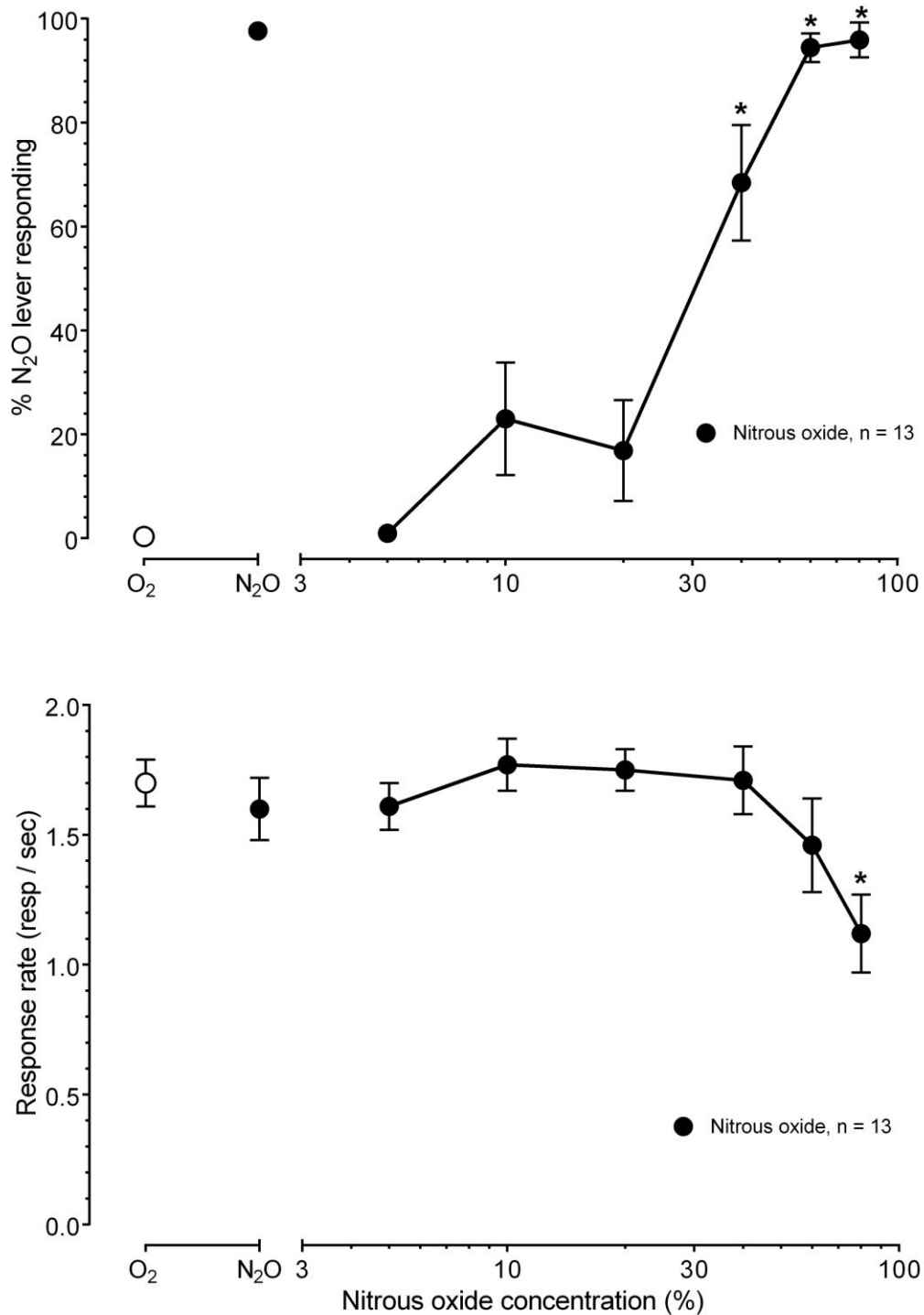
Figure 3. Nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice re-trained to discriminate 60% nitrous oxide from oxygen following 10 minutes of exposure and continued exposure through training/ testing.* indicates significant (p< 0.05) differences from oxygen control.

subjects acquired the discrimination in a mean of 38.2 (± 2.5) training sessions. Figure 4 shows the acquisition day of each subject plotted on a step graph. The shortest number of acquisition days was 24 and the most extended was 71 with a median of 34.5 training sessions for all 24 mice to meet acquisition criteria. Figure 5 shows the first fixed ratio (FFR) data from the first 71 training sessions separated by training condition. Initial FFR choice approximated 50% chance levels of discrimination accuracy. By N_2O training session 36, mean group FFR accuracy was over 90%. There was no difference in the speed of acquisition as a function of 60% N_2O+40% O_2 or 100% O_2 training conditions.

Nitrous oxide produced concentration-dependent full substitution for the 60% training concentration with an EC_{50} of 25% (CL 19 – 32%) (Figure 6, upper panel). Control tests of 100% O_2 and 60 % N_2O+40% O_2 produced a mean of 2% (±1) and 95% (±1) N_2O, respectively. Concentrations of 5% and 10% N_2O did not substitute for the training concentration. N_2O-lever selection was significantly greater than the vehicle condition [$F_{(23,138)}$=4.07, P<0.05] at concentrations of 20% N_2O and higher. Partial substitution for 60% N_2O+40% O_2 occurred at 20% and 40% N_2O concentrations. Full substitution occurred at the training concentration as well as the 80% N_2O exposure concentration. Nitrous oxide exposure produced a significant elevation of operant response rates compared to vehicle at the 20% concentration and a significant suppression of operant response rates at the highest 80% test concentration [$F_{(23,138)}$=6.8, P<0.05] (Figure 6, lower panel).

As a measure of continued discrimination accuracy following initial training, approximately two months after acquisition the number of days each mouse was eligible to test across 10 consecutive Tuesday and Friday testing opportunities (5 weeks x 2 test sessions/week) was tabulated as a percentage (Figure 7). On any given test day 87.5% or 21 of 24 subjects were

eligible to test. Though individual subjects tested between 40-100% of total testing opportunities, the median value was 90%.

To elucidate the onset kinetics of the stimulus properties of nitrous oxide, exposures of 1, 3, 7 and 10 minutes to 60% N_2O+40% O_2 were tested for their ability to substitute for the 10 minute 60% N_2O+40% O_2 exposure training condition (Figure 8, upper panel). Exposure durations of 1 and 3 minute produced 23% ($\pm$6) and 44% ($\pm$8) N_2O-appropriate responding, respectively. Seven minutes of exposure produced 93% ($\pm$3) N_2O-lever responding Only the 1 and 3 minute exposure durations resulted in significantly lower N_2O-appropriate responding as compared with the training exposure duration of 10 minutes [$F_{(20,60)}$=2.52, P<0.05]. There were no meaningful alternations is response rates as a function of exposure duration (Figure 8, lower panel).

To determine if the 10 minute training exposure duration produced the maximal possible stimulus effects at a given N_2O concentration, substitution tests were performed with 30% N_2O exposures of both 10 and 20 minutes, as well as the training condition of 60% N_2O+40% O_2 exposure for 10 minutes (Table 3). Extended exposure to 30% N_2O for 20 minutes did not significantly increase the mean percentage N_2O-lever responding compared with that produced by 10 minutes of exposure to 30% N_2O. However, both the 10 and 20 minutes exposures to 30% N_2O occasioned significantly less N_2O-appropriate responding than exposure to 60% N_2O+40% O_2 for 10 minutes [$F_{(10,20)}$=1.57, P<0.05]. Operant response rates following 10 and 20 minutes of exposure to 30% N_2O were not significantly different from each other but both were slightly but significantly greater than response rates after 10 minutes of exposure to 60% N_2O+40% O_2 [$F_{(11,22)}$=2.35, P<0.05].
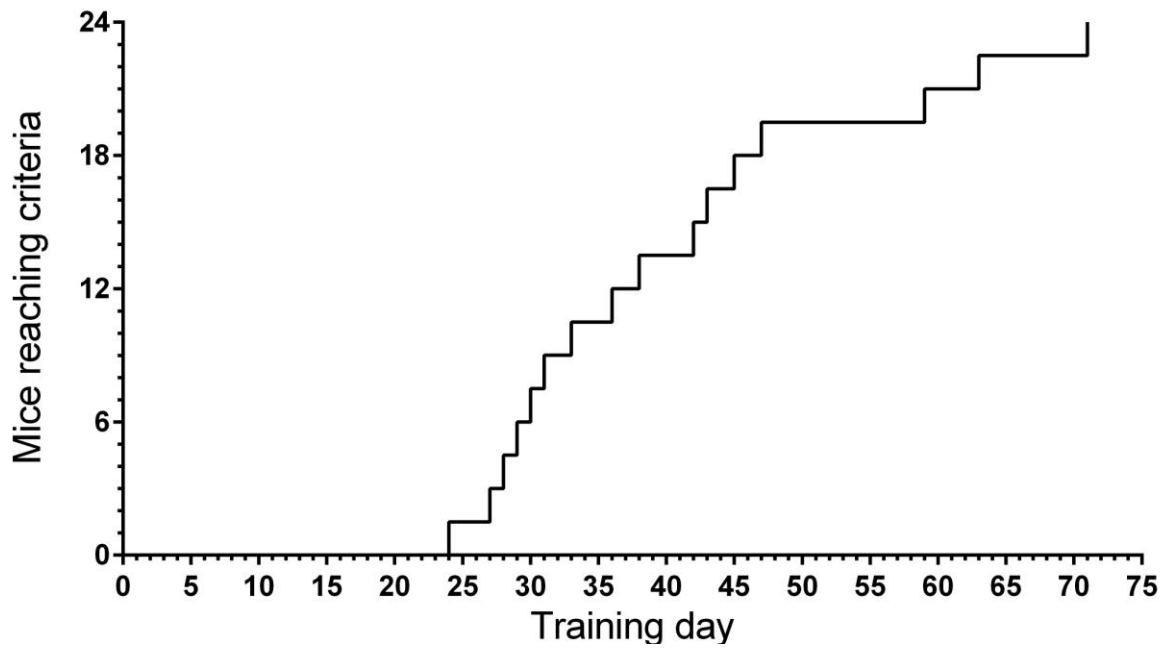
Figure 4. Stepwise acquisition plot showing the day acquisition criteria was met for each of 24 mice trained to discriminate 60% nitrous oxide + 40% oxygen from 100% oxygen. Data are from animals in which gas exposure was continued for the duration of the operant training session.

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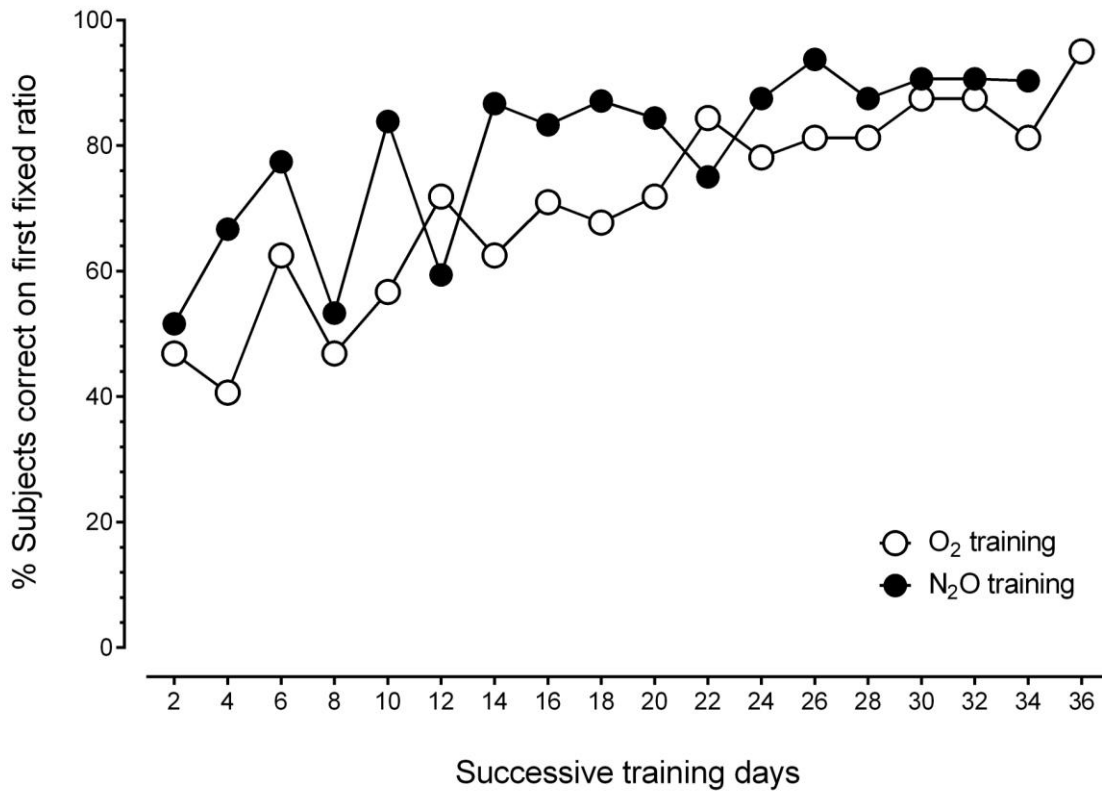
Figure 5. Learning curves of 24 naïve subjects trained to discriminate the stimulus effects of 60% nitrous oxide + 40% oxygen (closed circles) from 100% oxygen (open circles).
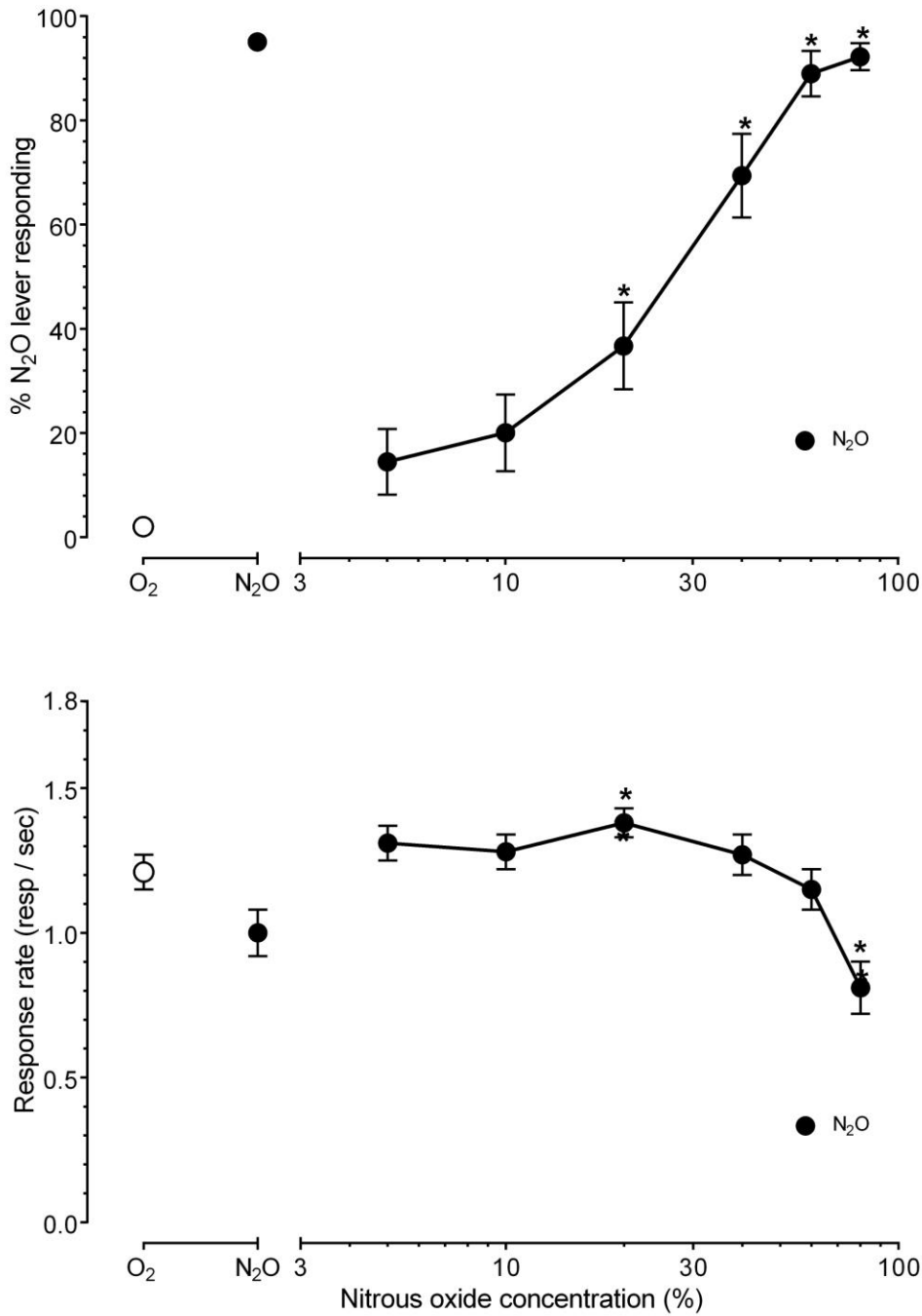
Figure 6. Nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 10 minutes of exposure to 60% nitrous oxide +40% oxygen from oxygen. Points above O_2 and N_2O reflect the 60% nitrous oxide + 40% oxygen and the 100% oxygen control conditions. * indicate significant ($p < 0.05$) differences from oxygen control.
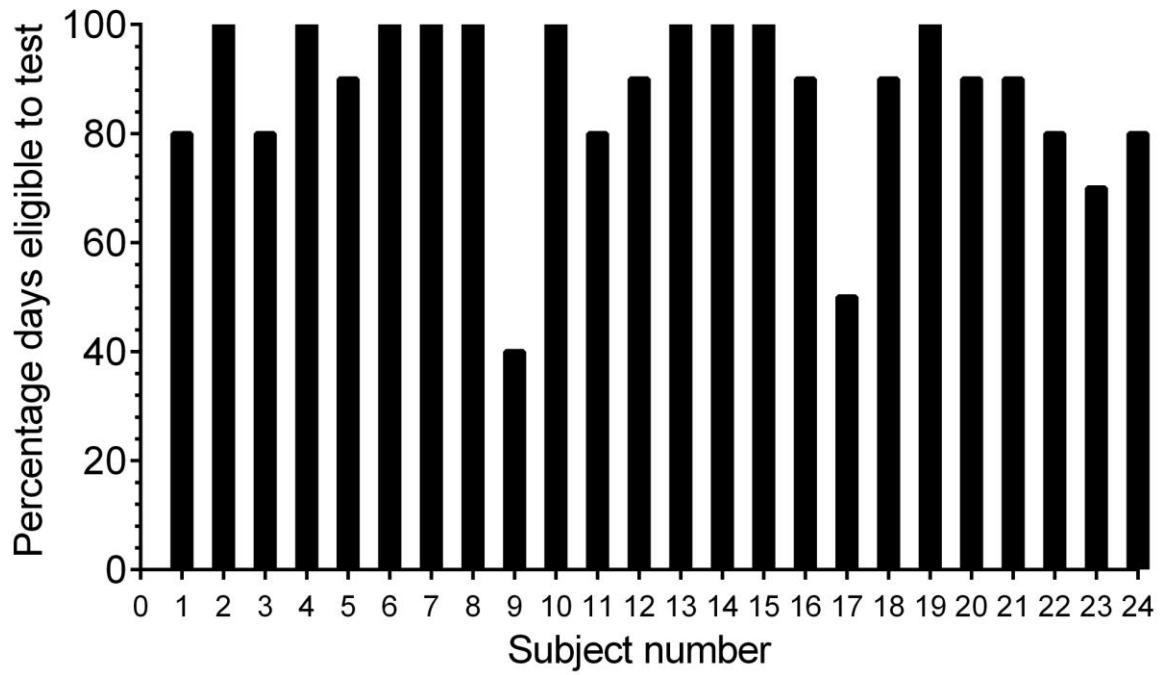
Figure 7. Percentage of 10 total Tuesday and Friday test sessions which each of 24 subjects met testing eligibility criteria.
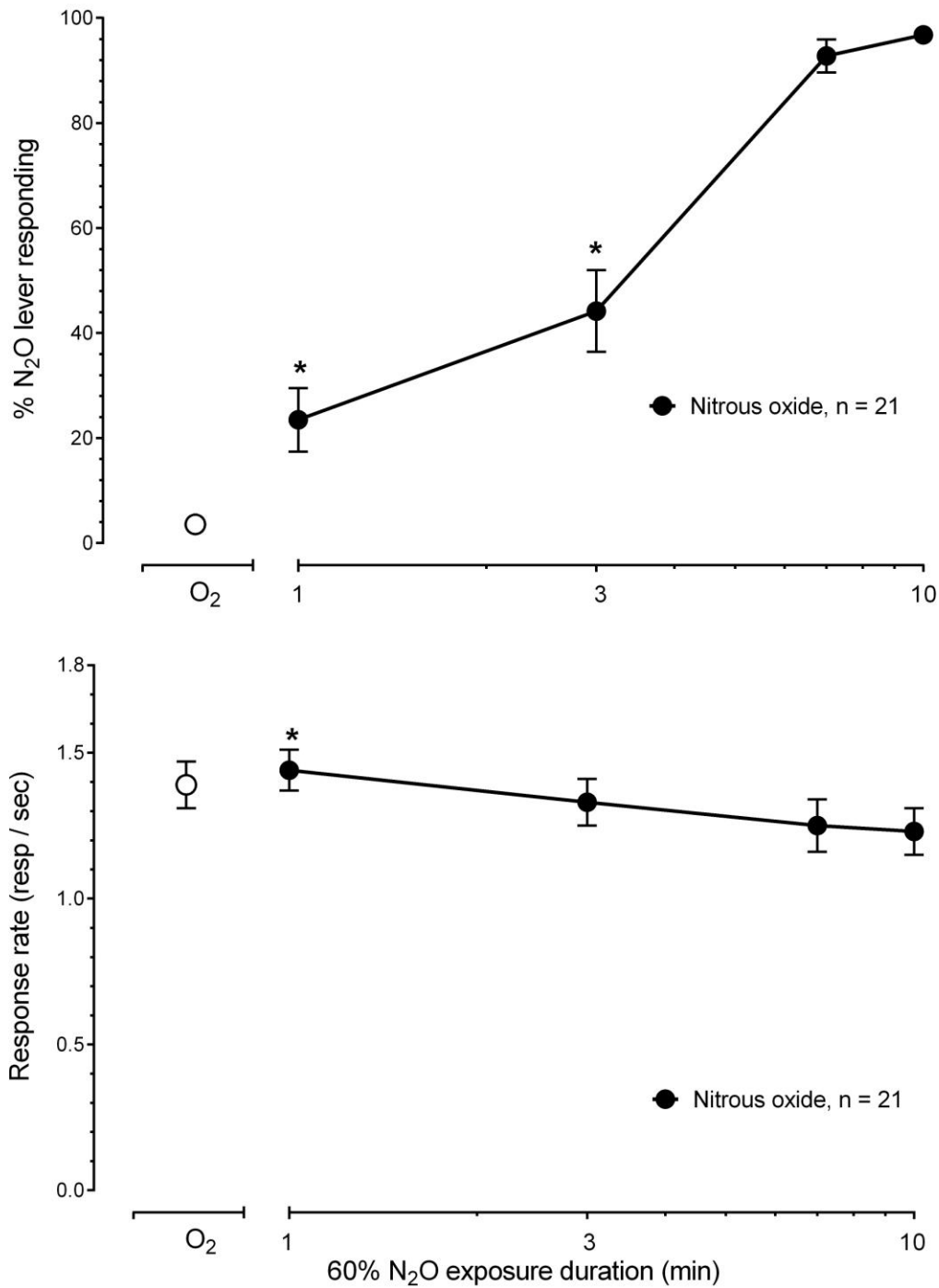
Figure 8. Onset of effects. Mean (±SEM) percentage nitrous oxide lever responding (upper panel) and operant response rates (lower panel) produced on varying the duration of 60% nitrous oxide +40% oxygen exposure. * indicates significant ($p < 0.05$) differences from the 10 minute 60% nitrous oxide + 40% oxygen exposure condition.

Table 3. Percentage N_2O lever responding (±SEM) and responses per second (±SEM) produced during exposure to training condition of 60% N_2O+ 40% oxygen for 10 minutes versus exposures to 30% N_2O for either 10 or 20 minutes. * indicates significant ($p < 0.05$) differences from the 10 minutes 60% N_2O exposure condition.

	100% O_2 10 min (±SEM)	60% N_2O 10 min (±SEM)	30 % N_2O 10 min (±SEM)	30% N_2O 20 min (±SEM)
% N_2O lever responding	1.3 (0.6)	91.3 (2.5)	40.6 (9.4) *	37.2 (10.3) *
Responses/sec	1.3 (0.1)	1.1 (0.1)	1.4 (0.1) *	1.5 (0.1) *

To assess the offset kinetics of N_2O discriminative stimulus the effects increased lengths

of delays in the start of the operant discrimination session were introduced following a 10 minute

exposure to 60% N_2O+40% O_2. The offset of nitrous oxide's discriminative stimulus effects

were rapid (Figure 9). Beginning the operant test session immediately after cessation of N_2O

exposure (0-minute delay) resulted in 88% ($\pm$7) N_2O lever responding (Figure 9, upper panel).

Delaying the start of the session by 1 and 3 minutes resulted in 61% ($\pm$12) and 44% ($\pm$13) N_2O

lever responding, respectively. Delaying the start of the test session by 5 minutes diminished

N_2O-appropriate responding to near vehicle levels. Both 3 and 5 minute delays prior to the start

of the operant session produced significantly lower N_2O-appropriate responding as compared

with the 0 minute delay condition [$F_{(12,36)}$=4.22, P<0.05].

Aim 2: Cross-substitution between N_2O, NMDA antagonists and GABAergic drugs

Given the *in vitro* evidence that N_2O attenuates NMDA receptor function, three NMDA

receptor channel blockers of varying affinities were tested for their ability to substitute for 60%

N_2O+40% O_2 (Figure 10). The high affinity NMDA receptor channel blocker (+)-MK-801

(closed circle) produced a dose-dependent and significant level of partial substitution for 60%

N_2O+40% O_2 [$F_{(7,35)}$=0.98, *P*<0.05]. The substitution ED_{50} of (+)-MK-801 for N_2O was 0.39

mg/kg (CL 0.20 - 0.77 mg/kg). Maximum substitution of 55% ($\pm$16) was produced by a (+)-

MK-801 dose of 0.75 mg/kg. (+)-MK-801 dose dependently attenuated operant responding with

an ED_{50} of 0.39 mg/kg (CL 0.30 – 0.50 mg/kg) [$F_{(7,42)}$=1.447, *P*<0.05]. Significant suppression

of operant response rates occurred at doses of 0.30 - 0.75 mg/kg of (+)-MK-801. The moderate

affinity NMDA receptor channel blocker ketamine (closed squares) also produced dose-

dependent partial substitution for N_2O [$F_{(6,18)}$=0.33, *P*<0.05]. Ketamine produced a maximum of

36% (±9) N₂O-lever responding at a dose of 15.6 mg/kg. Ketamine also dose dependently attenuated operant responding with an ED_{50} of 15.3mg/kg (CL 12.4 – 18.8 mg/kg) [$F_{(6,24)}$=2.21, P<0.05]. Significant suppression of responding occurred at ketamine doses of 15.6 mg/kg and 30 mg/kg. The low affinity NMDA receptor channel blocker memantine (closed triangles) produced dose-dependent partial substitution for 60% N₂O+40% O₂ [$F_{(6,30)}$=1.202, P<0.05] up to a maximum of 50% (±10) at a dose of 56 mg/kg. Memantine also dose dependently attenuated operant responding with an ED_{50} of 29.2 mg/kg (CL 24.9 – 34.3 mg/kg). Significant suppression of responding occurred at memantine doses of 30 and 56 mg/kg [$F_{(6,30)}$=3.88, P<0.05].

The NMDA receptor glutamate site competitive antagonist CGS-19755 (Figure 11, closed circle) did not produce significant substitution for N₂O [$F_{(7,21)}$=3.91, P=0.29]. A maximum of 11% (±5) N₂O-lever responding occurred at a dose of 17 mg/kg. However, CGS-19755 did dose dependently attenuate operant responding with an ED_{50} of 12.0 mg/kg (CL 8.1 – 17.9 mg/kg). Significant suppression of responding was produced by CGS-19755 doses of 10 and 17 mg/kg [$F_{(7,28)}$=4.59, P<0.05]. The NMDA receptor glycine site antagonist L-701,324 (Figure 11, closed square) also failed to significantly substitute for N₂O [$F_{(7,28)}$=1.22, P=0.13] producing no greater than 1% N₂O-lever selection at any dose. L-701,324 failed to significantly attenuate operant responding [$F_{(7,28)}$=9.28, P=0.44] up to the maximum dose tested of 30 mg/kg.

To further examine the role of NMDA receptor channel blockade in the discriminative stimulus of N₂O, I conducted a curve-shift experiment to determine if (+)-MK-801 pretreatment would enhance the discriminative stimulus of N₂O (Figure 12). A concentration-effect curve of N₂O+vehicle was compared to concentration-effect curves of N₂O combined with either 0.03 mg/kg or 0.17 mg/kg (+)-MK-801. N₂O+vehicle (closed circles) produced an EC_{50} of 32% (CL 25% – 41%). Pretreatment with a low dose of 0.03 mg/kg (+)-MK-801 (closed squares)
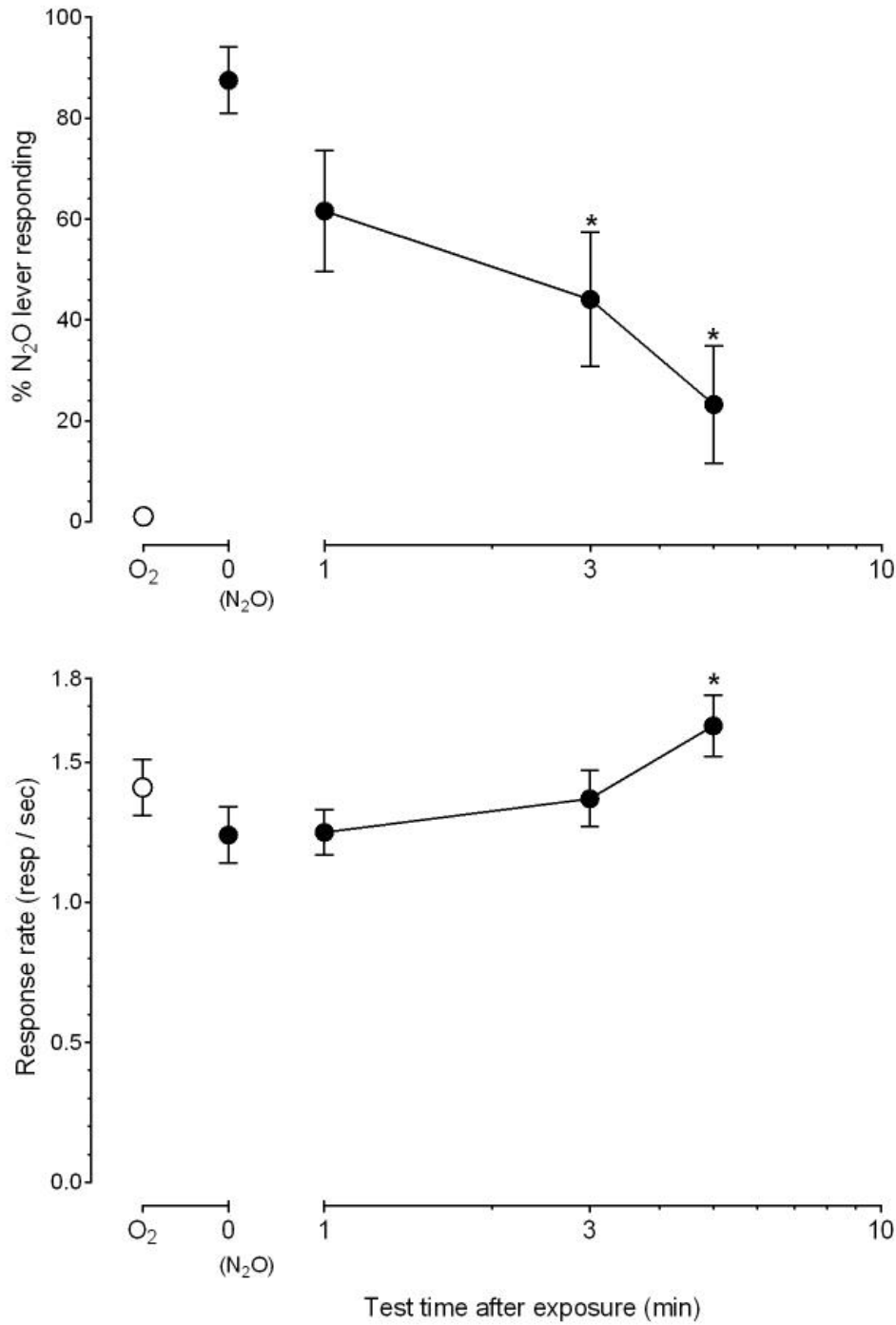
Figure 9. Offset of effects. Mean (±SEM) percentage nitrous oxide lever responding (upper panel) and operant response rates (lower panel) produced by a delay to the start of the 5 minute test session following cessation of 10 minutes of 60% nitrous oxide. Point above O_2 reflects the 100% oxygen control condition. Point above 0 minutes after exposure reflects the 60% nitrous oxide + 40% oxygen control condition. * indicates significant ($p < 0.05$) differences from N_2O control.

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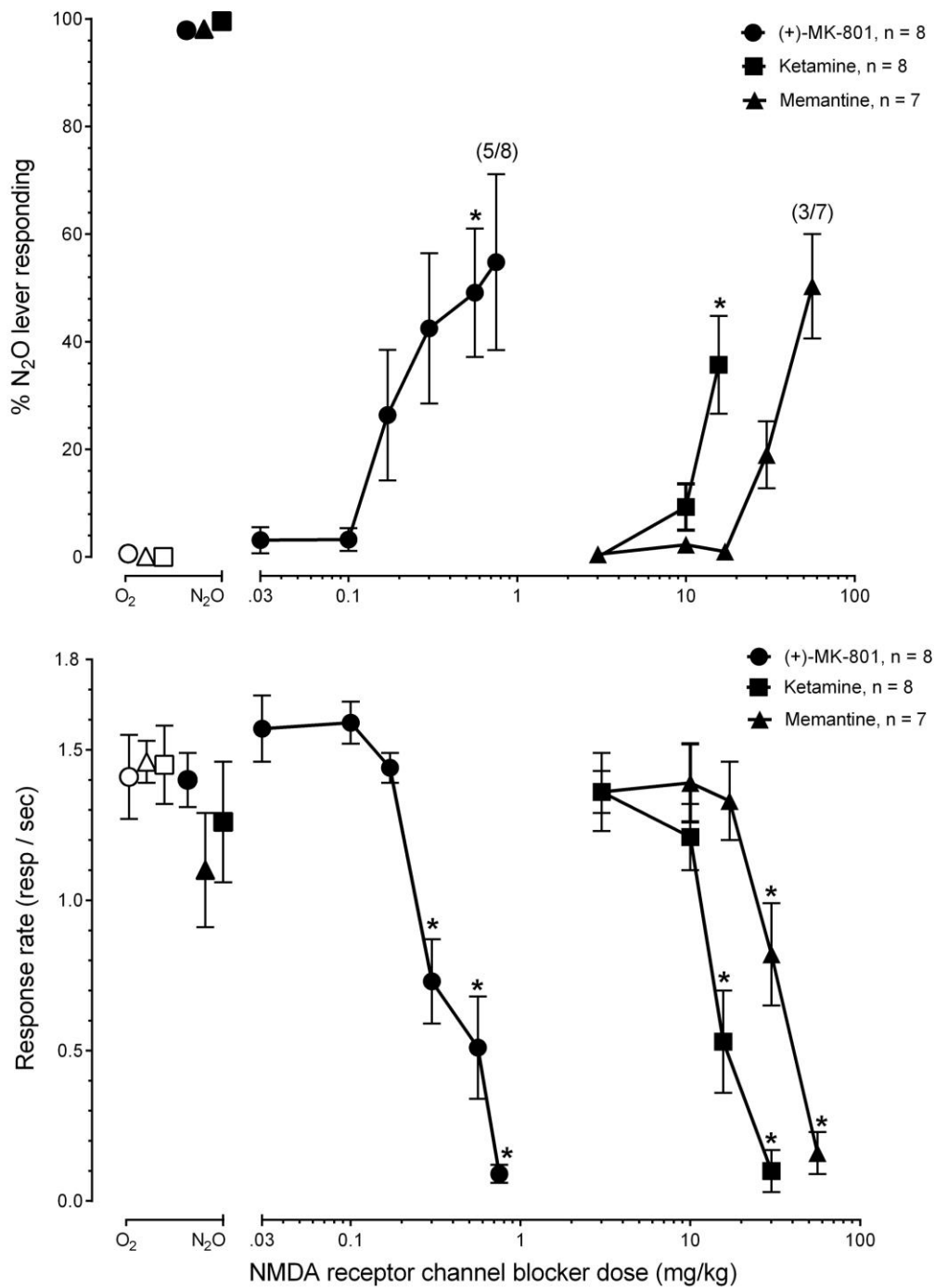
Figure 10. NMDA receptor channel blocker substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O +40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point. * indicates significant ($p < 0.05$) differences from oxygen control.
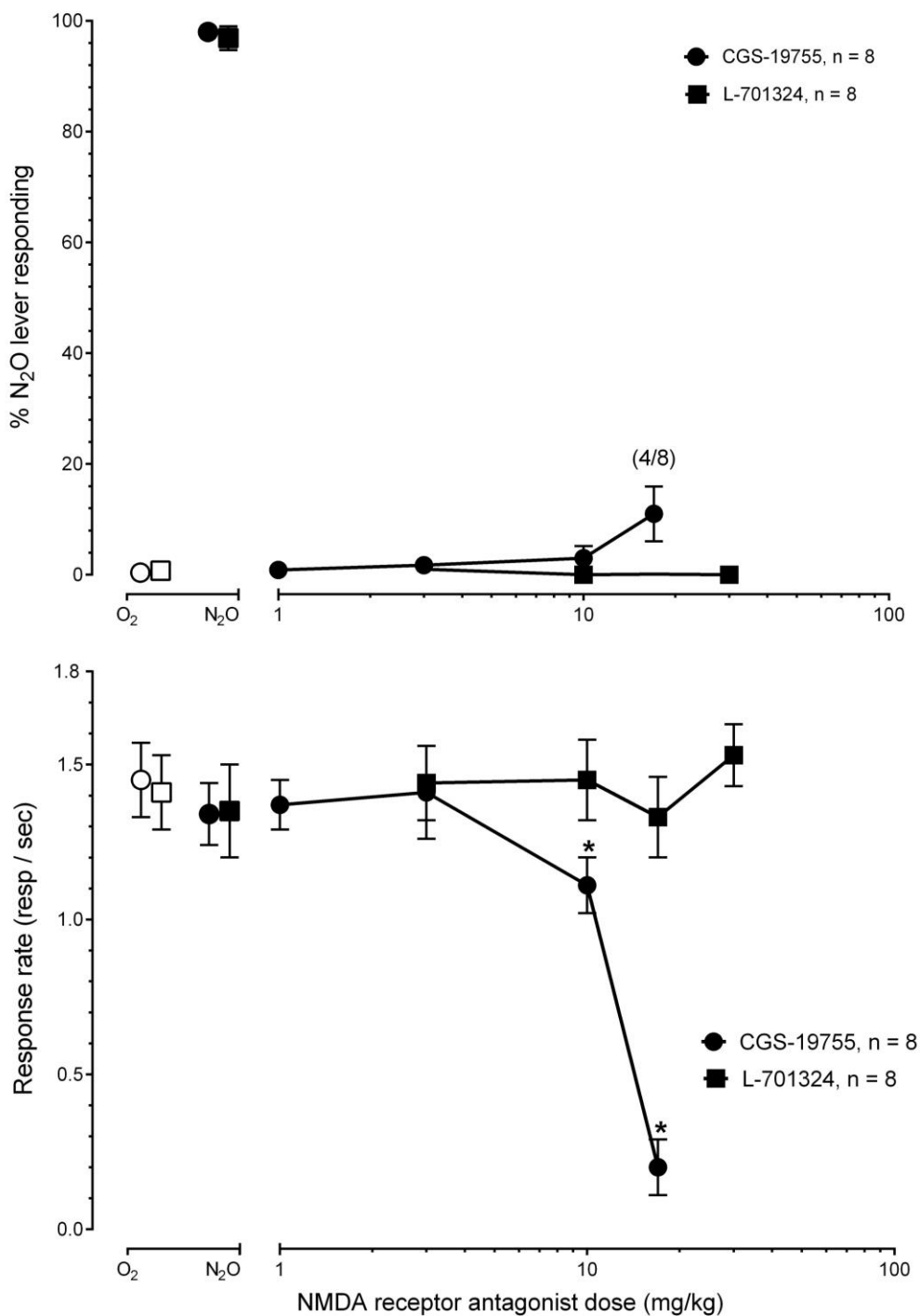
Figure 11. Glutamate and glycine site NMDA receptor antagonist substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O +40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point. * indicates significant ($p< 0.05$) differences from oxygen control.

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Figure 12. Effect of (+)-MK-801 pretreatment on nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O +40% oxygen and the 100% oxygen control conditions.* indicates significant ($p< 0.05$) differences from N_2O + vehicle concentration effect curve.

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produced a leftward shift of the N_2O concentration effect curve. However the EC_{50} of 26% (CL 17% – 39%) overlapped with the confidence limits of N_2O+vehicle. A moderate dose of 0.17 mg/kg (+)-MK-801 (closed triangles) in combination with N_2O produced a more pronounced 1.78 fold leftward shift in the N_2O concentration effect curve. The EC_{50} of N_2O when combined with 0.17 mg/kg (+)-MK-801 was 17% (CL 13% – 23%) which did not overlap with the EC_{50} of N_2O + vehicle. Further ANOVA analysis indicated that pretreatment with (+)-MK-801 significantly enhanced the discriminative stimulus effects of N_2O [$F_{(8, 48)}$=2.46, P<0.05]. Pretreatment with (+)-MK-801 also significantly enhanced the response-rate suppressing effects of N_2O [$F_{(10,60)}$=6.89, P<0.05]. Only N_2O combined with the moderate dose of 0.17 mg/kg dose of (+)-MK-801 produced concentration-dependent attenuation of operant responding sufficient to generate an EC_{50} which was 43% (CL 24% – 77%) (Figure 12, lower panel).

To determine if NMDA agonist could antagonize the discriminative stimulus of N_2O I conducted a series of tests in a subset of mice with the NMDA receptor glycine site co-agonist sarcosine. Sarcosine when administered alone (Figure 13) failed to significantly substitute for N_2O [$F_{(3, 12)}$=2.86, P=0.39]. Sarcosine produced a maximum of 25% (±25) N_2O-lever responding at a dose of 300 mg/kg. Sarcosine also produced no effects on operant responding [$F_{(12, 96)}$=3.91, P=0.47]. Next the concentration effect curve of N_2O+vehicle was compared to concentration-effect curves of N_2O preceded by pretreatment with 300 mg/kg or 600 mg/kg sarcosine (Figure 14). N_2O+vehicle (closed circles) produced concentration-dependent full substitution for the training concentration with an EC_{50} of 30% (CL 25% – 37%). The 300 mg/kg sarcosine pretreatment (closed squares) produced concentration-dependent full substitution for 60% N_2O+40% O_2 with an EC_{50} of 30% (CL 25% – 36%). Pretreatment with

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Figure 13. Sarcosine substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions.
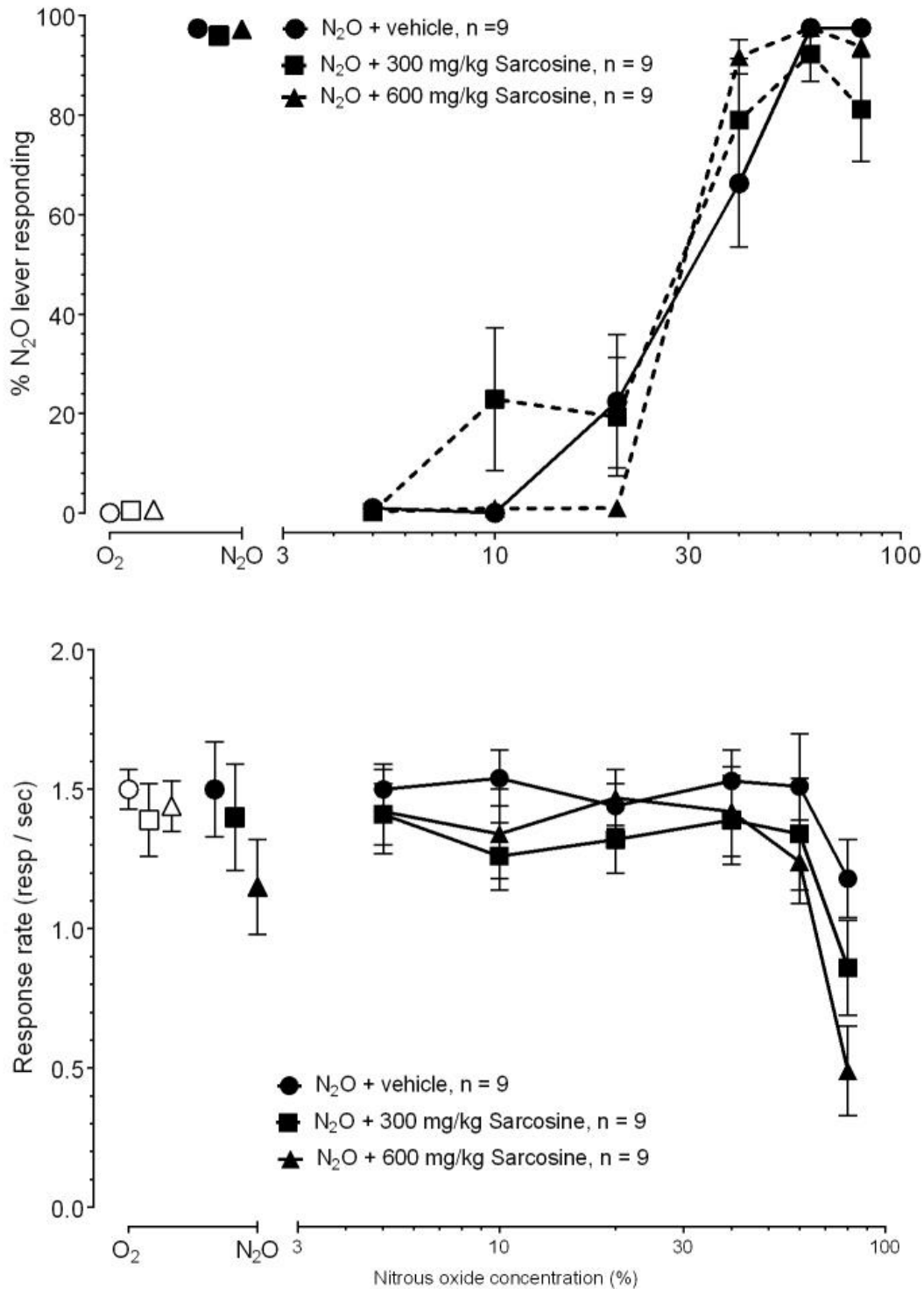
Figure 14. Effect of sarcosine pretreatment on nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N$_2$O+40% oxyg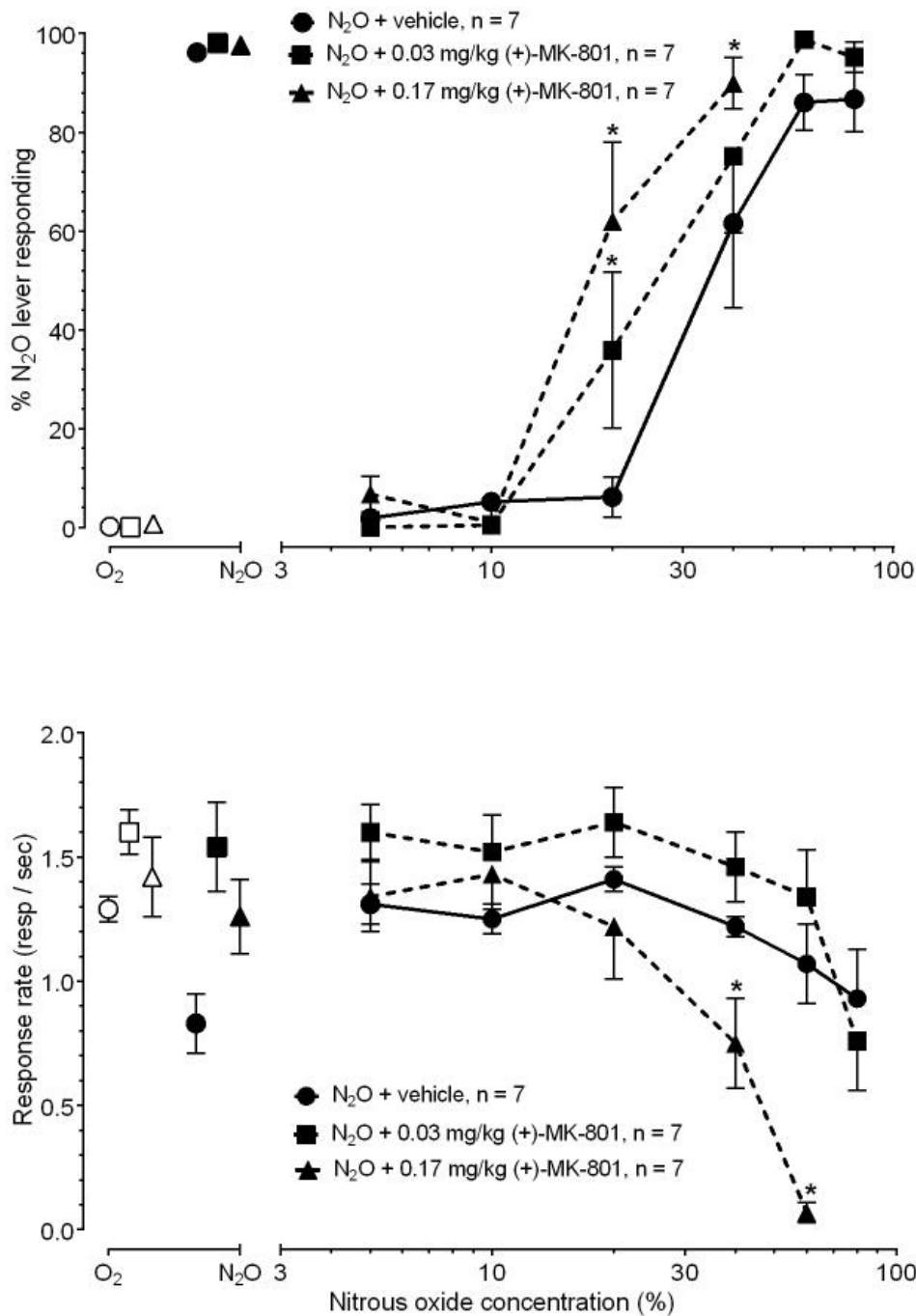en from 100% oxygen. Points above O$_2$ and N$_2$O reflect the 60% N$_2$O+40% oxygen and the 100% oxygen control conditions.

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600 mg/kg sarcosine (closed triangles) produced concentration-dependent full substitution for 60% N_2O+40% O_2 with an EC_{50} of 31% (CL 29% – 34%). The EC_{50} confidence limits for all three concentration-effect curves overlapped indicating that sarcosine does not significantly shift the N_2O concentration-effect curve under the conditions tested. Further analysis of variance testing confirmed that pretreatment with sarcosine did not significantly alter the discriminative stimulus effects of N_2O [$F_{(12, 96)}$=2.12, P=0.91]. Pretreatment with 300 mg/kg sarcosine did not have a significant effect on the rates of responding during N_2O exposure [$F_{(12, 96)}$=2.19, P=0.34]. However, pretreatment with 600 mg/kg dose of sarcosine prior to N_2O exposure produced a concentration-dependent attenuation of operant responding which was sufficient to generate an EC_{50} of 75% (69% – 82%).

To examine the role of $GABA_A$ receptors in the discriminative stimulus properties of N_2O, five GABAergic compounds were tested for the ability to substitute for 60% N_2O+40% O_2. The extrasynaptic $GABA_A$ receptor agonist gaboxadol (Figure 15, closed circle) failed to produce significant substitution for N_2O [$F_{(7,21)}$=2.59, P=0.27] resulting in a maximum of 4% ($\pm$3) N_2O-lever responding at a dose of 1.0 mg/kg. Gaboxadol attenuated operant responding with an ED_{50} of 6.4 mg/kg (CL 2.6 mg/kg - 15.7 mg/kg). Significant suppression of responding [$F_{(7,28)}$=5.27, P<0.05] occurred only at the 10.0 mg/kg dose of gaboxadol. The direct $GABA_A$ agonist muscimol (Figure 15, closed square) failed to significantly substitute for 60% N_2O+40% O_2 [$F_{(7,21)}$=1.83, P=0.06] producing a maximum of 22% ($\pm$22) N_2O-lever responding at a dose of 1.7 mg/kg. Muscimol produced dose-dependent attenuation of operant response rates with an ED_{50} of 1.2 mg/kg (CL 0.9 - 1.6 mg/kg). Muscimol significantly suppressed responding at doses of 1.7 and 3.0 mg/kg [$F_{(7,28)}$=2.68, P<0.05]. The GABA transaminase inhibitor valproic acid (Figure 15, closed triangle) also failed to elicit significant substitution for N_2O [$F_{(7,14)}$=19.0,

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P=0.35]. However, valproic acid did produce a maximum of 33% (±15) N_2O-lever responding at a dose of 560 mg/kg. Valproic acid dose-dependently suppressed operant responding with an ED_{50} of 430 mg/kg (CL 384 mg/kg - 481 mg/kg). Statistically significant suppression of responding [$F_{(7,21)}$=2.65, P<0.05] was produced by the highest valproic acid test dose of 560 mg/kg which completely suppressed operant responding in half of the subjects.

Two $GABA_A$ receptor positive allosteric modulators were tested for their ability to substitute for 60% N_2O+40% O_2 (Figure 16). The $GABA_A$ receptor benzodiazepine-site positive allosteric modulator midazolam (closed circles) produced a low (27%), but statistically significant level of partial substitution for 60% N_2O+40% O_2 at the highest dose tested of 56 mg/kg [$F_{(8,48)}$=1.92, P<0.05]. Midazolam dose dependently attenuated operant responding with an ED_{50} of 10.5 mg/kg (CL 3.2 – 34.8 mg/kg). Significant suppression of responding occurred at midazolam doses of 10 - 56 mg/kg [$F_{(8,48)}$=4.23, P<0.05]. The $GABA_A$ receptor barbiturate-site positive allosteric modulator pentobarbital (closed squares) failed to produce significant substitution for N_2O [$F_{(7,28)}$=1.91, P=0.17] generating a maximum of 10% (±3) N_2O-lever responding at a dose of 30 mg/kg. Pentobarbital produced dose-dependent suppression of operant responding with an ED_{50} of 28.9 mg/kg (CL 17 mg/kg – 49 mg/kg). Statistically significant suppression of responding was produced by pentobarbital doses of 30 and 50 mg/kg [$F_{(7,35)}$=2.27, P<0.05].

I also conducted a curve-shift experiment to determine if midazolam would enhance the discriminative stimulus of N_2O. A concentration effect curve of N_2O preceded by vehicle pretreatment was compared to concentration-effect curves of N_2O preceded by pretreatment with 0.3, 3 or 10 mg/kg midazolam (Figure 17). The highest pretreatment dose
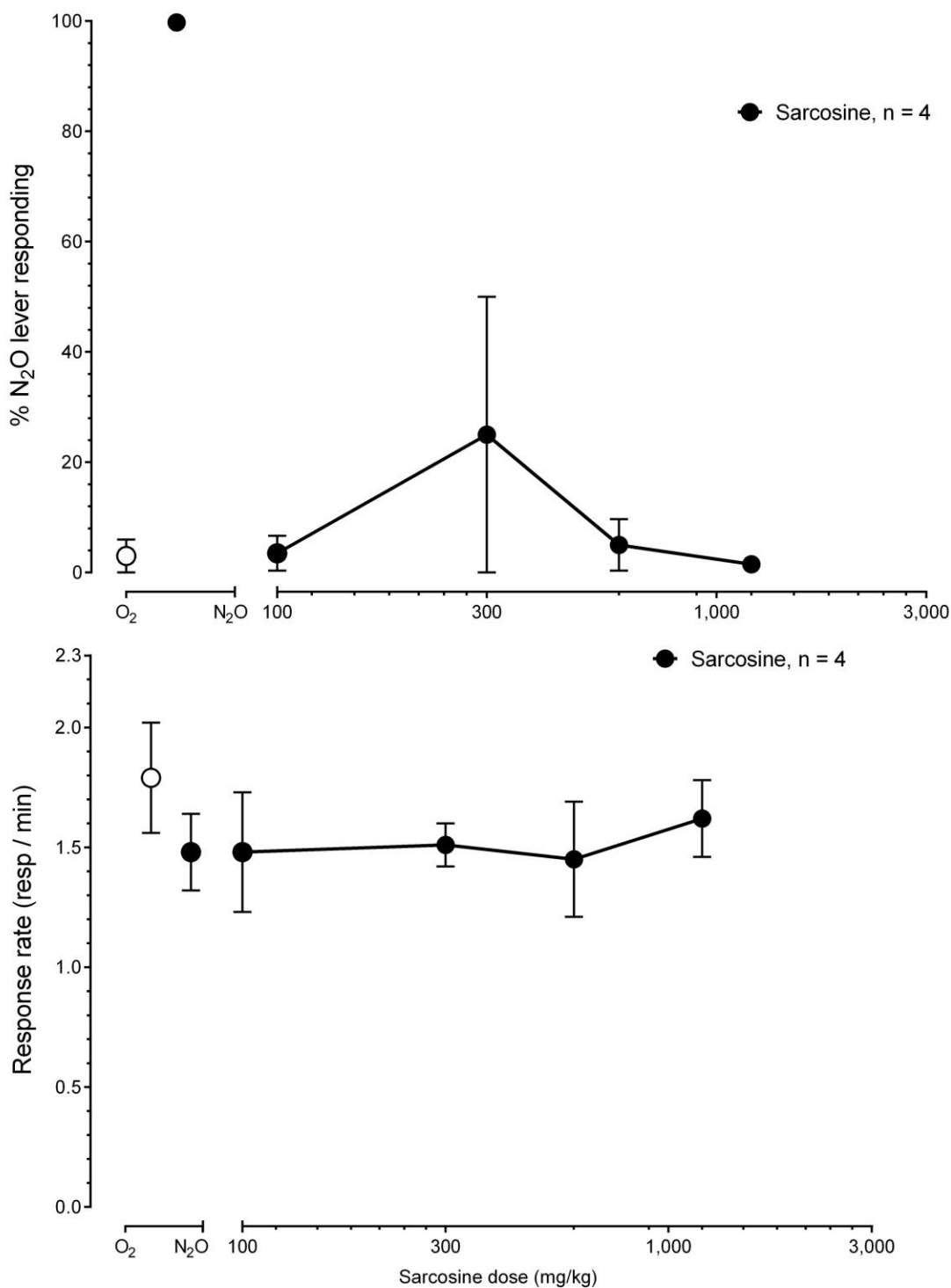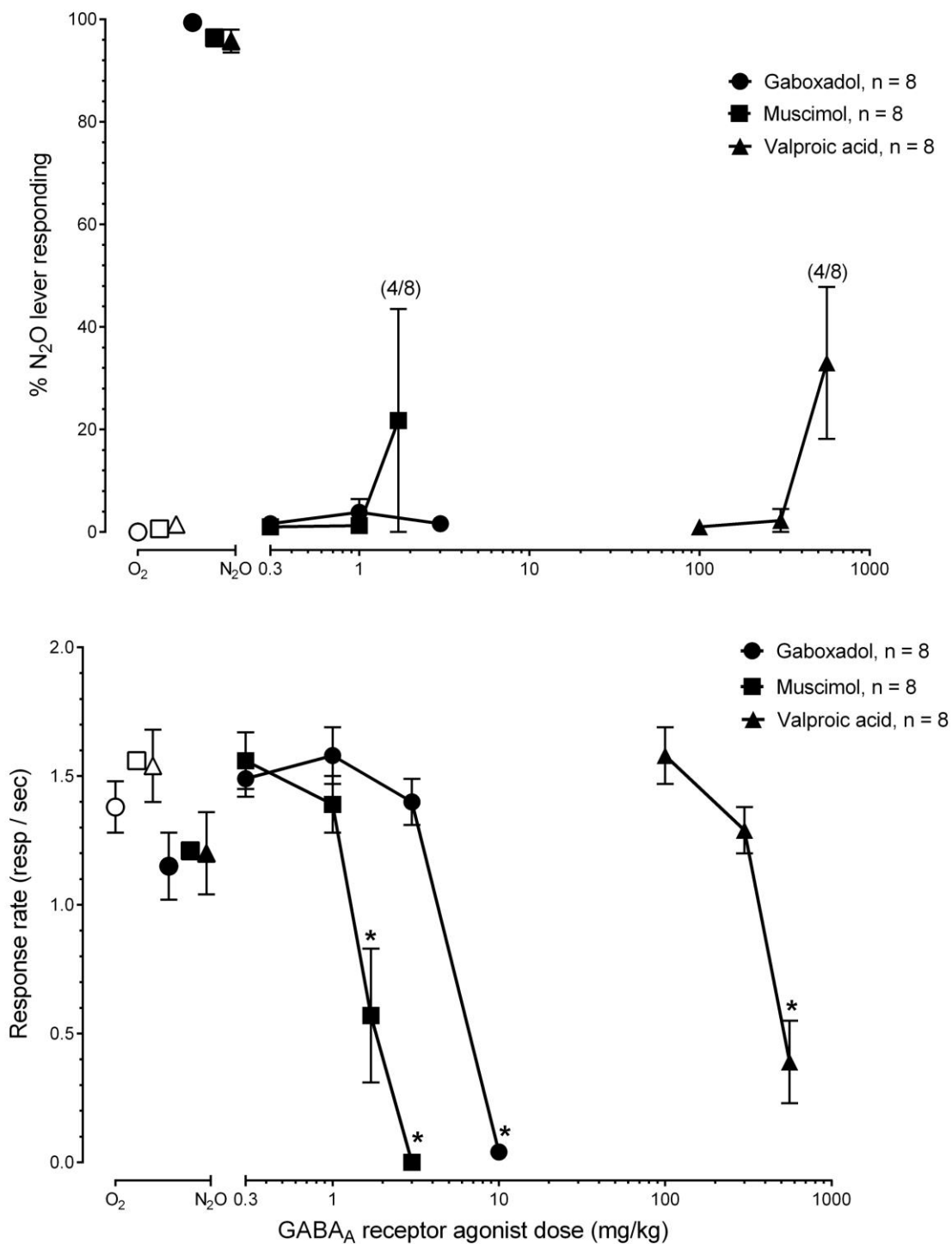
Figure 15. GABA$_A$ receptor agonist substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N$_2$O+40% oxygen from 100% oxygen. Points above O$_2$ and N$_2$O reflect the 60% N$_2$O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant (p< 0.05) differences from oxygen control.
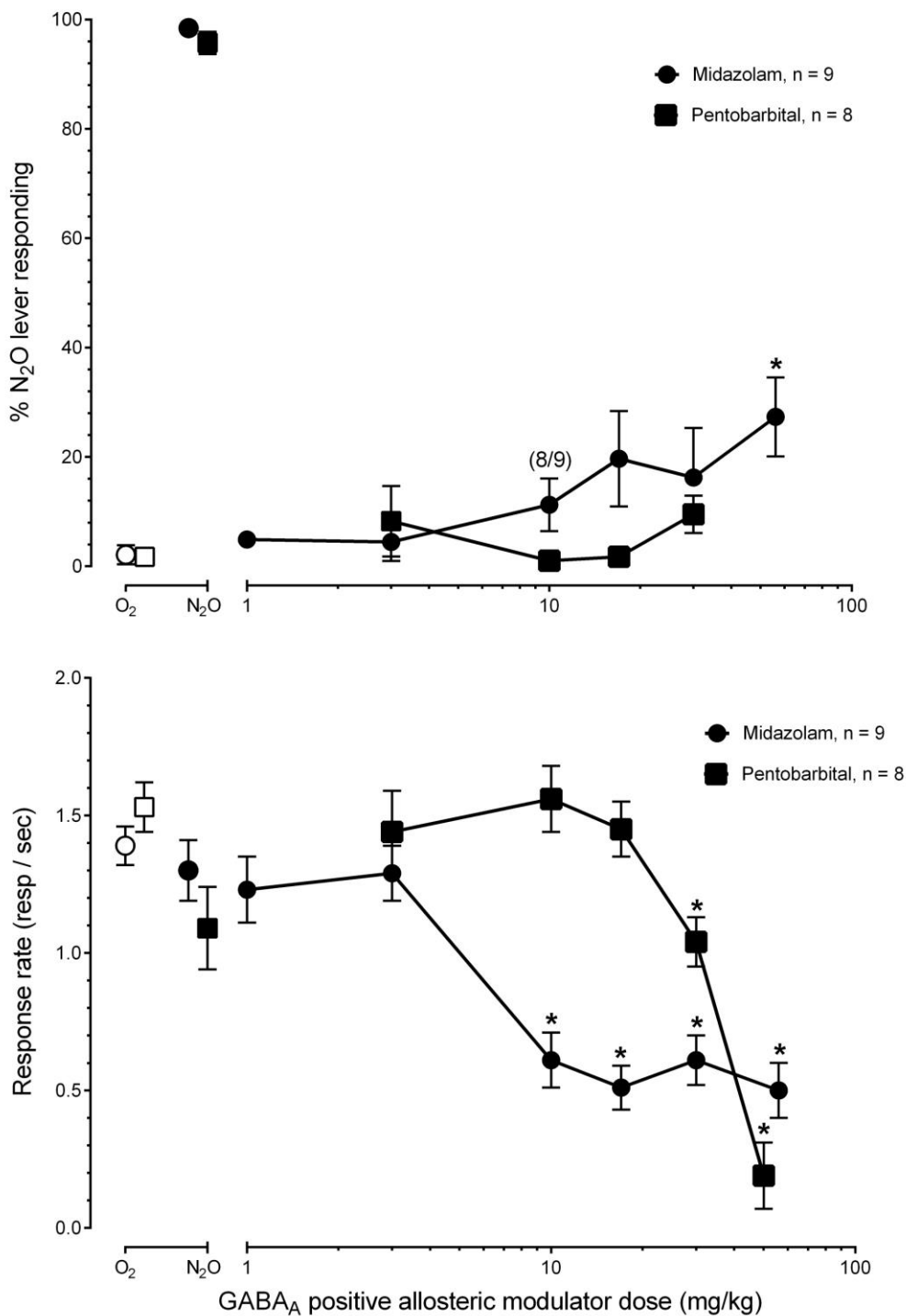
Figure 16. GABA$_A$ receptor positive allosteric modulator substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N$_2$O+40% oxygen from 100% oxygen. Points above O$_2$ and N$_2$O reflect the 60% N$_2$O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant (p< 0.05) differences from oxygen control.

Figure 17. Effect of midazolam pretreatment on nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N₂O+40% oxygen from 100% oxygen. Points above O₂ and N₂O reflect the 60% N₂O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant (p< 0.05) differences of N₂O + 3 mg/kg midazolam concentration effect curve from N₂O + vehicle concentration effect curve.

of 10 mg/kg midazolam was the minimum dose of midazolam which produced significant suppression of response rates when administered alone. N_2O+vehicle (closed circles) produced concentration-dependent full substitution for the 60% N_2O+40% O_2 training concentration with an EC_{50} of 25 % (CL 14% – 44%). Nitrous oxide combined with a low dose of 0.3 mg/kg midazolam (closed squares) produced concentration-dependent full substitution for the 60% N_2O+40% O_2 training condition with an EC_{50} of 35% (CL 25% – 47%). Nitrous oxide combined with 3.0 mg/kg midazolam (closed triangles) produced concentration-dependent full substitution for 60% N_2O+40% O_2 with an EC_{50} of 44% (CL 35% – 56%). Nitrous oxide combined with 10 mg/kg midazolam produced concentration-dependent partial substitution for 60% N_2O+40% O_2. Although midazolam pretreatment dose dependently shifted the N_2O concentration rightward, the ED_{50} confidence limits all overlapped. Further statistical analysis by ANOVA indicated that pretreatment with midazolam did not significantly affect the discriminative stimulus effects of N_2O [$F_{(10, 70)}$=0.63, P=0.78]. Pretreatment with midazolam did, however, significantly enhance the response-rate suppressing effects of N_2O [$F_{(12, 84)}$=5.56, P<0.05]. N_2O preceded by pretreatment with 3 mg/kg midazolam produced a concentration-dependent attenuation of operant responding with an EC_{50} of 22% (18% – 27%). N_2O combined with 10.0 mg/kg midazolam produced such pronounced suppression of operant responding at the lowest combination dose tested that an EC_{50} value could not be calculated.

Aim 3: Cross-substitution between N_2O and other abused inhalants

It was of interest to determine the degree to which the discriminative stimulus effects of nitrous oxide overlapped with those of other abused inhalants. Two halogenated ether

anesthetic vapors were tested for their ability to substitute for 60% N_2O+40% O_2 (Figure 18). The volatile halogenated anesthetic isoflurane (closed circles) produced a concentration-dependent and significant level of partial substitution for N_2O [$F_{(7,21)}$=2.91, P<0.05]. Maximum substitution of 39% (±11) was produced by 4000 ppm isoflurane. Isoflurane also produced a concentration-dependent attenuation of operant responding with an EC_{50} of 3803 ppm (CL 3218–4496 ppm). Significant suppression of operant responding occurred at isoflurane concentrations of 4000 ppm and 6000 ppm [$F_{(7,28)}$=4.814, *P*<0.05]. The volatile halogenated analgesic methoxyflurane (closed squares) produced a concentration-dependent and significant level of partial substitution for N_2O [$F_{(8,24)}$=2.59, P<0.05]. Maximum substitution of 47% (±14.2) N_2O-lever selection was produced by 2000 ppm methoxyflurane. Methoxyflurane also concentration-dependently attenuated operant responding with an EC_{50} of 1902 ppm (CL 1563 ppm – 2316 ppm). Significant suppression of operant responding was produced by 4000 ppm and 6000 ppm methoxyflurane [$F_{(8,32)}$=1.685, *P*<0.05].

Two abused vapors were also tested for their ability to substitute for N_2O (Figure 19). The chlorinated hydrocarbon vapor 1,1,1-trichloroethane (TCE) (closed circles), produced a concentration-dependent and significant level of partial substitution for nitrous oxide [$F_{(7,28)}$=1.76, *P*<0.05]. Maximum substitution of 44% (±18) N_2O-lever responding was produced by 12000 ppm TCE. TCE also resulted in a concentration-dependent attenuation of operant responding with an EC_{50} of 8264 ppm (CL 7295 ppm – 9361 ppm) [$F_{(7,28)}$=6.243, *P*<0.05]. Significant suppression of operant responding occurred at the highest tested concentration of 12000 ppm TCE. The aromatic hydrocarbon vapor toluene (closed squares) produced a concentration-dependent and significant level of partial substitution for N_2O [$F_{(7,28)}$=0.82, *P*<0.05] with an EC_{50} of 3174 ppm (CL 1970 ppm – 5117 ppm). Maximum

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substitution of 72% ($\pm$10) N_2O-lever selection was produced by 8000 ppm toluene. Toluene concentration dependently attenuated operant responding with an EC_{50} of 5192 ppm (CL 4509ppm – 5977 ppm) [$F_{(7,35)}$=8.85, P<0.05]. Toluene concentrations of 2000, 4000 and 8000 ppm significant suppressed operant responding.

As all of the volatile compounds produced some degree of substitution for 60% N_2O+40% O_2, I also examined if a strong odor cue devoid of CNS properties was sufficient to elicit N_2O-like stimulus effects (Figure 20). The sweet smelling odorant 2-butanol failed to significantly substitute for N_2O [$F_{(7,21)}$=26.74, P=0.43] producing no greater than 3% N_2O-lever selection at any concentration tested. The odorant also failed to significantly attenuate operant response rates [$F_{(7,21)}$=28.79, P=0.78] up to the maximum concentration tested of 100 ppm.

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Figure 18. Halogenated vapor anesthetic substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant ($p< 0.05$) differences from air control.
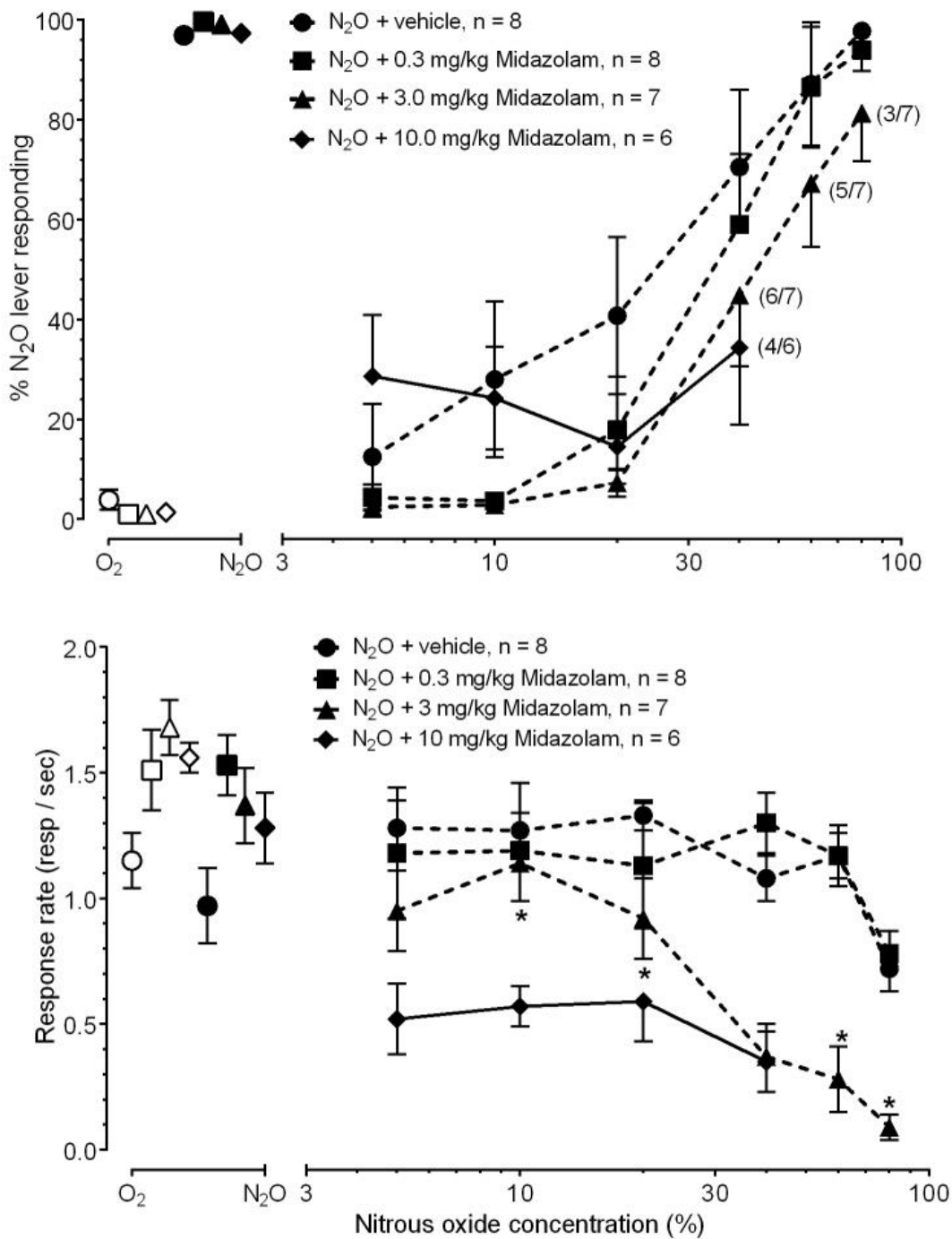
Figure 19. Abused inhalant vapor substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point. * indicates significant ($p< 0.05$) differences from air control.

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Figure 20. Odorant 2-butanol substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions.

<u>Aim 4: Other potential mediators of the stimulus effects of N_2O</u>

The discriminative stimulus effects of ethanol are mediated by both $GABA_A$ positive and NMDA antagonist effects (York, 1978, Grant, Waters, Green-Jordan, Azarov, & Szeliga, 2000, Kotlinska & Liljequist, 1997, Shelton & Grant, 2002). Therefore I also determined the degree to which the stimulus effects of ethanol overlap with those of nitrous oxide. Ethanol (Figure 21) produced partial substitution for N_2O up to a maximum of 45% ($\pm$12) at the highest test dose of 2.5 g/kg. Statistically significant substitution by ethanol for N_2O was produced by ethanol doses of 2 and 2.5 g/kg [$F_{(14,56)}$=3.30, $P<0.05$]. Ethanol also dose-dependently attenuated operant response rates with an ED_{50} of 2.23 g/kg (CL 1.97 g/kg– 2.73 g/kg). Ethanol significantly suppressed operant responding at doses of 1.5 g/kg – 2.5 g/kg [$F_{(14,56)}$=3.05, $P<0.05$].

To further examine if the discriminative stimulus properties of N_2O and ethanol are mediated by similar mechanism, I conducted a curve shift experiment to determine if ethanol would enhance the discriminative stimulus of nitrous oxide (Figure 22). The concentration-effect curve of N_2O+vehicle was compared to concentration-effect curves of N_2O preceded by pretreatment with 0.5 g/kg or 1.5 g/kg ethanol. N_2O+vehicle (closed circles) produced concentration-dependent full substitution for the 60% N_2O+40% O_2 with an EC_{50} of 31% (CL 27% – 36%). Nitrous oxide preceded by pretreatment with a low dose of 0.5 g/kg ethanol (closed squares) produced concentration-dependent full substitution for 60% N_2O+40% O_2 with an EC_{50} of 27% (CL 23% – 32%). Nitrous oxide preceded by pretreatment with a higher dose of 1.5 g/kg ethanol (closed triangles) produced a 2.84 fold leftward shift in the N_2O concentration effect curve and an EC_{50} of 11% (CL 7% – 18%) which did not overlap with the EC_{50} of N_2O+vehicle. Further analysis of variance indicated that pretreatment with 1.5 g/kg ethanol significantly enhanced the discriminative stimulus effects of nitrous oxide [$F_{(8,56)}$=4.43, $P<0.05$].

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Figure 21. Ethanol substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant ($p< 0.05$) differences from oxygen control.

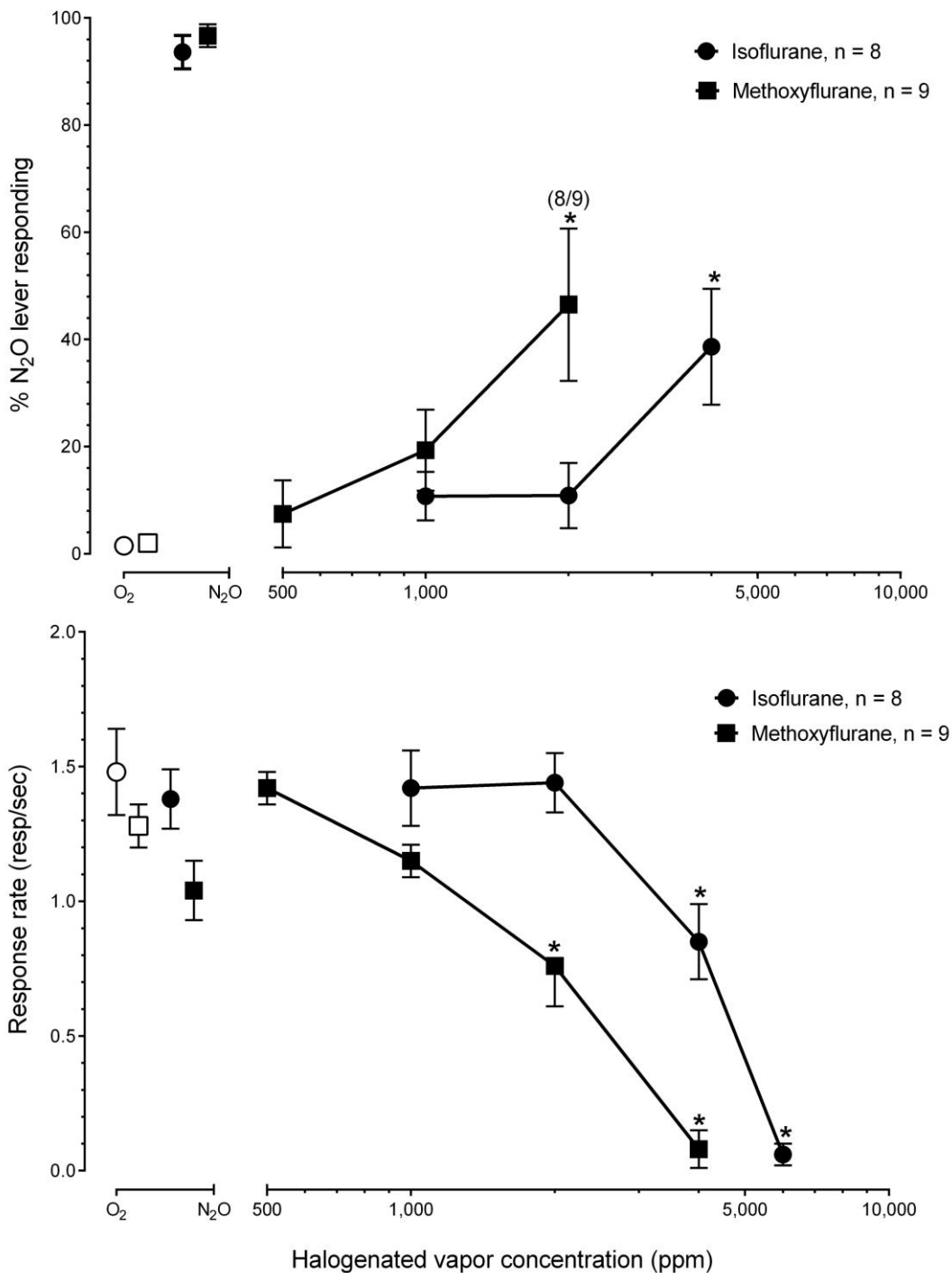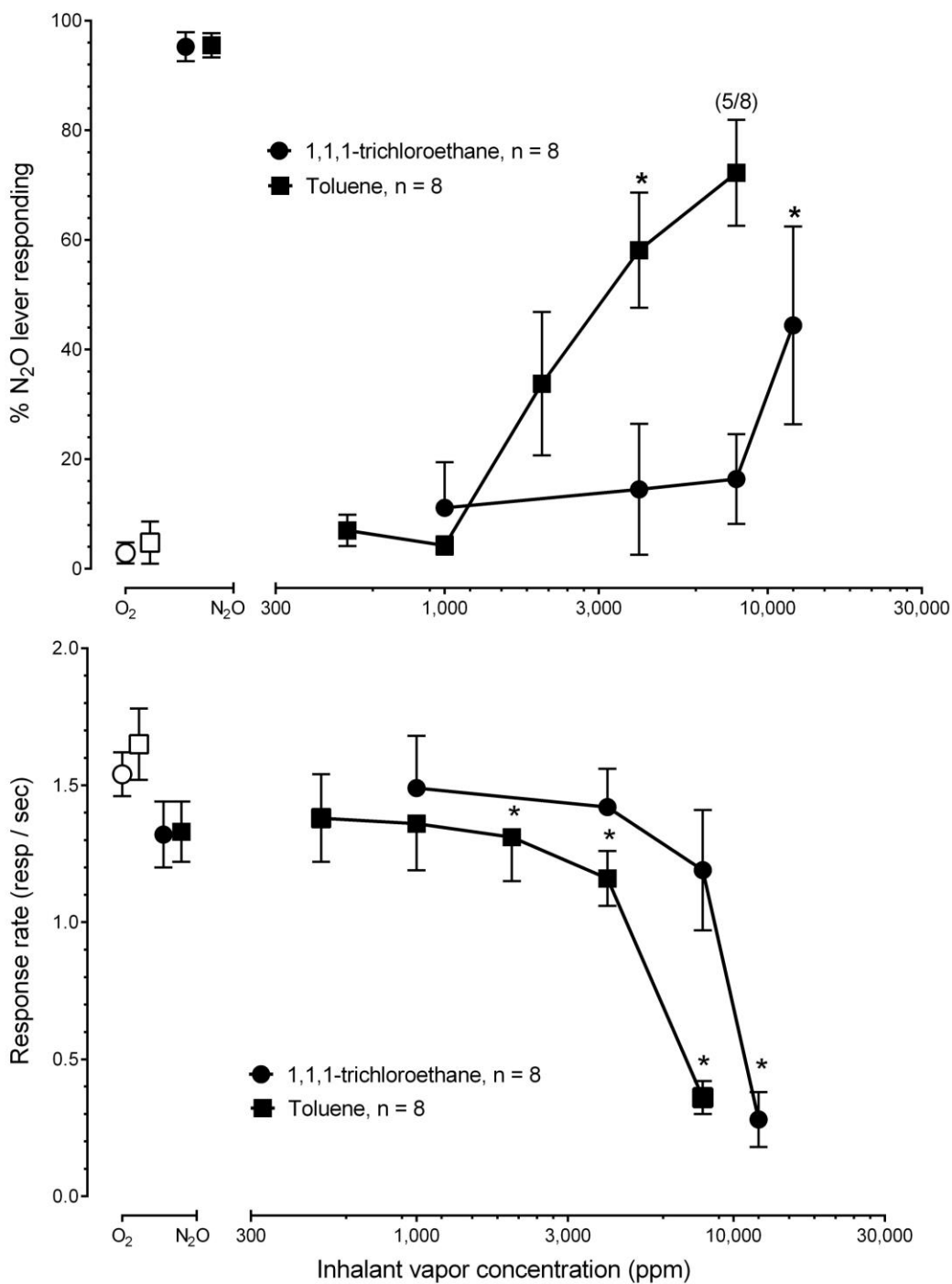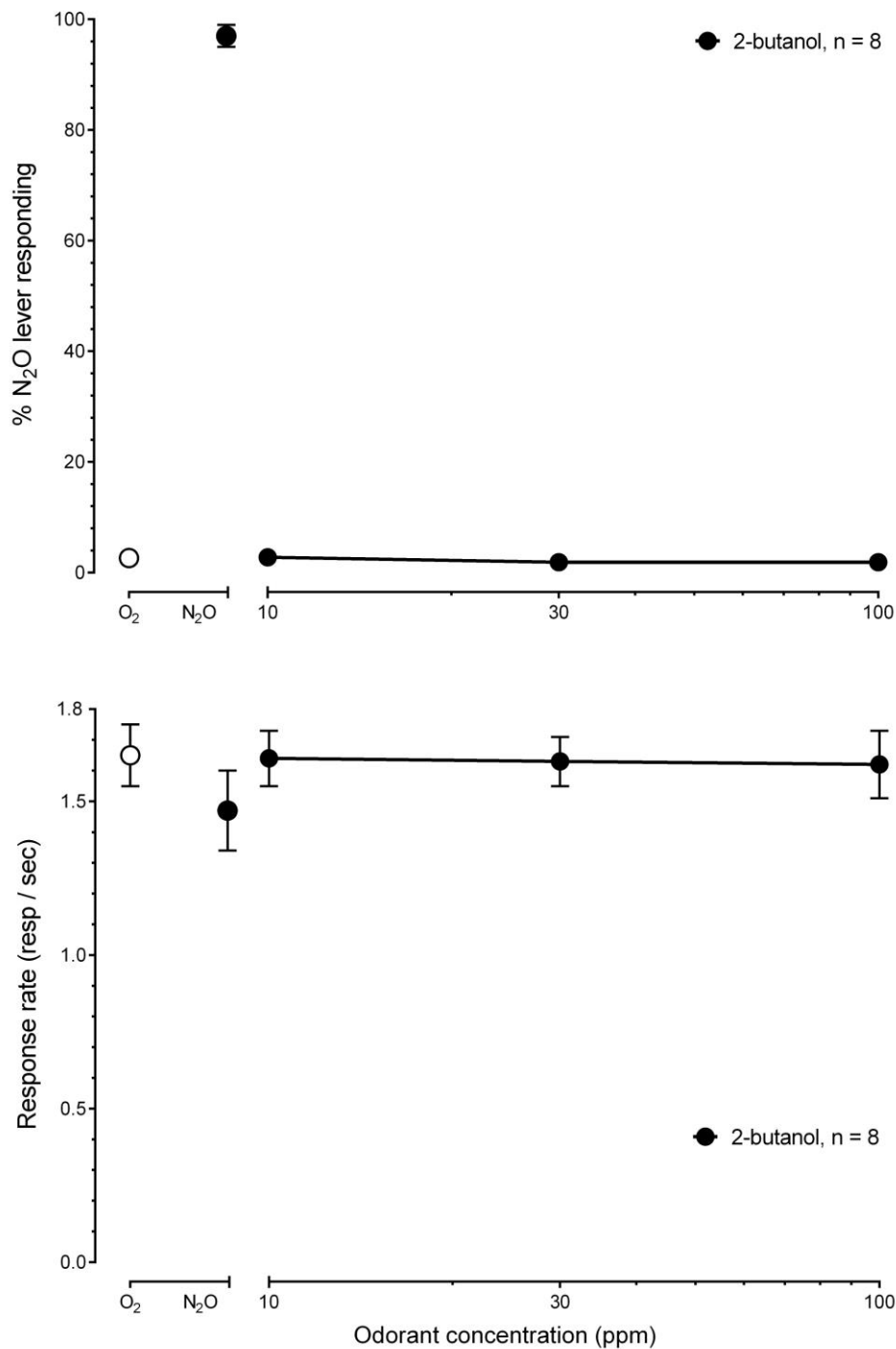Figure 22. Effect of ethanol pretreatment on N_2O substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. * indicates significant ($p < 0.05$) differences from N_2O + vehicle concentration effect curve through 60% N_2O.

Ethanol pretreatment also enhanced the response rate suppressing effects of N_2O [$F_{(10,70)}=6.62$, *P<0.05*]. Pretreatment with 0.5 g/kg ethanol produced a significant concentration-dependent attenuation of operant response rates. The 1.5 g/kg ethanol pretreatment produced a more pronounced concentration-dependent attenuation of operant responding with an EC_{50} of 24% (CL 17% – 36%) and full suppression of operant responding in the 1.5 g/kg ethanol+60% N_2O test condition.

The discriminative stimulus effects of ethanol are mimicked not only $GABA_A$ positive modulators and NMDA antagonists but also $5\text{-}HT_{1B/2C}$ agonists (Grant, Colombo, & Gatto, 1997). To examine the possibility that the partial substitution of ethanol for nitrous oxide was mediated by $5\text{-}HT_{1B/2C}$ receptors I examined if a $5\text{-}HT_{1B/2C}$ receptor agonist, mCPP, which substitutes for ethanol, would have N_2O-like discriminative stimulus effects. mCPP (Figure 23) failed to significantly substitute for nitrous oxide [$F_{(7,21)}=56.6$, *P*=0.053]. mCPP produced a maximum of 21% (±17) N_2O-lever responding at 10 mg/kg, a dose which also fully suppressed operant responding in three of eight subjects. mCPP dose-dependently attenuated operant responding with an ED_{50} of 3.7 mg/kg (CL 2.2 mg/kg – 6.5 mg/kg). Significant suppression of operant responding was produced by mCPP doses of 5.6 mg/kg and 10 mg/kg [$F_{(7,28)}=4.14$, *P<0.05*].

Nitrous oxide produces increased human subjective ratings on the LSD subscale of the ARCI questionnaire (Dohrn et al., 1993). To examine if these similarities were due to $5HT_{1A}$ agonist effects, I examined if the $5HT_{1A}$ agonist 8-OH DPAT would produce N_2O-like discriminative stimulus effects. 8-OH DPAT (Figure 24) failed to significantly substitute for N_2O [$F_{(7,28)}=7.98$, *P*=0.20] producing 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

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mg/kg – 0.71 mg/kg). 8-OH DPAT significantly suppressed operant responding at doses of 0.3 -
1.56 mg/kg [$F_{(7,28)}$=2.38, $P<0.05$].

It has been shown that toluene vapor will substitute for D-amphetamine (Bowen, 2006).
Given the high level of partial substitution of toluene for N$_2$O, this might suggest that facilitation
of dopamine release is involved in the discriminative stimulus effects of N$_2$O D-Amphetamine
(Figure 25) failed to significantly substitute for N$_2$O [$F_{(6,24)}$=1.29, P=0.36]. D-Amphetamine
produced no greater than 1% N$_2$O-lever selection at any dose. D-Amphetamine also failed to
significantly attenuate operant responding [$F_{(6,24)}$=8.89, P=0.30] up to the maximum dose tested
of 1.56 mg/kg.

Nitrous oxide has been shown to produce cross-substitution in rats trained to discriminate
a kappa but not a mu opioid receptor agonist (Hynes & Hymson, 1984). I tested a mu, kappa and
delta opioid receptor agonist for their ability to substitute for 60% N$_2$O+40% O$_2$ (Figure 26).
The mu opioid receptor agonist morphine (closed circles) produced a partial, but not statistically
significant level of substitution for N$_2$O [$F_{(7,28)}$=1.58, P=0.35]. A maximum of 33% ($\pm$33) N$_2$O-
lever responding was produced at the highest morphine test dose of 30 mg/kg, a dose which also
fully suppressed operant responding in 5 of 8 subjects. Morphine produced dose-dependent
attenuation of operant responding with an ED$_{50}$ of 7.9 mg/kg (CL 3.9 mg/kg - 16.2 mg/kg).
Significant suppression of responding occurred at the 10 and 30 mg/kg morphine doses
[$F_{(7,35)}$=6.29, $P<0.05$]. The kappa opioid receptor agonist U50-488H (closed squares) did not
significantly substitute for N$_2$O [$F_{(7,14)}$=6.41, P=0.66]. U50-488H produced a maximum of 11%
($\pm$11) N$_2$O-lever responding at a dose of 7 mg/kg. U50-488H dose dependently attenuated
operant responding with an ED$_{50}$ of 3.3 mg/kg (CL 2.7 mg/kg– 4.1 mg/kg). Significant
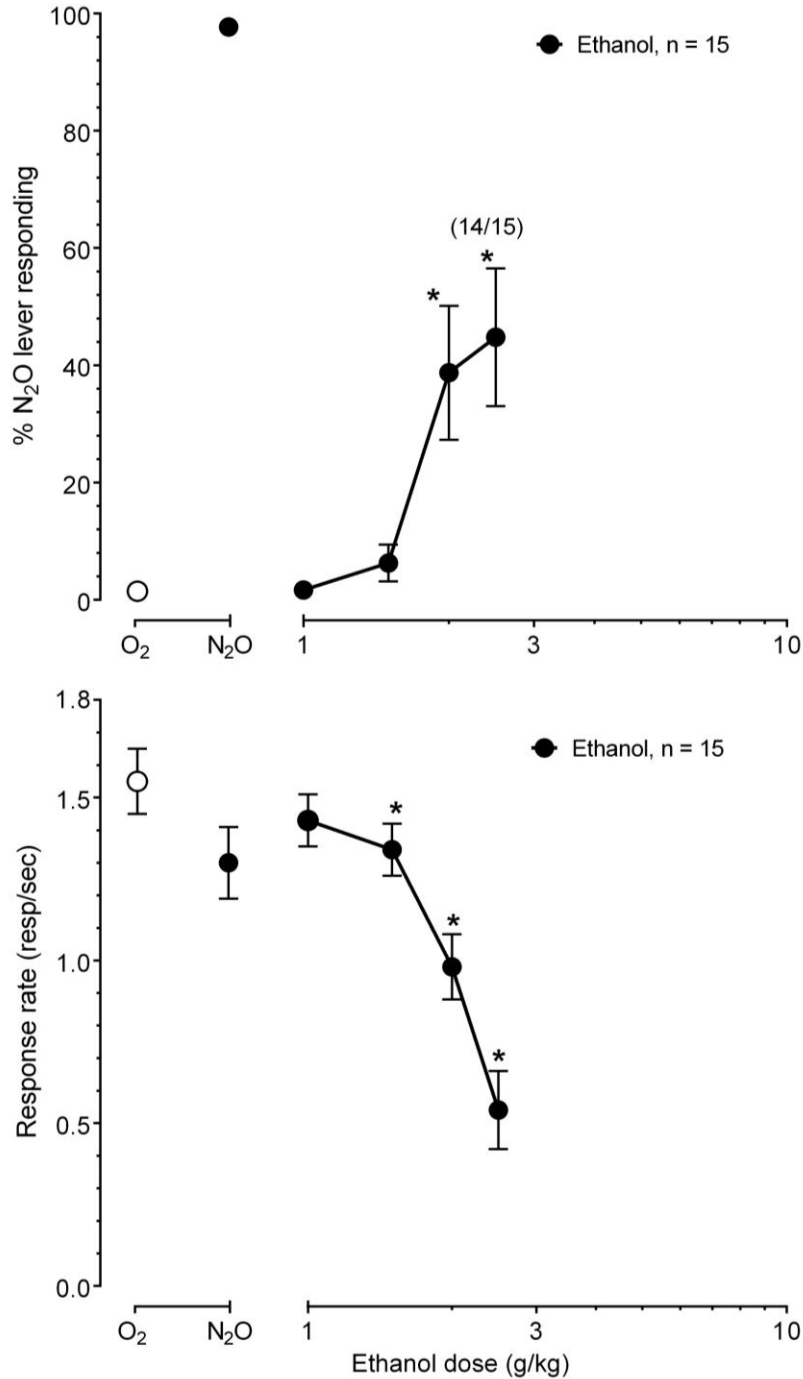suppression of

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Figure 23. mCPP substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant (p< 0.05) differences from oxygen control.
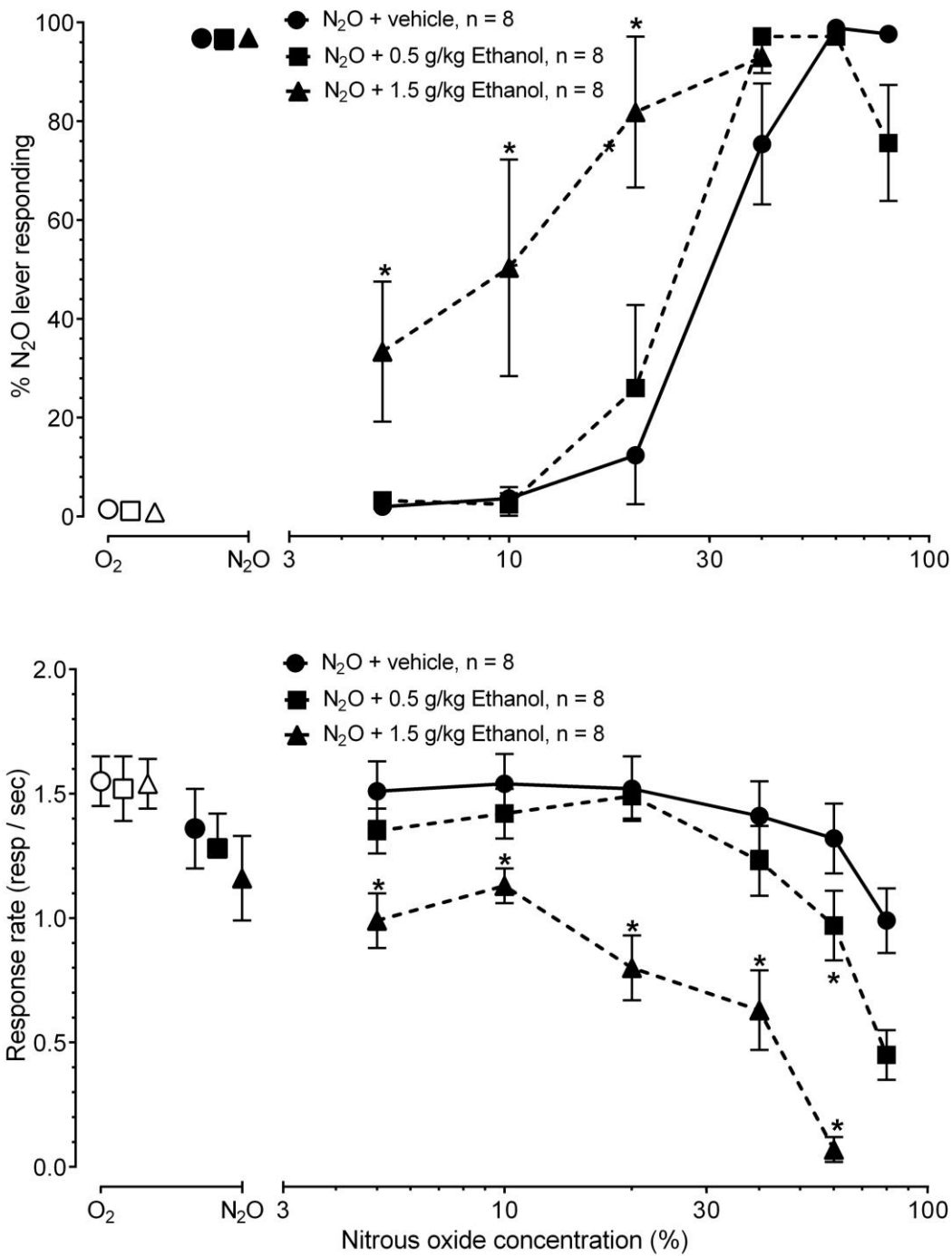
Figure 24. 8-OH DPAT substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. * indicates significant ($p< 0.05$) differences from oxygen control

Figure 25. D-amphetamine dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions.

Figure 26. Opioid receptor agonist substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant ($p < 0.05$) differences from oxygen control.

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operant responding [$F_{(7,28)}$=1.97, $P<0.05$] occurred at U-50488H doses of 1, 7 and 10 mg/kg. Lastly, the delta opioid receptor agonist SNC-80 (closed triangles) did not produce greater than 10% N_2O-lever responding at any dose tested. However due to the low level of variability, doses of both 10 mg/kg and 30 mg/kg SNC-80 resulted in significantly greater, but probably not meaningful higher, levels of N_2O-lever responding than vehicle [$F_{(7,42)}$=4.37, $P<0.05$]. SNC-80 produced a dose-dependent attenuation of operant responding with an ED_{50} of 28.6 mg/kg (CL 16.8 mg/kg – 48.6 mg/kg). Significant suppression of responding occurred at SNC-80 doses of 10 - 56 mg/kg [$F_{(7,49)}$=3.51, $P<0.05$].

There is limited evidence that nicotinic acetylcholine receptors may underlie some of the in vitro effects of nitrous oxide (Suzuki,et al., 2003, Yamakura & Harris, 2000). The nicotinic acetylcholine receptor agonist nicotine (Figure 27) failed to significantly substitute for N_2O [$F_{(6,24)}$=1.69, $P=0.19$] producing no greater than 1% N_2O-lever selection at any dose. Nicotine dose dependently attenuated operant responding with an ED_{50} of 1.1 mg/kg (CL 0.78 mg/kg – 1.7 mg/kg). Nicotine significantly suppressed operant responding at doses of 1.7 and 2.5 mg/kg [$F_{(6,30)}$=2.19, $P<0.05$].

To completely exclude the possibility that N_2O has interactions at nicotinic acetylcholine receptors I tested if a behaviorally active dose of nicotine could shift the nitrous oxide concentration effect curve (Figure 28). N_2O+vehicle (closed circles) produced concentration-dependent full substitution for the 60% training with an EC_{50} of 35% (CL 27% − 44%). Pretreatment with 1.0 mg/kg nicotine produced no significant alteration [$F_{(6,36)}$=0.35, $P=0.91$] in the N_2O concentration effect curve (closed squares) resulting in an almost identical EC_{50} of 36% (CL 27% − 48%). Neither N_2O+vehicle nor 1.0 mg/kg nicotine+ N_2O significantly attenuated operant response rates [$F_{(6,36)}$=0.40, $P=0.87$].
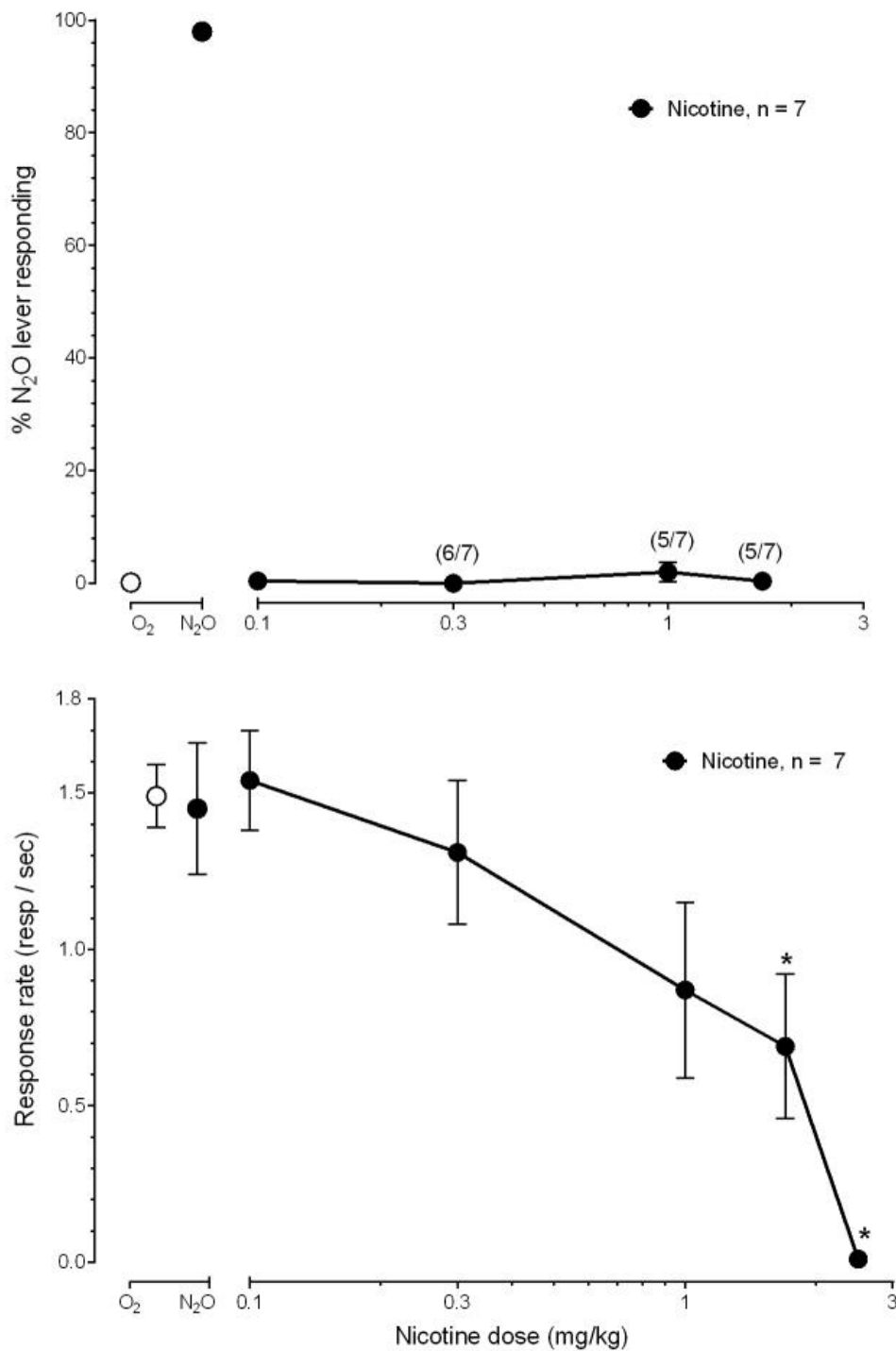
Figure 27. Nicotine substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions. Numbers in parenthesis indicate total number of subjects out of those tested which emitted response rates sufficient to be included in that point.* indicates significant ($p< 0.05$) differences from oxygen control.

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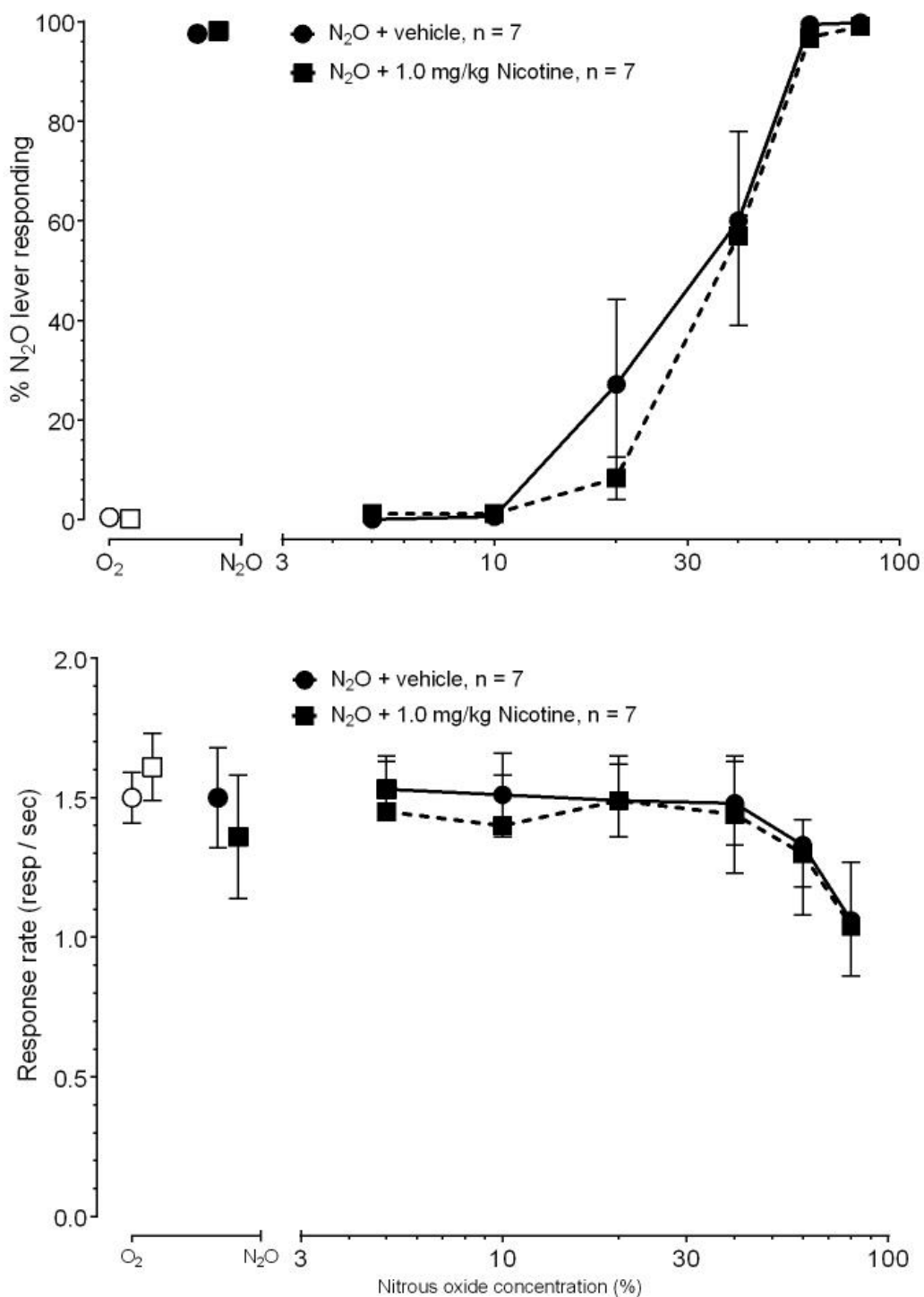
Figure 28. Effect of 1.0 mg/kg nicotine on nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N$_2$O+40% oxygen from 100% oxygen. Points above O$_2$ and N$_2$O reflect the 60% N$_2$O+40% oxygen and the 100% oxygen control conditions.

Studies have shown that neuronal nitric oxide synthase (nNOS) enzymes may play a role in N_2O anxiolysis [review see (Emmanouil & Quock, 2007)]. The nNOS inhibitor L-NAME (Figure 29) failed to substitute for N_2O [$F_{(3,9)}$=1.73, P=0.49] producing no greater than 2% N_2O lever selection at any dose. L-NAME did not significantly reduce operant response rates [$F_{(3,9)}$=2.85, P=0.49]. To explore if nNOS inhibition plays any role in the discriminative stimulus of N_2O I also conducted a curve-shift experiment to determine if L-NAME would antagonize the discriminative stimulus of N_2O (Figure 30). N_2O+vehicle (closed circles) produced a concentration-dependent full substitution for the 60% N_2O training concentration with an EC_{50} of 31% (CL 25% − 37%). Nitrous oxide preceded by pretreatment with 30 mg/kg L-NAME (closed squares) produced concentration-dependent full substitution for 60% N_2O with an EC_{50} of 35% (CL 32% - 39%) and did not significantly shift the nitrous oxide concentration effect curve [$F_{(6,42)}$=0.25, P=0.95]. No dose of N_2O alone attenuated operant responding by more than 25% of the O_2 control response rate. However, pretreatment with 30 mg/kg L-NAME prior to N_2O exposure produced a significant [$F_{(6, 42)}$=7.34, P<0.05] and concentration-dependent attenuation of operant responding sufficient to generate an EC_{50} of 73% (CL 61% - 86%).

As a final series of experiments, after sixteen months of training and testing, a full N_2O concentration-effect curve was reassessed to determine if tolerance or sensitization has occurred as a result of daily training and testing (Figure 31). The initial N_2O concentration-effect curve had resulted in an EC_{50} was 25% (CL 19% − 32%). The N_2O substitution curve from month sixteen (closed squares) resulted in an EC_{50} of 25% (CL 20% − 32%). A two way ANOVA revealed a significant main effect of N_2O concentration but there was no significance test date effect and no significant interaction [$F_{(6,138)}$=3.06, P=0.11] showing that tolerance did not develop to nitrous oxide's discriminative stimulus effects. There was, however, a significant

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increase in operant response rates between the initial concentration-effect curve and the

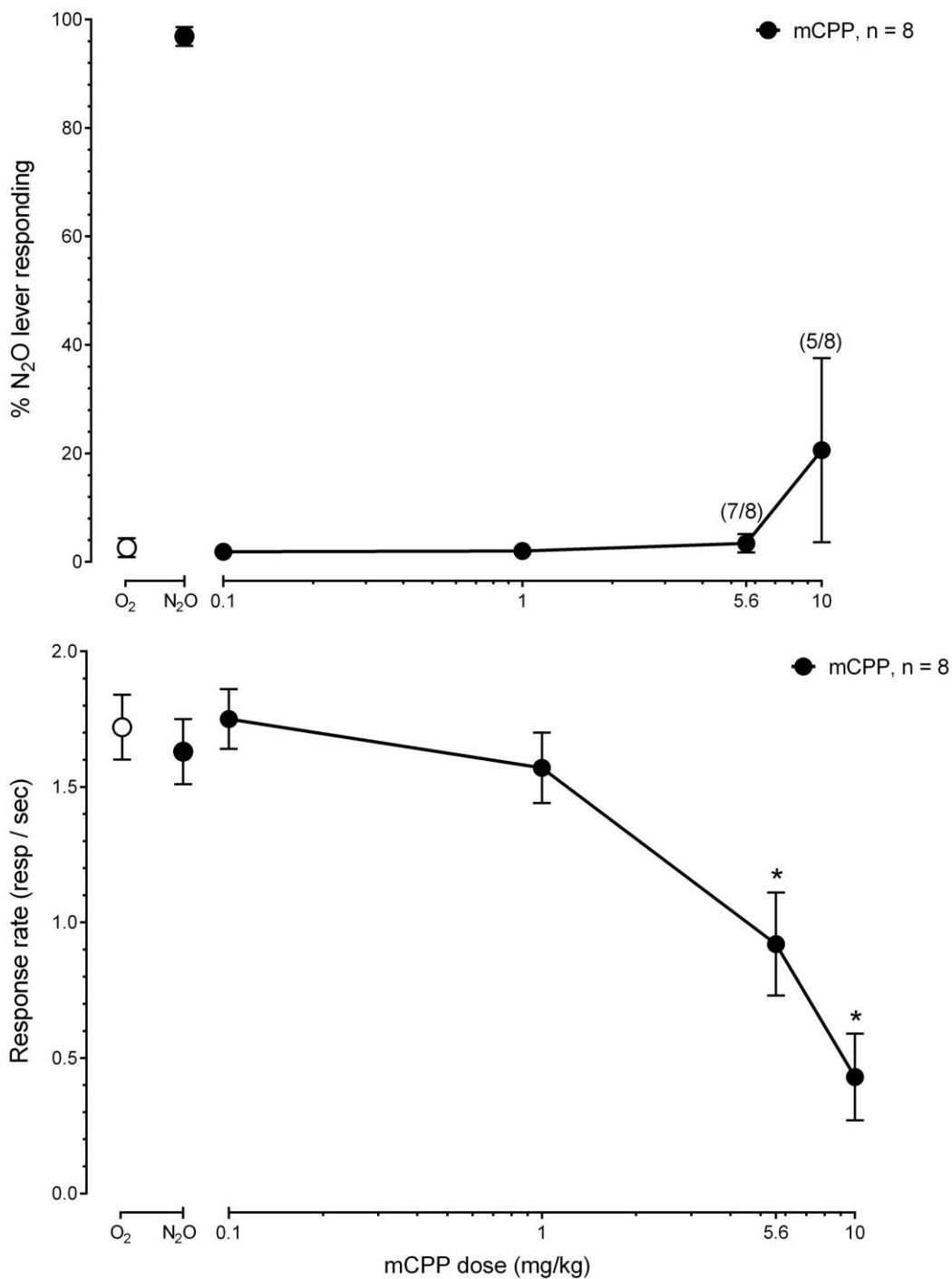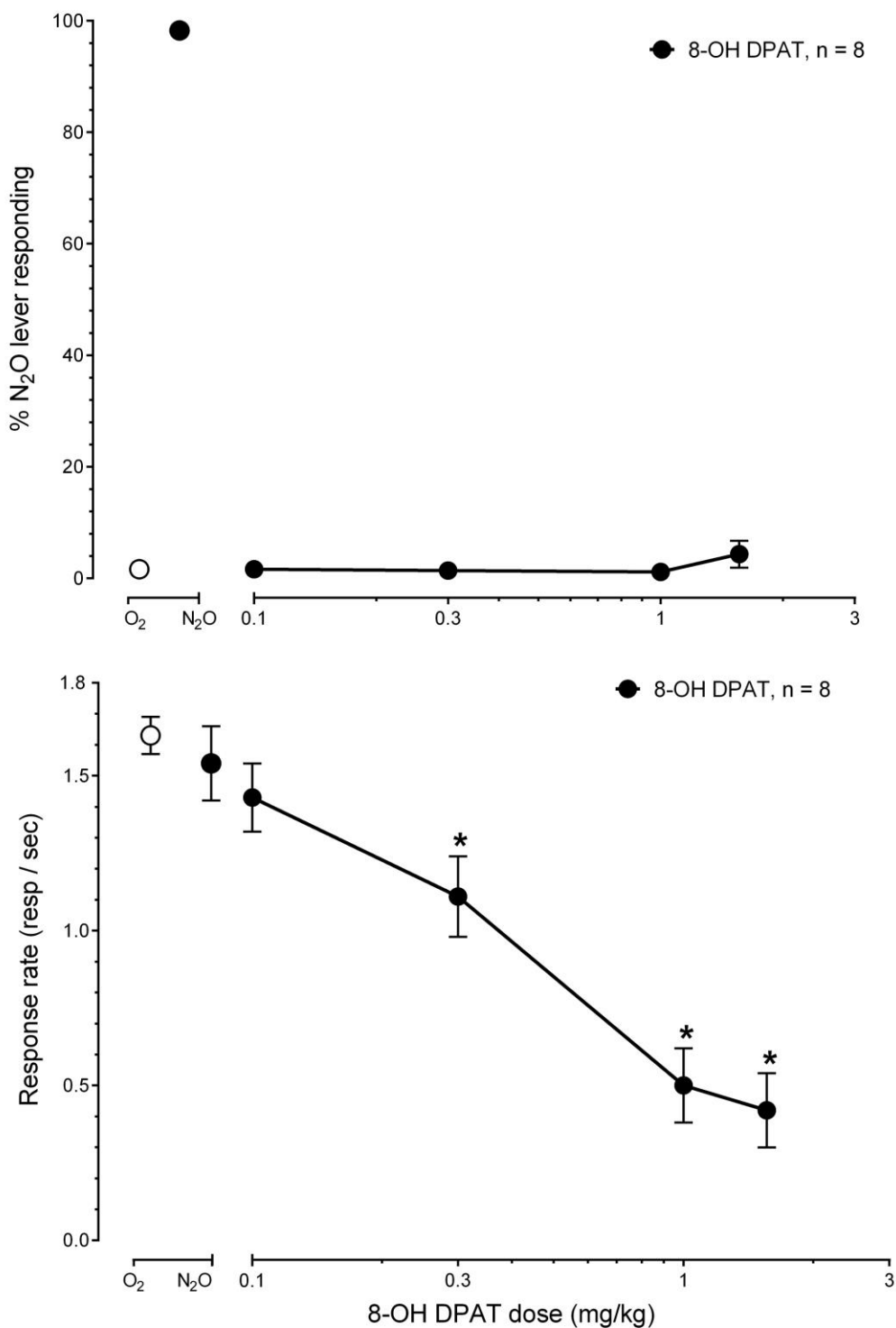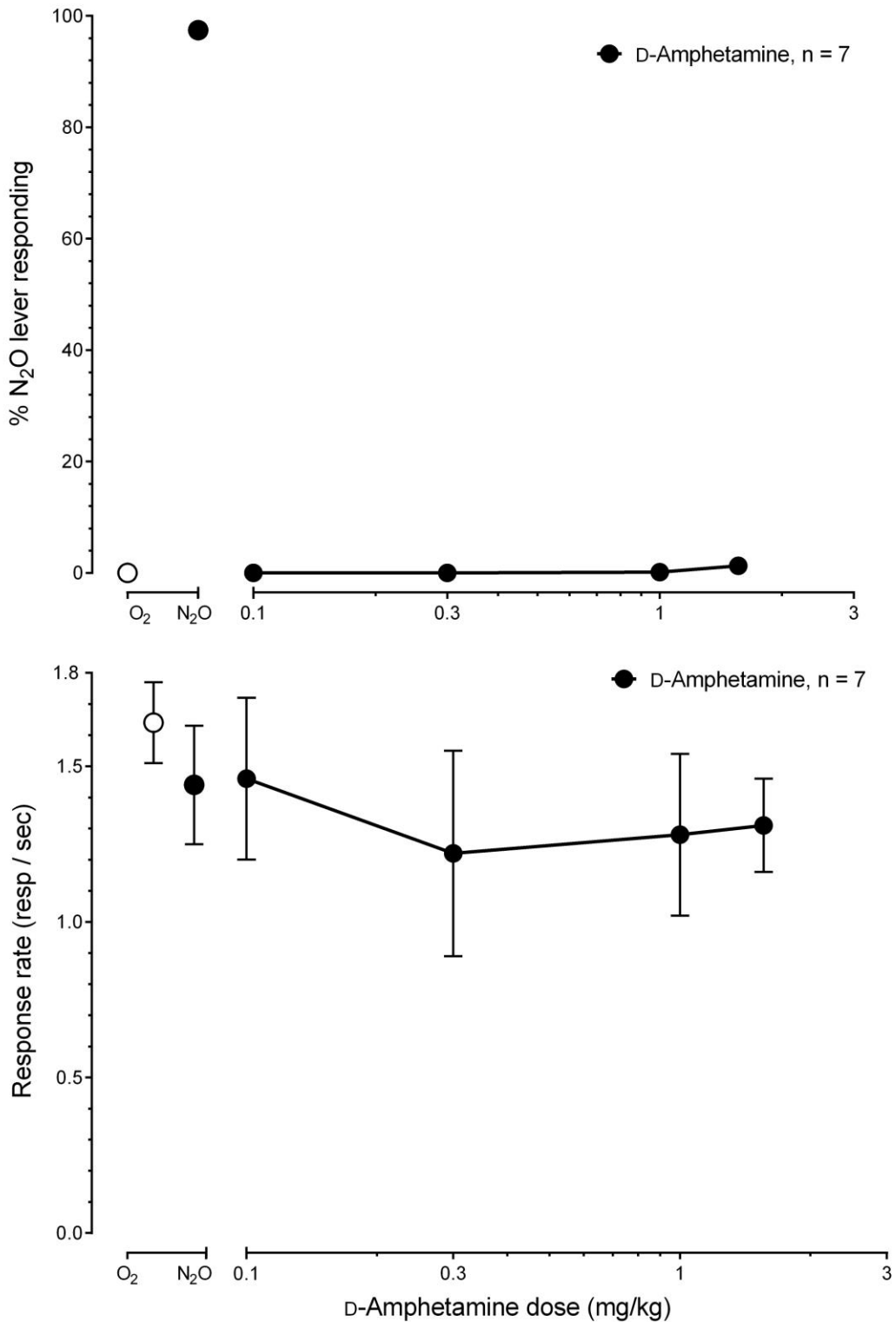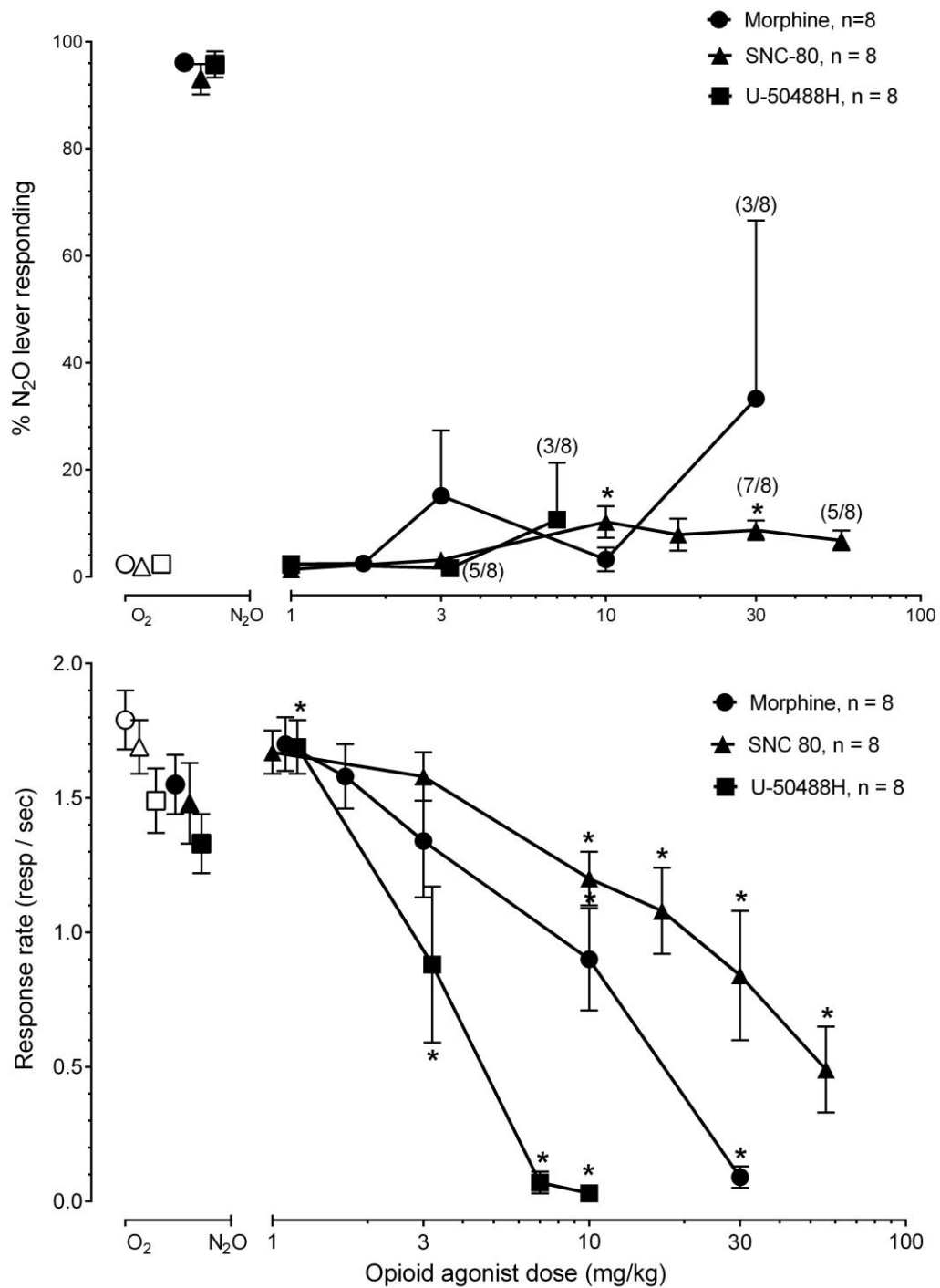concentration-effect curve generated in month sixteen [$F_{(6,138)}$=0.98, P<0.05].
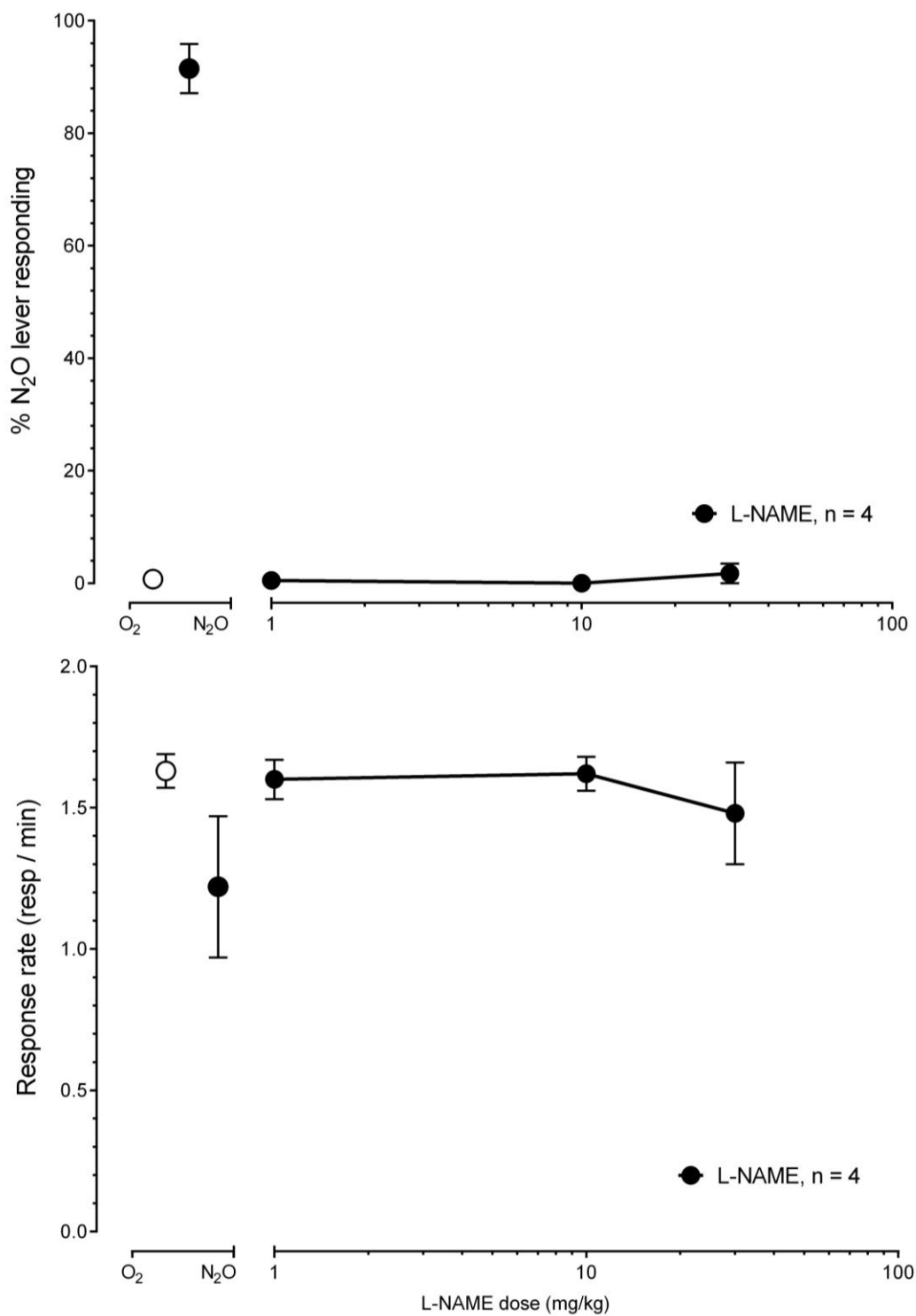
Figure 29. L-NAME substitution dose response (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions.

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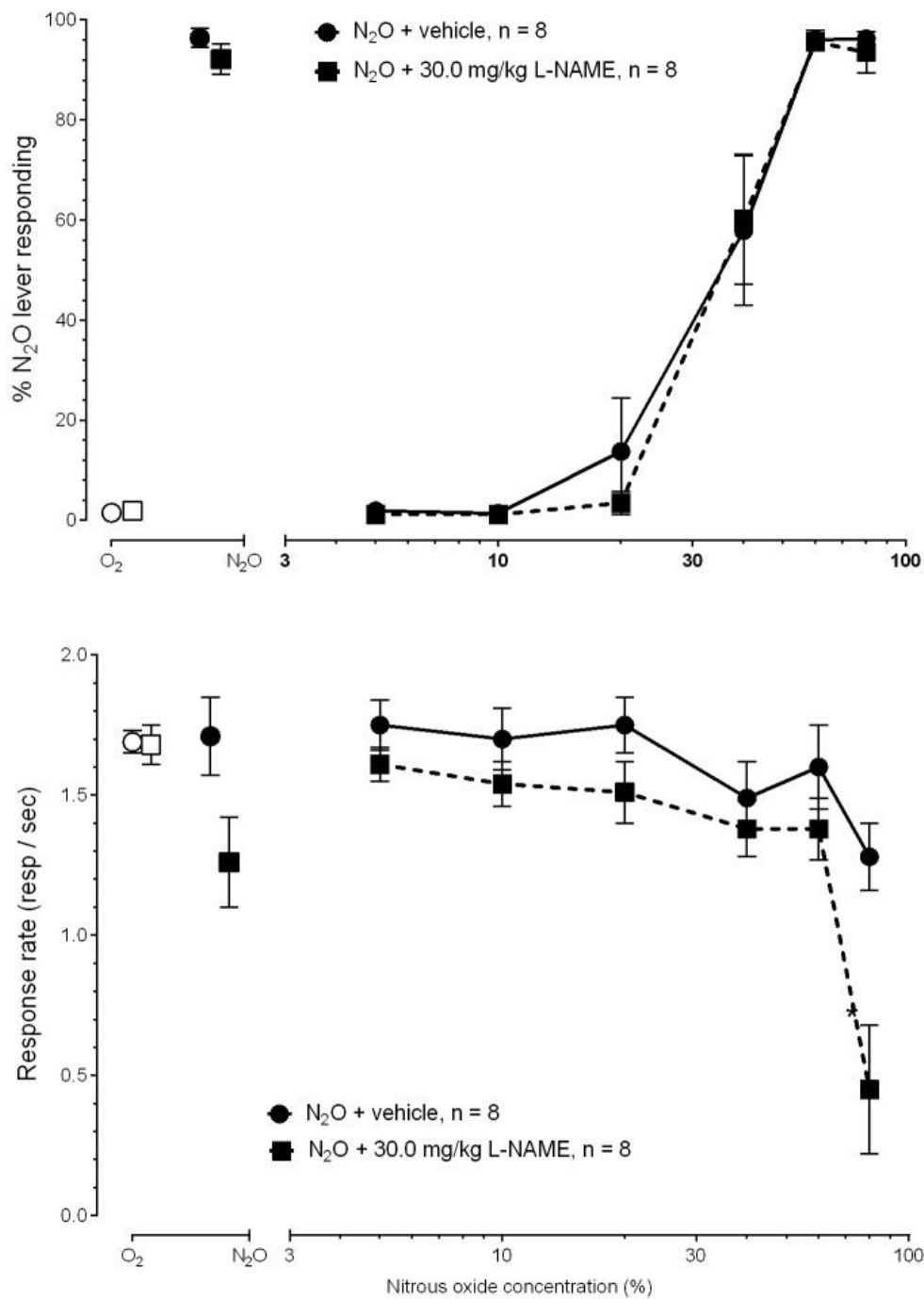
Figure 30. Effect of 30.0 mg/kg L-NAME on nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves in mice trained to discriminate 60% N_2O+40% oxygen from 100% oxygen. Points above O_2 and N_2O reflect the 60% N_2O+40% oxygen and the 100% oxygen control conditions.* indicates significant ($p < 0.05$) differences from N_2O + vehicle concentration effect curve.

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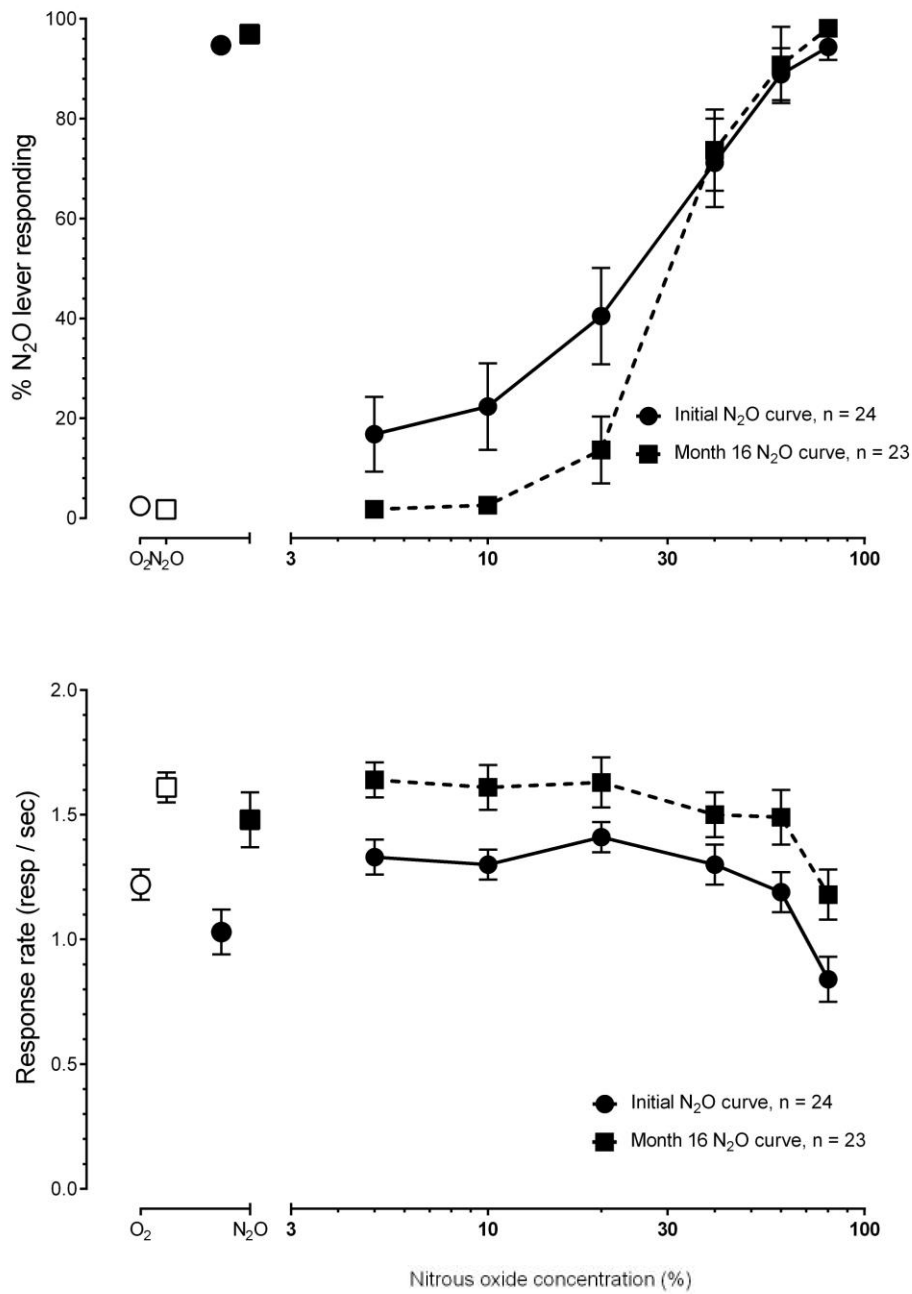
Figure 31. Nitrous oxide substitution concentration effect (upper panel) and operant response rate (lower panel) curves generated approximately one month after acquisition and after sixteen months of training and testing.

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Table 4. Inhalant exposure chamber concentrations and t_{99} calculations for the 26.0-L dual purpose exposure chamber. Applicable to N=16 subjects.

Desired concentration (%)	N$_2$O Scale	Q (LPM)	O$_2$ Scale	Q (LPM)	V (L)	Q total (LPM)	Concentration Start (%)	End (%)	t_{99} (min)
5	5	0.32	72	7.27	25.96	7.59	0.04	0.04	15.73
10	8	0.60	72	7.27	25.96	7.87	0.07	0.08	15.17
20	10	0.79	40	3.69	25.96	4.48	0.14	0.16	26.64
40	20	1.75	30	2.61	25.96	4.36	0.33	0.37	27.41
60	30	2.72	20	1.58	25.96	4.30	0.51	0.58	27.80
80	40	3.70	10	0.63	25.96	4.33	0.69	0.78	27.58

Table 5. Inhalant exposure chamber concentrations and t_{99} calculations for the 26.0-L dual purpose exposure chamber after optimization. Applicable to N=24 subjects.

Desired concentration (%)	N_2O Scale	Q (LPM)	O_2 Scale	Q (LPM)	V (L)	Q total (LPM)	Concentration Start (%)	End (%)	t_{99} (min)
5	5	0.32	150	4.58	25.96	4.90	0.06	0.06	24.37
10	8	0.6	146	4.52	25.96	5.12	0.10	0.11	23.33
20	14	1.17	105	3.61	25.96	4.78	0.21	0.23	24.99
40	30	2.72	97	3.39	25.96	6.11	0.40	0.43	19.56
60	42	3.89	50	1.92	25.96	5.81	0.60	0.65	20.56
80	73	6.93	44	1.71	25.96	8.64	0.77	0.80	13.82

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Discussion

Aim 1

Establishing a discrimination based on N₂O

My first aim was to train a N₂O discrimination in mice. Nitrous oxide also produces abuse-related subjective effects in humans (Beckman, Zacny, & Walker, 2006, Walker & Zacny, 2001, 2002, Zacny et al., 1994). Previous studies have also demonstrated that abused volatile vapors can serve as discriminative stimuli in rodents either when administered by injection or inhalation (Knisely, Rees, & Balster, 1990, Rees, Knisely, Jordan, & Balster, 1987, Shelton, 2007, 2009, 2010, Shelton & Nicholson, 2010, 2012, Shelton & Slavova-Hernandez, 2009). In the same respect I expected that, provided suitable training conditions were established, an inhaled N₂O gas discrimination could be trained in mice.

Given that nitrous oxide has never been trained as a discriminative stimulus in rodents, developing suitable training conditions was an important consideration. As with any drug discrimination assay the training concentration needed to be salient enough to produce discriminable CNS effects. Human data provided the most insight on appropriate conditions. In a choice study, human subjects chose 20% N₂O versus placebo in less than 25% of trials (Dohrn et al., 1993). In contrast, after 20 minutes of nitrous oxide exposure, ratings of both "high" and euphoria were significantly greater than vehicle ratings at a concentration of 30% N₂O but not 15% N₂O (Beckman, Zacny, & Walker, 2006). Further, 40% but not lower N₂O concentrations increased morphine-benzedrine group (MBG) ARCI ratings significantly above baseline in human volunteers (Dohrn et al., 1992). Based on these investigations of human subjective effects N₂O I hypothesized that concentrations above 40% should be both discriminable in mice

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as well as within the range of those which produce abuse-related effects in humans. A 10 minute N_2O exposure duration was chosen as the training exposure duration based on human choice paradigms which utilized a 10 minute sampling inhalation period prior to permitting subjects to choose N_2O or placebo (Walker & Zacny, 2001, Zacny, Walker, & Derus, 2008). In these studies subjective effects were reported as soon as 5 minutes into the 10 minute exposure (Zacny, Walker, & Derus, 2008). I believed that facilitation of training in mice would be improved by using concentrations and exposure times which exceeded the minimums that demonstrated effects in humans. Therefore, in the present study B6SJLF1/J mice were trained to discriminate 10 minutes of inhaled 60% N_2O+40% O_2 gas mixture from 10 minutes of exposure to 100% O_2.

Nitrous oxide is generally inhaled alone when abused. However, extended exposure such as that in the above mentioned studies or when utilized in clinical practice necessitates the addition of oxygen to prevent hypoxia. For simplicity, in the present study 100% oxygen rather than a variable nitrogen/oxygen mixture was used to dilute N_2O and 100% oxygen was chosen as the vehicle condition. These choices bear some discussion as it is possible that if 100% oxygen itself has stimulus effect what might have been trained in the present study was a drug versus drug rather than a drug versus vehicle discrimination. Although drug versus drug discriminations have been successfully trained the interpretation of cross-substitution results becomes more complex (Overton, 1982, Overton et al., 1989). However, when compared with air, 100% oxygen does not appear to affect mood or psychomotor performance (Dohrn et al., 1992). Further, oxygen as a vehicle to nitrous oxide is not unprecedented (Quock, Emmanouil, Vaughn, & Pruhs, 1992) and no other studies have noted confounds related to using an oxygen vehicle (Dohrn et al., 1992, Dohrn et al., 1993, Zacny et al., 1994, Walker and Zacny, 2003, Kangas and Walker, 2008). In the present series of studies, air was used as a control for

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experiments in which volatile compounds were administered and it produced exclusively oxygen-lever responding. Therefore it was likely that under the present training conditions, the stimulus effects of oxygen were negligible.

The discriminative stimulus effects of solvent and anesthetic vapors have been successfully trained in our laboratory (Shelton, 2007, 2009, 2010, Shelton & Nicholson, 2010, 2012, Shelton & Slavova-Hernandez, 2009). In each of these studies the subjects were exposed to the training inhalant and then removed from the exposure chamber for testing. These studies demonstrated that the stimulus effects of other inhalants were short but training was generally not problematic (Shelton, 2007, 2009, Shelton & Nicholson, 2010). The primary manifestation of the dissipation of the behavioral effects of the training inhalants was an underestimation of the potency of these compounds for suppressing operant behavior. In these studies, the authors only examined the first minute of behavior during each 5-minute test sessions in order to compensate for any loss of potency of the training inhalant over the course of the test session.

Since these methods were previously successful in my first study, I mimicked these prior inhalant discrimination training conditions. Subjects were exposed to 60% N_2O+40% O_2 or 100% O_2 for 10 minutes. They were then removed from the exposure chamber and trained in standard operant chambers in room air. Applying this approach it required a mean of 137.3 (±8.8) training sessions (88-192 range) to reach acquisition criteria of >80% total responses on appropriate lever and correct FFR in 8 of 10 training sessions (Figure 1). This was well beyond the number of training sessions required for other inhalants such toluene and TCE which were trained in our laboratory in a mean of 26 (±2.8) and 27 (±1.8) training sessions, respectively (Shelton, 2007, 2009). However, studies reporting the acquisition of other discriminative stimuli requiring an excess of 75 training sessions are not unprecedented. For instance, in rats

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trained to discriminate 2 hours food restriction from 22 hours food restriction the hunger cue versus satiety cue required 82 training sessions (Jewett et al., 2009). There were several possible reasons the initial nitrous oxide versus oxygen discrimination acquisition was so lengthy. First, long acquisition periods of drugs active in the CNS may be attributed to a weakly discriminable training dose (Overton, 1982). The problem can often be overcome by increasing the training dose of a drug (Willetts & Balster, 1989). Further the maximum concentration of N_2O which does not produce hypoxia, even when diluted in 100% oxygen, is approximately 79%. Therefore, if 60% N_2O is simply weakly discriminable in mice it would have been difficult to take corrective action. Other possible reasons for the lengthy training could have been an overly strict training criteria which seems unlikely given the criteria were identical to that used in a number of other inhalant drug discrimination studies in the laboratory (Shelton, 2007, 2009, Shelton & Nicholson, 2010). Lastly, it might have been that N_2O disrupted cognitive performance to such as extent that the discrimination could not be established. This too seemed unlikely given data showing the drugs which have demonstrated memory impairing effects such as the uncompetitive NMDA antagonist (+)-MK-801 could be trained in mice in a mean of 50 training session (Shelton & Balster, 2004).

In aggregate the data suggested that the most likely reason for the extended acquisition was that the stimulus effects of N_2O were so short that even a very brief 5-minute training session which was conducted after the cessation of exposure still resulted in excessive diminution of stimulus effects and poor stimulus control. This conclusion is supported by the N_2O generalization curve collected under these conditions. Specifically, the training concentration of 60% N_2O failed to fully substitute for itself (Figure 2). It required an even higher concentration of 66% N_2O to fully substitute for the 60% training concentration. Further,

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the discriminative reliability of the subjects was also quite poor resulting in few testing opportunities even after the acquisition criteria had been reached. I subsequently examined the hypothesis that the stimulus effects of nitrous oxide were simply too brief to train effectively under conditions used for other inhalants by revising our exposure/test apparatus and training new subjects.

Development and optimization of dynamic exposure/test apparatus

The revised apparatus consisted of four standard two lever mouse operant chambers housed inside individual acrylic exposure cubicles. This new system allowed continuous inhalant exposure through discrimination training and subsequent generalization testing. The development of a new dynamic system posed several additional challenges relative to the previous exposure apparatus. The most pertinent of which were chamber dynamics at different gas concentrations and flow rates. In volatile vapor and solvent discriminations conducted previously a fixed volume of volatile liquid was injected and circulated within a static exposure chamber. This permitted exposure concentrations to be calculated using the ideal gas law. It also allowed chamber concentrations to be achieved rapidly as the inhalant vapor was simply diluted and dispersed within the static chamber atmosphere rather than the existing atmosphere having to be displaced by a test atmosphere. In a dynamic system the rise in chamber concentration over time and the point at which a steady state concentration is achieved become additional critical parameters. Since the internal volume in the revised continuous exposure/test apparatus was more than double that of the smaller exposure chamber used in the initial experiment it was necessary to calculate and adjust the flow rate such that the chamber

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concentration was at the target training concentration of 60% for as long as possible prior to the start of the operant session.

Appendix 3 shows the application of equations which were used to model the exposure chamber concentration (C) at any time and the time required for the chamber to reach 99% steady state (t_{99}) in an open circuit dynamic exposure system (Cheng et al., 2010). The t_{99} can be mathematically determined when the volume of the chamber and total flow rate of gases are available. The estimated chamber concentration at any point during exposure can be approximated if the initial concentration, chamber volume, flow rate and flow time are known. In the smaller 9.9 liter exposure apparatus employed in initial training fixed proportional flow rates of N_2O and O_2 (60% or a 6:4 ratio) were used with the assumption being the exposure chamber would rapidly equilibrate at a steady state 60% N_2O training concentration. I later determined using the application of the above summarized formulas that this was an incorrect assumption and the 60% N_2O training concentration was reached only at the end of the training exposure period. When the same mice were retrained in the larger combined exposure/operant test chamber the usage of same proportional flow rates yielded an exposure concentration which never reached the target 60% N_2O concentration (Table 4). Therefore it was also possible that the previously extended training may have been exacerbated by insufficient exposure concentrations and durations. To alleviate this problem in the subsequent groups of mice trained exclusively in the combination exposure/operant test chamber and used for the bulk of the reported experiments, I increased gas flow rates to insure that the chamber concentration reached 60% N_2O prior to the start of the operant session (Table 5).

While these mathematical calculations provide expected values at differing flow rates I believed it was essential to develop an empirical method to confirm they were in fact accurate in

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my system. This was especially important given the highest test concentration of 80% N_2O would have produced hypoxia if the calculations overestimated the amount of oxygen available during 80% N_2O testing. Although specialized equipment can be used to directly measure N_2O concentrations that equipment was unavailable for the present series of studies. Therefore I chose to indirectly test N_2O concentration by monitoring oxygen concentrations during a mock 80% N_2O exposure using an inexpensive personal wearable safety oximeter (model PGM-1100; ToxiRAE, San Jose, CA, USA). Appendix 5 shows a plot of oxygen levels for an 80% mock test session using concentrations which were employed prior to flow rate optimization. Since the chamber was initially filled with air rather than 100% oxygen at the start of the session the oximeter revealed that the mice would have been exposed to hypoxic conditions as early as the third minute of exposure if the proportion of gas flows were not adjusted. I therefore altered the gas flow rates to insure that available oxygen levels never fell below 20% during the actual 80% N_2O exposure test sessions (Appendix 6).

Following the studies to optimize the revised exposure/test apparatus new mice were obtained and were trained under conditions in which the animals continued to be exposed to N_2O during discrimination training. The results confirmed my hypothesis that the most likely cause of the prior extended training was the rapid diminution of stimulus effect of N_2O following the cessation of exposure. Specifically the new system and exposure regimen resulted in a dramatic reduction in the number of days required for subjects to meet the acquisition criteria. When gas exposure continued for the duration of the operant training sessions the naïve subjects acquired the 60% N_2O+40% O_2 discrimination in a mean of 38.2 ($\pm$ 2.5) training sessions (Figure 4). This more rapid rate of acquisition is consistent with that required for other inhalants (Shelton, 2007, 2009) as well as drugs with multiple component cues (Shannon et al., 2004). My hypothesis is

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also supported by the greater discriminability of N_2O during the generalization curve in which the 60% N_2O+40% O_2 fully substituted for itself (Figure 3, upper panel) where it hadn't previously (Figure 2, upper panel) as well as the data showing that the mice generally tested on the majority of available opportunities after acquisition (Figure 7).

<u>N_2O concentration effect curve</u>

N_2O produced concentration-dependent full substitution for the 60% training concentration with an EC_{50} of 25% (CL 19% – 32%) (Figure 6, upper panel). In drug discrimination studies, full substitution of a training drug for itself typically occurs at the training dose as well as at higher doses (Johansson & Jarbe, 1976, Rees, Coggeshall, & Balster, 1985, Stolerman, Naylor, Elmer, & Goldberg, 1999). Likewise full substitution in the present study was engendered by both the 60% training concentration as well as by 80% N_2O exposure. Test doses of a training drug which are higher than the training dose often result in suppression of operant responding (Harris & Balster, 1968, Heffner, Drawbaugh, & Zigmond, 1974). However, in the present study the maximum test concentration of 80% N_2O only slightly and non-significantly attenuated operant responding (Figure 3 and Figure 6). In humans the N_2O minimum alveolar concentration required for anesthesia has been estimated to be 105% (Steffey et al., 1974) therefore the limited ability of N_2O to produce CNS depressant effects is not surprising.

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<u>Onset, offset and duration of discriminative stimulus effects</u>

The onset of the N_2O cue was fairly rapid. Seven minutes of 60% N_2O exposure was necessary to engender full substitution for the 10 minute training exposure (Figure 8). Previous choice paradigms in humans indirectly estimated a 5 minute onset of stimulus effect of N_2O (Walker and Zacny, 2003; Kangas and Walker, 2008; Zacny et al., 2008) and subjective effects ratings on the PCAG-ARCI (pentobarbital– chlorpromazine–alcohol group) and LSD-ARCI scales were significantly increased 15 minutes after the initiation of N_2O inhalation (Dohrn et al., 1993). Thus the onset of effects in mice was consistent with human data.

In contrast, the offset of stimulus effects of N_2O was more rapid in mice than estimates extrapolated from human subjective effects and choice data. In humans, drug liking was still elevated 40 minutes after one hour of exposure to 30% or 40% N_2O (Zancy et al., 1996). In mice, the stimulus effects were near vehicle levels only 5 minutes after the cessation of exposure (Figure 9). The more rapid offset but similar onset of nitrous oxides effects in mice and humans was unexpected. One potential factor in the human/mouse discrepancy may be exposure duration; in the previous human studies, the exposure duration was one hour but here the training exposure duration was only 10 minutes. It is therefore possible that 10 minutes is insufficient time to fully saturate the tissue of mice, resulting in a more rapid offset. However, the possibility that extended gas exposure would have lengthened offset of effects of nitrous oxide is unlikely based on my data (Table 3) which showed that doubling the exposure duration of 30% N_2O from 10 to 20 minutes failed to increase the degree of substitution it engendered. Instead, factors like the increased rate of respiration in mice and smaller total body volume are more likely responsible for the species difference in the offset of nitrous oxide's effects. These data further support my hypothesis that the fast offset kinetics of nitrous oxide's stimulus properties in mice

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probably heavily influenced the extended acquisition period observed when mice performed

discrimination training under room air.

Aim 2

The goal of my second aim was to examine the receptor systems underlying the stimulus effects of N_2O. Based on existing *in vitro* and *in vivo* data, I hypothesized that the stimulus effects of N_2O are based on multiple mechanisms. The nature of its subjective effects and strong *in vitro, ex vivo* and *in vivo* evidence of interactions at NMDA receptors implicated NMDA antagonism as the primary mediator of nitrous oxide's discriminative stimulus effects. *In vitro, ex vivo* and *in vivo* evidence also suggested an interaction of N_2O with $GABA_A$ receptors. Therefore, I hypothesized that $GABA_A$ receptor positive allosteric modulation may also play a role in the abuse related subjective effects of nitrous oxide. If these hypotheses were accurate I predicted that both NMDA antagonists as well as $GABA_A$ positive allosteric modulators would mimic nitrous oxides discriminative stimulus effects at least to some degree.

NMDA antagonism

Nitrous oxide attenuated agonist mediated NMDA receptor current in amygdalar slices (Ranft et al., 2007), substantia nigra cells (Balon et al., 2003), hippocampal preparations (Jevtović-Todovorić et al., 1998, Mennerick et al., 1998) as well as in heterologous expression systems (Ogata et al., 2006, Petrenko et al., 2010, Sato et al., 2005). Due to the strong evidence of interactions at NMDA receptors I hypothesized that NMDA antagonism was the primary mediator of nitrous oxides discriminative stimulus effects. There are several sites which N_2O may bind to the NMDA receptor to exert its discriminative stimulus effects: the glutamate binding/competitive site, glycine-binding site, channel blockade and polyamine sites.

I conducted cross-substitution tests with a representative NMDA receptor competitive antagonist (CGS-19755), a glycine site antagonist (L-701,324) as well as with three

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uncompetitive channel blockers (memantine, ketamine and (+)-MK-801 or dizocilpine). Drugs which antagonize the NMDA receptor via polyamine site-binding such as spermine, spermidine, arcaine (Nicholson & Balster, 1998), eliprodil (Balster, Nicholson, & Sanger, 1994) and ifenprodil hemitartrate (Sanger & Zivkovic, 1989) were not tested as I was interested in the abuse-related effect of nitrous oxide and this class of drugs appears to have little abuse liability.

In the present study, CGS 19755 (Figure 11, closed circle) produced a maximum of 11% (±5) nitrous oxide-lever responding at a dose of 17.0 mg/kg. CGS 19755 is a well-established and selective competitive NMDA antagonist which cross-substitutes with other competitive antagonists such as AP5, AP7 and NPC 12626 (Baron & Woods, 1995, Willetts et al., 1993). It is unlikely that the inability of CGS 19755 to produce nitrous oxide-like effects was due to insufficient test doses. CGS-19755 produced full substitution for isoflurane at 17 mg/kg CGS-19755 accompanied by reductions in operant response rates (Shelton & Nicholson, 2010). In the present study doses up to 30 mg/kg which also significantly attenuated operant responding were examined (Figure 11). Therefore, the poor substitution of a glutamate site antagonist for 60% N_2O is consistent with the conclusion that the discriminative stimulus properties of N_2O are not mediated by competitive antagonism of the NMDA receptor.

I probed glycine site NMDA receptor antagonism as a mechanism for the discriminative stimulus effects N_2O by conducting a cross-substitution test with L-701,324. L-701,324 produced no greater than 1% nitrous oxide-lever responding up to the maximum dose tested of 30 mg/kg. Across the dose range used in the present study L-701,324 did not produce attenuation of response rates. Higher doses of 45 mg/kg (Shelton & Nicholson, 2013) and 50 mg/kg (Shelton & Nicholson, 2012) L-701,324 also failed to suppress of operant response rates in mice previously. These data suggest that the behavioral effects of L-701,324 may require

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even higher doses or that it simply does not possess behavioral activity at all. However, L-701,324 has anticonvulsant activity (Wlaz & Poleszak, 2011) and will reduce forced-swim immobility time in mice (Poleszak et al., 2011) at doses as low as 2 mg/kg and 4 mg/kg, respectively, showing that it does have behavioral effects in some assays. Further, maximum substitution levels were produced by L-701,324 doses of 10 mg/kg in NPC 17742 trained rats and 3 mg/kg in PCP trained rats (Nicholson & Balster, 2009). These data support the tentative hypothesis that the discriminative stimulus effects of N_2O are not mediated by NMDA glycine site antagonism. However, it may be that L-701,324 is a poor probe drug for glycine site NMDA receptor antagonism (Nicholson & Balster, 2009, Witkin, Steele, & Sharpe, 1997) and different results would have been produced by a more potent and selective agent.

I examined NMDA receptor channel blockade as a mechanism underlying the discriminative stimulus effects N_2O by conducting cross-substitution test with the open channel blockers memantine, ketamine and (+)-MK-801. Three channel blockers with a range of affinities were tested because it was previously shown that drugs with the low affinity for the channel did not substitute in subjects trained to substitute 2.0 mg/kg PCP from saline, which has a high affinity for the channel (Nicholson & Balster, 2003). The NMDA receptor channel blockers produced greater N_2O appropriate responding than either the competitive or glycine-site NMDA antagonists. The low affinity NMDA receptor channel blocker memantine (Figure 10, closed triangle) produced dose-dependent partial substitution for 60% N_2O up to a maximum of 50% (±10) drug-lever responding. The moderate affinity NMDA receptor channel blocker ketamine (Figure 10, closed square) produced dose-dependent partial substitution for N_2O up to a maximum of 36% (±9) N_2O lever responding. The high affinity NMDA receptor channel blocker (+)-MK-801 (Figure 10, closed circle) produced a maximum of 55% (±16) N_2O-lever

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responding at the highest test dose of 0.75 mg/kg. In summary, the data suggested that NMDA receptor channel blockade might play some role in the stimulus effects of N_2O but there was little difference in the degree to which any individual open channel blocker could mimic the discriminative stimulus effects of N_2O. These data suggest that relative affinity of NMDA receptor channel blockers is not critical for modulating their relative similarity to N_2O.

The near 50% responding of the NMDA channel blockers is consistent with two additional alternative explanations. The first is that NMDA receptor channel blockers simply disrupted discrimination performance, producing roughly chance levels of substitution. NMDA antagonists disrupt glutamatergic neurotransmission in long term potentiation (Manahan-Vaughan et al., 2008), which can interrupt memory recall (Florian & Roullet, 2004). (+)MK-801 (Sanger & Zivkovic, 1989, Shelton & Balster, 2004) and other channel blockers (Beardsley et al., 2002, Bowen et al., 1999, Nicholson & Balster, 2003, 2009) can be easily trained in drug discrimination therefore this hypothesis is unlikely. However, approximately 50% levels of generalization are well below that which one can confidently infer mechanism.

Prior studies have shown that additive or synergistic discriminative stimulus effects may detect interactions between receptor systems (Young et al., 2006). Investigations of discriminative stimuli as well as other behavioral phenomena (Young et al., 2006) have used potentiation of effects at sub-maximal doses by a second compound as evidence of involvement in the mechanism of the behavioral effect. Based on these and similar studies I hypothesized that if NMDA receptor channel blockade was involved in the stimulus effects of N_2O it should be possible to shift the N_2O concentration effect curve leftward by (+)-MK-801 pretreatment. In the present study (+)-MK-801 did indeed enhance the discriminative stimulus of nitrous oxide. N_2O+vehicle (Figure 12, closed circle) produced full substitution for the training concentration

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with an EC_{50} of 32% (CL 24.8% – 41.3%). A low dose of (+)-MK-801 (Figure 12, closed square) produced a slight leftward shift of the N_2O concentration effect curve however the EC_{50} of 25.7% (CL16.8% – 39.3%) overlapped with the confidence limits of N_2O+vehicle. A moderate dose of 0.17 mg/kg (+)-MK-801 (Figure 12, closed triangle) in combination with nitrous oxide produced a significant [$F_{(10,60)}$=6.89, P<0.05] 1.78 fold leftward shift in the N_2O concentration effect curve. These data further support the hypothesis that the discriminative stimulus effects of nitrous oxide are at least partially mediated by NMDA receptor channel blockade.

Attenuation of the stimulus properties on N_2O by co-administration of a potential blocking agent such as an NMDA agonist is an alternative method to validate a role for interactions at or downstream of NMDA receptor antagonism in the discriminative stimulus effects of N_2O. However, previous literature indicated it might be difficult to antagonize the stimulus effects of drugs that have multiple receptor mechanisms (Bienkowski, Stefanski, & Kostowski, 1997). For instance, the administration of the agonist NMDA could not antagonize the discriminative stimulus effects of ethanol (Bienkowski, Stefanski, & Kostowski, 1997). Further complicating this strategy it has been demonstrated that only competitive NMDA antagonists but not channel blockers could attenuate the discriminative stimulus effects of NMDA without significant reduction in rates of responding (Willetts & Balster, 1989).

Nonetheless I attempted to antagonize the NMDA antagonist component of N_2O cue with the glycine site co-agonist sarcosine. There is some data to suggest it may be possible to attenuate some of the effects of NMDA antagonists by administration of co-agonists acting at the glycine recognition site. Specifically, the glycine site co-agonist sarcosine significantly reduced toluene-induced cognitive impairment in the novel object recognition test and motor

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incoordination in the rotarod test but did not alter response latency or threshold in toluene ICSS (Chan et al., 2012). In the present study sarcosine did not substitute for N_2O nor did it alter response rates (Figure 16). More importantly, pretreatment with the same 300 mg/kg dose of sarcosine which attenuated toluene-induced behavioral deficits (Chan et al., 2012) failed to significantly shift the nitrous oxide concentration effect curve (Figure 17, closed squares) nor did it alter response rates. Pretreatment with and even higher 600 mg/kg sarcosine dose also failed to alter the nitrous oxide concentration-effect curve producing an almost identical EC_{50}. However, pretreatment with 600 mg/kg sarcosine actually enhanced, rather than reduced the rate suppressing effects of N_2O. Unfortunately, these experiments with sarcosine neither provide additional evidence in support of nor refute my hypothesis that the discriminative stimulus effects of nitrous oxide are mediated by NMDA receptor antagonism. To provide any useful information it might have been productive to determine if the nitrous oxide-like stimulus effects of (+)-MK-801 could be attenuated by sarcosine, but these studies were unfortunately not conducted.

Overall, uncompetitive NMDA antagonists had a greater ability than competitive and glycine site NMDA antagonists to mimic the discriminative stimulus effects of N_2O. However, cross-substitution has been reported between channel blockers and competitive antagonists (Baron & Woods, 1995, Nicholson & Balster, 2002, Wiley & Balster, 1994) which leaves open the possibility that a probe drug with another site of action on NMDA receptors may produce appreciable substitution results. This hypothesis is somewhat less likely given the completely negative results produced by the other classes of NMDA antagonists in N_2O-trained mice. The conclusion that N_2O acts through mechanisms similar to channel blockade is further strengthened by data from another study demonstrating that the competitive antagonist NPC 17742 did not

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elicit substitution in rats trained to discriminate 2.0 mg/kg of the uncompetitive NMDA antagonist PCP from vehicle (Nicholson & Balster, 2009) as well as data showing that glycine site NMDA antagonists with very few exceptions (Baron & Woods, 1995) at best partially substitute for PCP (Balster et al., 1995, Beardsley, Ratti, Balster, Willetts, & Trist, 2002, Nicholson & Balster, 2009).

GABA$_A$ receptor positive allosteric modulation

Nitrous oxide potentiates agonist mediated GABA$_A$ current in hippocampal preparations (Dzoljic & Van Duijn, 1998) and in heterologous expression systems (Hapfelmeier et al., 2001, Hapfelmeier et al., 2000, Yamakura & Harris, 2000). Further, nitrous oxides subjective effects are attenuated by administration of a benzodiazepine site antagonist, flumazenil (Zacny et al., 1995). These data suggest that the discriminative stimulus effects of nitrous oxide may also be at least partially mediated by GABA$_A$ receptor positive modulatory effects. To address this possibility I tested the ability of five different site-selective GABA-positive drugs for their ability to substitute for nitrous oxide. These drugs included the direct GABA$_A$ agonist muscimol, the extrasynaptic GABA$_A$ agonist gaboxadol (THIP), the GABA transaminase inhibitor valproic acid as well as positive allosteric modulators midazolam and pentobarbital.

The direct GABA$_A$ agonist muscimol failed to significantly substitute for 60% N$_2$O (Figure 13, closed squares). Gaboxadol a direct partial agonist at extrasynaptic $\alpha4\beta3\delta$ and $\alpha6\beta1\gamma2$ GABA$_A$ receptors also failed to produce significant substitution for 60% N$_2$O (Figure 13, closed circles). These data suggest that subjective effects of nitrous oxide are not mediated by direct GABA$_A$ agonist effects at either synaptic or extrasynaptic GABA$_A$ receptors and are

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consistent with studies showing N_2O does not potentiate $GABA_A$ current without the presence of agonist (Hapfelmeier et al., 2000).

Of the potential GABAergic mechanisms, the possibility that nitrous oxide acts as a positive allosteric modulator at $GABA_A$ was the most strongly implicated in the literature. A concentration of 29.2 mM nitrous oxide did not activate $\alpha1\beta2\gamma2L$ recombinant $GABA_A$ receptors without the presence of agonist GABA but did enhance activation in the presence of GABA (Hapfelmeier et al., 2000). Furthermore, the benzodiazepine chlordiazepoxide produced behavioral cross-tolerance with 75% nitrous oxide in an anxiolytic-sensitive staircase task (Quock et al., 1992). Positive $GABA_A$ allosteric modulatory sites have been identified for the binding of benzodiazepines, barbiturates and GABA positive neurosteroids. In the present study neither the benzodiazepine-site positive allosteric modulator midazolam nor the barbiturate pentobarbital produced meaningful levels of cross-substitution with 60% N_2O. Midazolam did however (Figure 14, closed circle) produce a low but statistically significant level of partial substitution for 60% N_2O. This data is likely an anomaly attributable to the extremely low variability across subjects but to completely rule out the possibility of a GABA positive component in the discriminative stimulus effects of nitrous oxide I conducted a curve-shift experiment to determine if midazolam would enhance the discriminative stimulus of 60% N_2O. N_2O+vehicle (Figure 15, closed circles) produced concentration-dependent full substitution for the 60% N_2O training concentration with an EC_{50} of 25 % (CL 14% – 44%). Pretreatment with 0.3 as well as 3 mg/kg midazolam failed to potentiate the N_2O concentration effect curve. Pretreatment with 10.0 mg/kg midazolam also failed to potentiate the N_2O concentration-effect curve but it did increase the potency of N_2O for suppressing responding.

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Lastly, it was remotely possible that N_2O might function in some manner to increase extracellular GABA levels through interference with GABA reuptake or degradation mechanisms. One of the major actions of valproic acid is to increase GABA levels by blocking GABA transaminase. In the present study valproic acid (Figure 13, closed triangles) did not significantly substitute for N_2O. Therefore it is very unlikely nitrous oxides subjective effects are mediated by this mechanism either.

In summary these negative findings with GABAergic probe compounds were surprising especially considering the *in vitro* literature as well as human data showing that flumazenil attenuates the subjective "high" produced by 30% N_2O (Zacny et al., 1995). One explanation could be GABAergic involvement in nitrous oxides cue is only prevalent or even detectable at certain training doses. This would somewhat parallel the findings from studies in which ethanol has been trained as a discriminative stimulus. In these experiments it was found that the GABAergic component of ethanol's discriminative stimulus was present at low ethanol training doses but higher training doses were required to reveal the NMDA antagonist-like stimulus effects of ethanol (Grant & Colombo, 1993, Shelton & Grant, 2002, Vivian, Waters, Szeliga, Jordan, & Grant, 2002). In N_2O trained mice it may be that the exact opposite is the case in that the 60% N_2O training concentrations only produce NMDA antagonist-like stimulus effects whereas a higher training concentration might reveal a GABAergic component. This seems somewhat unlikely given that it appears that the stimulus effects of N_2O are produced at similar concentrations in humans and mice and the 30% N_2O concentration which was attenuated by flumazenil in humans was lower, not higher than that trained in the present study. It is also possible that there is a GABAergic component present in the stimulus effects of N_2O but the relatively high 60% training concentration resulted in such a strong NMDA antagonist

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component that the GABAergic component was overshadowed. Lastly, it is also possible that GABAergic mechanisms are not involved in the stimulus effects of N_2O but may be implicated in other behavioral effects such as anxiolysis (Czech & Quock, 1993, Czech & Green, 1992, Li & Quock, 2001). Regardless in sum the present data strongly suggest that GABAergic positive modulation is not an important mechanism in transducing the subjective effects of N_2O under the present training conditions.

Aim 3

The purpose of Aim 3 was to determine, as has been speculated, if nitrous oxide represents a unique entity within the broader abused inhalant drug class (Balster, 1998). To explore this question the stimulus effects of N_2O were compared to other representative abused inhalants from a variety of chemical classes.

Comparison of the stimulus effects of N_2O to volatile halogenated anesthetics

Isoflurane is an inhalational anesthetic described as sedating at sub-anesthetic concentrations in human volunteers (Zacny et al., 1994) while N_2O is described as producing a more pronounced "high" accompanied with a "dreamy, detached reverie" state (Zacny et al., 1994; Beckman et al., 2006). In the present study isoflurane (Figure 18, closed circle) produced a maximum of 39% (±11) N_2O-lever selection. The limited degree of overlap between N_2O and isoflurane is consistent with previous results from our laboratory in which N_2O produced a maximum of 31% (±18) isoflurane appropriate responding (Shelton & Nicholson, 2010). In this regard isoflurane and N_2O substitute symmetrically, albeit partially, for one another. Unlike isoflurane, methoxyflurane has pronounced analgesic effects in humans (Tomi et al., 1993; Abdullah et al., 2011; Caldicott, 2011); methoxyflurane may have been more N_2O-like given that it too produces analgesic effects. Methoxyflurane (Figure 18, closed square) produced a maximum of 47% (±14) N_2O-lever selection. While somewhat greater than the substitution produced by isoflurane, the mean difference in substitution between the two volatile inhalants are probably not sufficient to make any conclusions given the normal variability in drug discrimination data. Additional studies with much larger subject sizes would be required to determine if this difference is meaningful.

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The discriminative stimulus properties of isoflurane are mimicked by both $GABA_A$ positive modulators and NMDA antagonists. Specifically, benzodiazepine, barbiturates and valproic acid produced robust, dose-dependent substitution for 6,000 ppm isoflurane. The competitive NMDA antagonist CGS 19755 was also isoflurane-like while an open channel blocker produced partial substitution in isoflurane trained subjects. Based on the poor substitution of GABAergic compounds for 60% N_2O, the degree to which isoflurane was able to produce overlapping discriminative stimulus effects with N_2O is probably due to the contributions of NMDA antagonism to its discriminative stimulus. The limitations of isoflurane's ability to produce a greater degree of N_2O appropriate responding despite having a common NMDA antagonist component could be speculated to be attributable to overshadowing of that component by the GABAergic effects of isoflurane. Additional experiments will be required to more fully explore this possibility.

Comparison of the stimulus effects of N_2O and abused volatile inhalants.

Toluene is perhaps the prototypic abused inhalant and has been speculated to be in a subclass of volatile inhalants which are distinct from N_2O. Like N_2O, toluene has numerous molecular targets including NMDA, $GABA_A$, glycine, $5HT_3$, neuronal nicotinic acetylcholine, dopaminergic and muscarinic receptors as well as sodium, calcium and potassium channels [for review see (Bowen et al., 2006)]. In the present study toluene (Figure 19, closed square) produced a concentration-dependent and significant level of partial substitution for N_2O up to a maximum of 72% (±10) N_2O-lever responding. In individual subjects, the degree of similarity between toluene and N_2O was even more apparent in that toluene produced full substitution for N_2O in seven of eight subjects at one or more test concentrations. Of all the inhalants tested,

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toluene produced the most robust N_2O-like stimulus effects. The high degree of substitution of toluene for 60% N_2O would suggest the neurochemical substrates underlying their stimulus properties are very similar. Several studies have characterized toluene's discriminative stimulus (Knisely et al., 1990, Rees et al., 1987, Shelton, 2007, Shelton & Nicholson, 2013, Shelton & Slavova-Hernandez, 2009). Toluene has overlapping discriminative stimulus effects with pentobarbital and ethanol (Rees et al., 1987). Further, benzodiazepines produce robust substitution in toluene-trained subjects (Knisely, Rees, & Balster, 1990, Shelton & Nicholson, 2013). In contrast, NMDA antagonism does not appear to be a strong component of toluene's discriminative stimulus effect (Shelton & Nicholson, 2013). These data have been interpreted as evidence that the stimulus effects of toluene are primarily $GABA_A$ receptor mediated. It is difficult to reconcile the toluene cross-substitution data with my prior results demonstrating that N_2O has NMDA antagonist-like but little to no $GABA_A$ positive modulator-like stimulus effects. It could be that these data are simply an aberration. That seems unlikely given that TCE also produced some degree of cross-substitution in the present study. Like toluene, the stimulus effect of TCE have been shown to overlap with $GABA_A$ positive modulators but not NMDA antagonists (Shelton, 2010, Shelton & Nicholson, 2012). Given the large number of potential targets the most parsimonious explanation is that some other as yet unidentified stimulus commonality may exist between volatile inhalants and N_2O and that component may be responsible for the robust cross-substitution data. This is, however, purely speculation as no data has yet been generated to support this hypothesis.

Lastly, it is worth noting that an alternative interpretation of the ability of all of the volatile compounds to produce some degree of N_2O-like stimulus effects may be due to their strong odors. To determine if a strong odor alone was sufficient to elicit N_2O-like stimulus

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effects, a sweet smelling odorant devoid of CNS properties was examined for cross-substitution

(Figure 20). 2-butanol produced no greater than 3% N_2O lever responding at any concentration

tested. This data is consistent with prior studies from the laboratory showing that odor does not

produce inhalant-like discriminative stimulus effects in TCE or toluene trained mice (Shelton,

2007, 2009) and therefore support the conclusion that the stimulus effects of abused inhalants are

CNS mediated.

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Aim 4

The goal of Aim 4 was to explore some of the less likely molecular mechanisms which might have been involved in transducing the discriminative stimulus properties of nitrous oxide. These molecular mechanisms were considered less likely due to either a limited number of studies in the literature suggesting their involvement or the presence of overtly conflicting reports. For several of the drugs chosen positive substitution results of drugs tested in Aims 2 and 3 influenced the choice of drugs tested in Aim 4.

Dopamine involvement in the stimulus effects of nitrous oxide

One study has shown that toluene elicits partial substitution in D-amphetamine-trained mice (Bowen, 2006). Given the overlap between the discriminative stimuli of toluene and N_2O I speculated that the stimulus effects of N_2O might also have a dopaminergic component. In the present study, D-amphetamine produced only vehicle-appropriate responding (Figure 25) across a dose range which, while not able to suppress operant behavior, has been shown to be active in other behavioral procedures such as drug discrimination (Porter et al., 2008) and locomotor activity (Marquez, Hamid, & Lutfy, 2013). This data suggests that facilitation of dopaminergic neurotransmission is probably not involved in the discriminative stimulus effects of N_2O despite data showing that dopamine may be involved in the antinociceptive effects N_2O (Koyanagi, Himukashi, Mukaida, Shichino, & Fukuda, 2008).

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<u>Effects of ethanol and serotonin agonists</u>

Ethanol has been found to have many common behavioral properties with inhalants including stimulus effects (Rees, Knisely, Breen, & Balster, 1987), low dose locomotor activation and high dose respiratory depression (Bowen & Balster, 1998) and anxiolytic properties (Lapin, 1993). A drinking history also increases N_2O choice in humans and a N_2O exposure history reduces ethanol drinking in rats (Kosobud, Kebabian, & Rebec, 2006, Zacny, Walker, & Derus, 2008). The stimulus effects of ethanol have been shown to be mediated by primarily by NMDA antagonism (Kotlinska & Liljequist, 1997, Shelton & Balster, 1994) and $GABA_A$ receptor positive modulation (Grant, Waters, Green-Jordan, Azarov, & Szeliga, 2000, Helms, Rogers, & Grant, 2009, Shelton & Balster, 1994). I had originally postulated that N_2O, like ethanol, was a compound cue primarily composed of a NMDA antagonist and a $GABA_A$ positive allosteric modulator component. Therefore, I speculated that ethanol would robustly substitute for 60% N_2O. Rather than elicit full substitution, ethanol produced a maximum of 45% (±12) N_2O-lever selection at a dose of 2.5 g/kg, the highest dose tested. This partial level of substitution suggested some overlap in stimulus effects of ethanol and N_2O but, as was the case with MK-801, the data were equivocal. To increase my confidence that the data were not simply an anomaly I conducted a curve shift experiment to determine if ethanol would enhance the discriminative stimulus of N_2O. Pretreatment with a low dose of 0.5 g/kg ethanol failed to significantly increase the stimulus potency of N_2O. However, a moderate pretreatment dose of 1.5 g/kg ethanol significantly enhanced the discriminative stimulus effects of N_2O [$F_{(8,56)}=4.43$, $P<0.05$] producing a 2.84 fold leftward shift in the N_2O concentration-effect curve.

These data supported my hypothesis that N_2O and ethanol have a least one common stimulus component. The failure of ethanol to fully substitute for N_2O is probably not surprising

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given that GABAergic drugs had little to no overlap with the stimulus properties of N_2O (Figures 13-15). It is most likely that only interactions at NMDA receptors are responsible for the shared stimulus properties between ethanol and N_2O. Comparing Figure 10 and Figure 21 reveal almost identical N_2O lever responding; ethanol alone produced 45% ($\pm$12) and MK-801 alone produced 50% ($\pm$10). However, comparing Figure 12 and Figure 22 suggest that ethanol may produce a slightly more robust leftward shift in the N_2O concentration effect curve than (+)-MK-801. Specifically, the most efficacious dose of 0.17 mg/kg (+)-MK-801 (Figure 12, closed triangle) produced a 1.78 fold leftward shift in the N_2O concentration effect curve and an EC_{50} value of 17% (CL 13% − 23%). In contrast pretreatment with 1.5 g/kg ethanol (Figure 22, closed triangle) produced a more pronounced 2.84 fold leftward shift in N_2O concentration effect curve and an EC_{50} 11% (CL 7% − 18%). It is therefore possible that there is another common mechanism underlying the stimulus effects of both compounds.

The most likely additional common receptor mediator of the stimulus effects of both N_2O and ethanol are serotonin receptors. The discriminative stimulus effects of ethanol also have an additional serotonergic component which is manifested most strongly at low training doses (Grant, Colombo, & Gatto, 1997). The $5HT_{1B/2C}$ agonist mCPP substitutes for ethanol (Grant, Colombo, & Gatto, 1997). In the present study mCPP failed to substitute for N_2O. mCPP produced a maximum of 21% ($\pm$17) N_2O lever responding at the highest dose of 10 mg/kg, which also fully suppressed operant responding in three of eight subjects. This would suggest that the discriminative stimulus of N_2O does not have a $5HT_{1B/2C}$ agonist-like component. However, the most robust substitution of mCPP for ethanol occurred at a low, rather than high training doses (Grant, Colombo, & Gatto, 1997). Another serotonergic agonist 8-OH DPAT which is more selective for $5HT_{1A}$ receptors produced less robust substitution for ethanol overall

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but showed stronger ethanol-like stimulus effects at a high training dose. In the present study 8-OH DPAT (Figure 24) failed to significantly substitute for N_2O [$F_{(7,28)}=7.98$, $P=0.20$] producing no greater than 4% N_2O-lever selection at any dose. These data suggest that the discriminative stimulus effects of N_2O are not mediated by $5HT_{1B/2C}$ or $5HT_{1A}$ receptors. However, only a single training concentration of N_2O was used in the present study. The 60% N_2O training concentration was probably relatively high given it had robust stimulus effects and relatively short training duration. It is therefore possible that N_2O does have some serotonergic component which might be revealed with a lower training concentration. Additional studies would be necessary to address this hypothesis.

In summary, the discriminative stimulus effects of ethanol and N_2O have a limited degree of similarity. Of the three major mediators of ethanol's discriminative stimulus, only NMDA antagonists produced N_2O-like stimulus effects. Further the most robust level of substitution produced by any NMDA antagonist was nearly identical to that produced by ethanol. These data support the conclusion that the overlap in stimulus effects are due to the uncompetitive NMDA antagonist-like stimulus effects of both compounds.

Mu, kappa and delta opioid agonist effects

The analgesic and antinociceptive effects of N_2O have been hypothesized to be mediated by interactions with mu opioid receptors (Emmanouil et al., 2008). In the present study morphine (Figure 26, closed circles) produced a maximum of 33% (± 33) N_2O lever responding at a dose which fully suppressed operant responding in five of eight subjects. The inability of morphine to produce appreciable substitution in these subjects is consistent with lack of

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antagonism of 30% N_2O's subjective effects by opioid antagonist naloxone (Zacny et al., 1999, Zacny, Coalson, Lichtor, Yajnik, & Thapar, 1994) as well as data showing that N_2O does not produce morphine-like discriminative stimulus effects in guinea pigs (Hynes & Hymson, 1984). These data suggest that mu opioid receptors may mediate the analgesic and antinociceptive effects of N_2O but are probably not involved in transducing the discriminative stimulus effects of N_2O. Like morphine, the delta opioid agonist SNC-80 (Figure 26, closed triangles) also failed to produce greater than 10% N_2O lever responding. The failure of the delta opioid agonist to substitute in these subjects is consistent with reports that the delta opioid receptor agonist naltrindole does not attenuate N_2O analgesia (Koyama & Fukuda, 2010).

Unlike the negative substitution results in morphine-trained guinea pigs, N_2O substituted in guinea pigs trained to discriminate the purported kappa opioid agonist ethylketocyclazocine from vehicle (Hynes & Hymson, 1984). In the present study the selective kappa opioid agonist U50-488H (Figure 26, closed squares) did not substitute for 60% N_2O, producing a maximum of only 11% ($\pm$11) N_2O-lever responding. The failure of U50-488H to elicit significant substitution in the present study was surprising given the prior ethylketocyclazocine data (Hynes & Hymson, 1984). However, recent data suggests 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, Li, & McMillan, 2011). This discrepancy does not explain why N_2O has ethylketocyclazocine but not morphine-like stimulus effects in guinea pigs but, unlike ethylketocyclazocine, U50-488H is a more selective kappa opioid agonist. It may have been the case that a mixed mu/kappa opioid agonist would have produced more robust substitution for N_2O than either compound alone but that possibility was not examined. Alternatively it may be that the differences between studies were species dependent although this

seems unlikely given mice and guinea pigs both express all three opioid receptor subtypes. Overall, the low levels of N_2O appropriate responding engendered by cross substitution tests of opioid agonists would imply that opioid agonism does not play a role in N_2O cue.

Nicotinic involvement in the stimulus effects of nitrous oxide.

Human homomeric α_7 neuronal nicotinic acetylcholine (nACh) receptors in *Xenopus oocytes* are modestly inhibited by N_2O (Suzuki et al., 2003). Similar results have also been found using heteromeric nACh receptors preparations (Yamakura & Harris, 2000). In the present study, nicotine (Figure 27) produced no greater than 1% N_2O-lever responding up to doses which significantly suppressed operant response rates. This was not surprising given the *in vitro* data would suggest that N_2O functions to negatively modulate nicotinic receptors. In a subsequent curve-shift experiment, a 1 mg/kg dose of nicotine also failed to produce any attenuation of the N_2O concentration-effect curve (Figure 28). The inability of nicotine to substitute for or antagonize the stimulus effects of N_2O is consistent with data showing that it does not alter the discriminative stimulus effects of TCE (Shelton, 2010). Taken together the data support the conclusion that an interactions with nACh receptors does not mediate the discriminative stimulus effects of N_2O.

Role of nitric oxide in the stimulus effects of nitrous oxide.

Several reports have linked the analgesia effects of N_2O with nitric oxide production. In particular, neuronal nitric oxide synthase (nNOS) inhibitors can attenuate N_2O analgesia [review see (Emmanouil & Quock, 2007)]. Figure 29 shows that the nNOS inhibitor L-NAME does not substitute for N_2O nor does it alter operant response rates. This result was unsurprising and I

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was primarily interested in determining if L-NAME would antagonize the discriminative stimulus of 60% N_2O (Figure 30). N_2O+vehicle produced concentration-dependent full substitution with an EC_{50} of 31% (CL 25% − 37%). N_2O+30 mg/kg L-NAME (closed squares) produced concentration-dependent full substitution with an almost identical EC_{50} of 35% (CL 32% - 39%). Interestingly, no dose of N_2O alone attenuated operant responding by more than 25% of the O_2 control response rate. However, pretreatment with 30 mg/kg L-NAME prior to N_2O exposure produced a significant $[F_{(6, 42)}=7.34, P<0.05]$ and concentration-dependent attenuation of operant responding sufficient to generate an EC_{50} of 73% (CL 61% - 86%). Therefore I would conclude that while N_2O analgesia in rodents is antagonized by nNOS inhibition, nitric oxide is not involved in the discriminative stimulus effects of N_2O. However, based on the response rate data there may be some other interaction between nitric oxide and nitrous oxide that is synergistic rather than antagonistic in nature.

Summary

The present series of experiments showed that under the proper training conditions N_2O can serve as a discriminative stimulus in mice. Like other drugs the stimulus effects of N_2O are exposure concentration-dependent and orderly. The extensive series of cross substitution experiments demonstrated that the stimulus effects of N_2O are probably not mediated by $GABA_A$, opioid, 5-HT or nicotinic acetylcholine receptors. The only receptor system of those tested which appears to be involved in transducing the discriminative stimulus effects of N_2O is the NMDA receptor. The data in this regard suggest that N_2O may function as a NMDA receptor channel blocker.

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The less than complete substitution engendered by the uncompetitive NMDA antagonists suggest that N_2O may have a compound cue composed of multiple components. Of the two types of compound cues defined by the cross-substitution of individual cue components N_2O appears to mirror a conditional compound cue rather than a redundant compound cue (Stolerman, Rauch, and Norris, 1987). In a conditional compound cue a singular component would not be expected to fully substitute for the training condition. Only a singular drug or drug mixture which presents both components jointly would produce full substitution for the training condition. If indeed N_2O is a conditional compound cue it differs in this regard from the stimulus of ethanol which has consistently been shown to produce a redundant compound cue when trained in a drug versus vehicle discrimination (Helms, Rogers, & Grant, 2009). Another possibility is that the cue complex of N_2O like ethanol is redundant but that the failure of NMDA antagonists to elicit full substitution is due to stimulus overshadowing by another, as yet unidentified, major mediator of its stimulus effects. This is certainly plausible given the fact that no drug tested fully substituted for N_2O.

It has been speculated that N_2O represents a unique subclass of abused inhalants. The present data at least partially refute this conclusion. Of all the compounds tested toluene produced the most robust N_2O-like effects, eliciting full substitution at one or more test concentrations in seven of eight subjects tested. The overlap between N_2O and toluene is very interesting but difficult to reconcile with current published data especially the data showing that of the mechanisms tested which included NMDA antagonism, only $GABA_A$ positive modulators produced toluene-like discriminative stimulus effects (Shelton & Nicholson, 2013). However toluene like N_2O has a number of molecular targets which have yet to be explored and it is certainly possible that one of these mechanisms will provide the necessary common link between

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these two abused inhalants. For instance future studies might include substitution tests of $5HT_3$ antagonists, glycine agonists or TREK-1 potassium channel activators. $5HT_3$ antagonists reduce anxiety-like behaviors (Costall, Kelly, Naylor, Onaivi, & Tyers, 1989) and N_2O inhibits $5HT_3$ receptor current (Takahiro Suzuki et al., 2002, Yamakura & Harris, 2000). To my knowledge $5HT_3$ antagonists have not even been investigated for their effects on N_2O mediated anxiolysis however it is possible there is some overlap with the discriminative stimulus effects of N_2O. Second, N_2O potentiates glycine receptors (Yamakura & Harris, 2000). Since ethanol has also been shown to potentiate glycine receptors [for review see (Perkins, Trudell, Crawford, Alkana, & Davies, 2010)] perhaps β-alanine, L-alanine or taurine may have positive cross-substitution results in N_2O trained subjects. Finally, TREK-1 or $K_{2P}2.1$ channels are leak channels activated by N_2O (Gruss et al., 2004). BL 1249 is a specific ligand with biological activity tested *in vivo* (Tertyshnikova et al., 2005) but has not been tested in drug discrimination. Although N_2O has interactions with these receptors it is not known if TREK-1 channel activation has discriminable effects. In sum the information derived from these studies may lead to the discovery of the secondary mechanism/mechanisms responsible for this discriminative stimulus effects of N_2O.

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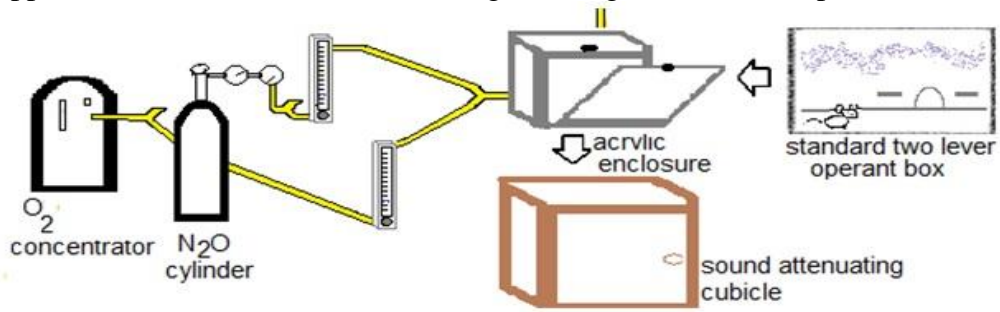*Pharmacology and Experimental Therapeutics*, *280*(1), 46–52.

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Appendix 1. Image of the apparatus. Each 26.0-L acrylic exposure chamber encased one modified two-lever mouse operant conditioning chamber and one 80mm 24-Volt DC fan.

Appendix 2: Schematic of the flow of gas through the 26.0-L exposure chamber.

standard two lever operant box

acrvlic enclosure

O_2 concentrator

N_2O cylinder

sound attenuating cubicle

Appendix 3. Application of rise of chamber concentration (top) and t_{99} (bottom) calculations for optimization of the training concentration in the dual purpose exposure chamber.

$C = C_O [1 - \exp (t (-Q/V)]$

      C_O = steady state concentration = 0.60

      V = volume of chamber = 25.96 L

      Q = flow rate = 5.81 L/min

      t = time = x

               $C = C_O [1 - e^{(t)(-Q/V)}]$

               $C = 0.6 [1 - e^{(time)(-5.81\ LPM\ /\ 25.96L)}]$

               If x = 10 $C = 0.6 [1 - e^{(10)(-0.22/min)}] = 0.60$

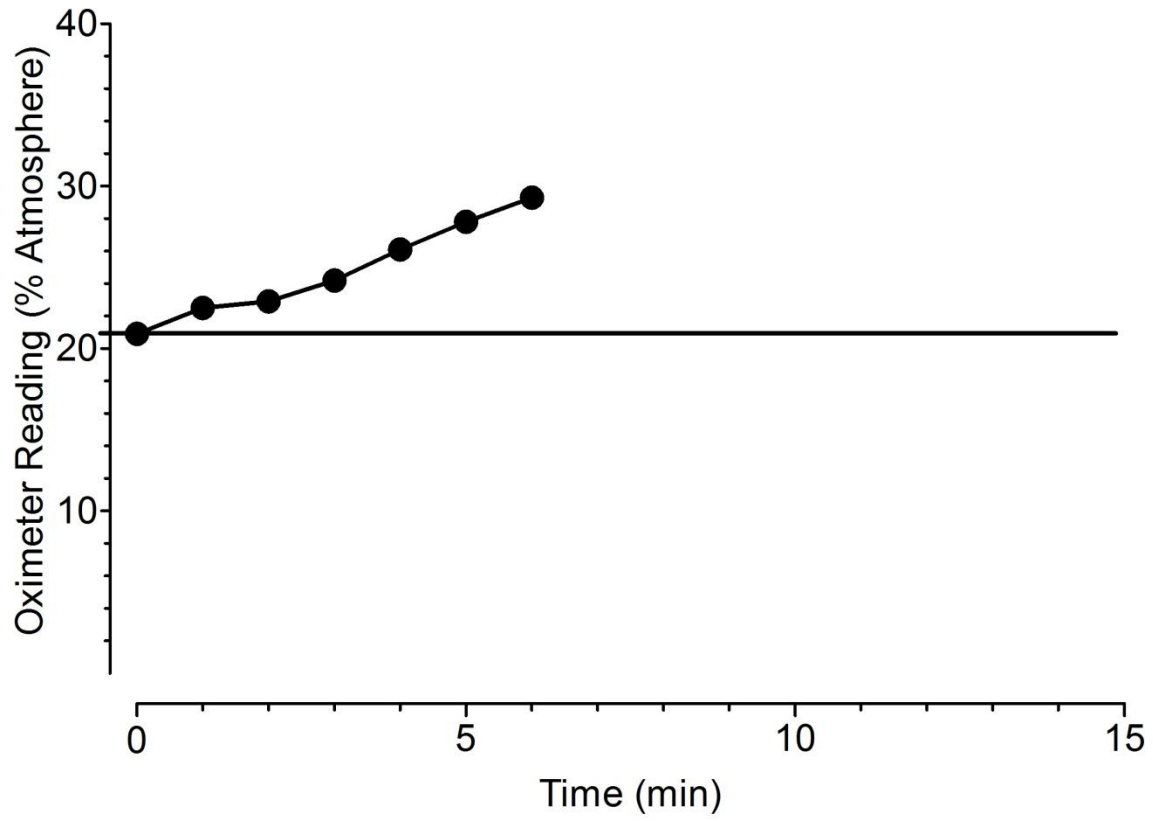   If x = 15 $C = 0.6 [1 - e^{(15)(-0.22/min)}] = 0.65$

$t_{99} = 4.6\ V/Q$

     V = volume of chamber = 25.96 L
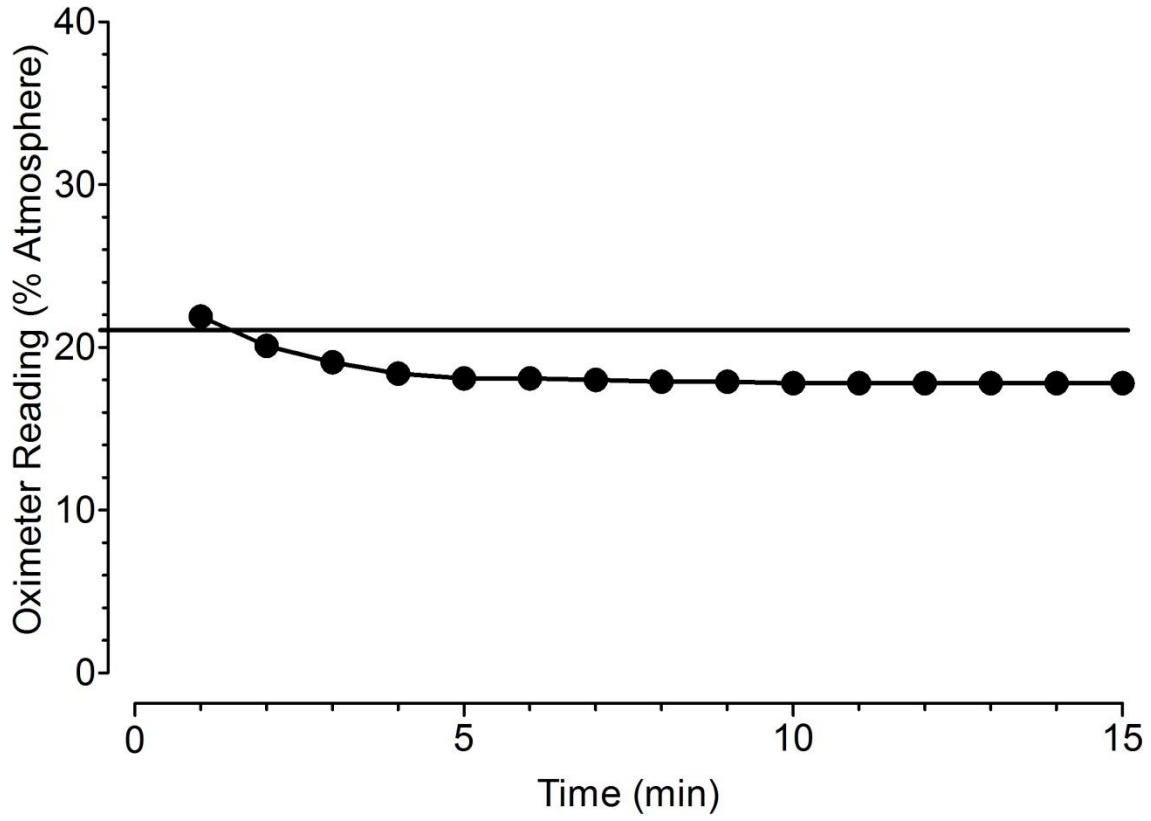
     Q = flow rate = 5.81 L/min

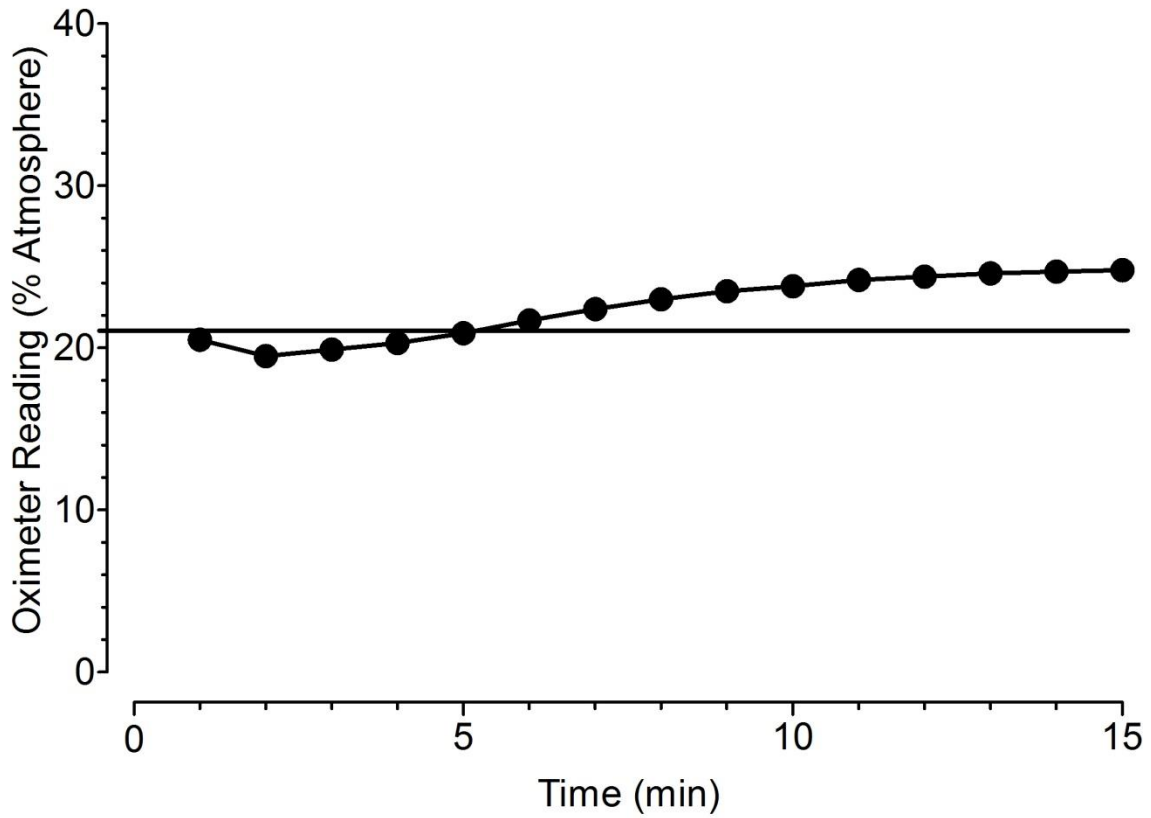 $t_{99} = 4.6\ V/Q = 4.6\ (25.96\ L\ /\ 5.81\ LPM) = 20.56\ min$

Appendix 4. Oximeter readings during a mock exposure to 60% N_2O

Appendix 5. Oximeter readings during a mock exposure to 80% N_2O in the 26.0-L chamber before optimized calculation.

Appendix 6. Oximeter readings during an optimized mock exposure to 80% N_2O in the 26.0-L chamber after optimized calculations.

Vita

Kellianne Jean Richardson née Muse was born on May 21, 1984, in Washington, DC, and is an American citizen. She graduated from West Potomac High School in Alexandria, Virginia in 2002. Kellianne earned her Bachelor of Arts from Oberlin College in Oberlin, Ohio in 2006. At Oberlin College she was an undergraduate researcher in the laboratory of Dr. William Fuchsman in the Department of Chemistry. She worked at Rotech Healthcare Inc. 2007 to 2009. In 2009 she joined the Department of Pharmacology & Toxicology at Virginia Commonwealth University in Richmond, Virginia. Spring 2010 she joined the laboratory of Dr. Keith L. Shelton. Kellianne won travel awards from both the American Society for Pharmacology and Experimental Therapeutics (ASPET) and Federation of American Societies for Experimental Biology-Minority Access to Research Careers (FASEB MARC) for expenses related to presenting her work at Experimental Biology April 2013. Her work establishing the abused inhalant nitrous oxide as a discriminative stimulus was published in the February 2014 issue of *Behavioural Pharmacology.*