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GABA_A-positive modulator and NMDA antagonist-like discriminative stimulus effects of isoflurane vapor in mice

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Abstract

Rationale—Several neurotransmitter systems have been hypothesized to be involved in the *in vivo* effects of volatile anesthetic. Drug discrimination may represent a novel procedure to explore the neurochemical systems underlying the sub-anesthetic behavioral effects of these compounds.

Objectives—The purpose of the present study was to examine the contribution of GABA_A and NMDA receptors to the discriminative stimulus effects of a behaviorally-active sub-anesthetic concentration of isoflurane vapor.

Methods—Sixteen B6SJLF1/J mice were trained to discriminate 10 min of exposure to 6000 ppm isoflurane vapor from air. Substitution tests were conducted with volatile anesthetics, abused vapors, GABA_A positive modulators, NMDA antagonists and nitrous oxide.

Results—The volatile anesthetics, enflurane and halothane as well as the abused vapors toluene and 1,1,1-trichloroethane fully substituted for isoflurane. The GABA_A positive modulators, pentobarbital, midazolam and zaleplon but not the direct GABA_A agonist, muscimol, produced high levels of partial substitution for isoflurane. The anticonvulsant, valproic acid fully substituted for isoflurane but a second, tiagabine, did not substitute. The competitive NMDA antagonist, CGS-19755, fully and the non-competitive NMDA antagonist, dizocilpine, partially substituted for isoflurane. The glycine site NMDA antagonist, L-701,324 did not substitute for isoflurane. GHB and nitrous oxide gas also failed to substitute for isoflurane.

Conclusions—The discriminative stimulus effects of subanesthetic concentrations of isoflurane vapor are shared by other vapor anesthetics and abused inhalants. The discriminative stimulus effects of isoflurane vapor appear to be mediated by both positive allosteric modulation of GABA_A receptors as well as antagonism of NMDA receptors.

Keywords

drug discrimination; isoflurane; volatile anesthetics; inhalants; GABA; NMDA

Introduction

Halogenated volatile anesthetics including isoflurane, desflurane, sevoflurane, enflurane and methoxyflurane are an important and widely used class of anesthetic agents. Clinically these compounds differ primarily in their pharmacokinetics but there is limited evidence that they may also differ to some degree in their neurochemical sites of action (Westphalen and Hemmings 2006). For many decades the mechanism of action of volatile, indeed all anesthetics, was believed to reside in their ability to nonspecifically perturb the lipid bilayer

of cellular membranes. The Meyer-Overton correlation, as it came to be known, held that the potency of an anesthetic for producing immobility was inversely related to that agent's lipid solubility expressed as an oil:gas partition coefficient [for review see (Kopp Lugli et al. 2009)]. This hypothesis was reevaluated when it was noted that some volatile anesthetics exhibited stereoselective effects which could not be accounted for by the Meyer-Overton correlation (Harris et al. 1992; 1994). More recent *in vitro* studies have demonstrated that volatile anesthetic vapors interact fairly specifically with a host of ligand-gated ion channel receptors [for review see (Grasshoff et al. 2006)]. Considerable research has focused on attempting to determine which receptor-mediated effects of volatile anesthetics are responsible for their *in vivo* actions.

Volatile anesthetics share with benzodiazepines and barbiturates the ability to produce hypnosis and like benzodiazepines can induce anterograde amnesia (Dutton et al. 2002). Therefore, it is not surprising that positive modulatory effects of volatile anesthetics on GABA_A receptors have been suggested as underlying their hypnotic and amnestic properties (Rau et al. 2009). A number of studies have shown interactions between volatile anesthetics and benzodiazepines. For instance, co-administration of benzodiazepines lowered the minimal alveolar concentration (MAC) necessary to induce anesthesia in dogs (Hellyer et al. 2001). The benzodiazepine antagonist, flumazenil, significantly improved cortical and neuromotor functions following isoflurane anesthesia in humans (Weinbroum and Geller 2001) as well as prevented the development of isoflurane tolerance (Flaishon et al. 2003). In humans, flumazenil binding was concentration-dependently enhanced by isoflurane inhalation (Gyulai et al. 2001). However, isoflurane did not alter flumazenil binding in rat cerebral cortex (Hansen et al. 1991) nor did flumazenil antagonize the anesthetic effects of isoflurane in dogs (Schwieger et al. 1989). A combination of naloxone and flumazenil also failed to antagonize isoflurane-induced suppression of cat dorsal horn neurons (Okuda et al. 1993). While it is unclear if volatile anesthetics directly interact with the benzodiazepine binding site, there is little doubt they positively modulate GABA_A receptor activity. For instance, isoflurane enhanced the binding of the direct GABA_A agonist muscimol in mouse cerebral cortex (Harris et al. 1994) and mutations of GABA_A receptors expressed in oocytes diminished their sensitivity to the positive modulatory effects of isoflurane (Greenblatt and Meng 2001). GABA_A $\alpha 4$ subunit knockout mice are resistant to the amnestic effects of isoflurane (Rau et al. 2009) and thalamic neurons from these mice exhibited reduced sensitivity to the positive modulatory effects of isoflurane (Jia et al. 2008).

Halogenated volatile anesthetics also alter NMDA-receptor function. For instance, isoflurane inhibits NMDA receptor activity in rat hippocampal neuron cultures (de Sousa et al. 2000) as well as hippocampal (Wakasugi et al. 1999), amygdala (Ranft et al. 2004) and striatal slices (Keita et al. 1999). Isoflurane, sevoflurane and desflurane all attenuated the function of mutant NMDA receptors expressed in oocytes (Ogata et al. 2006). Intrathecal administration of the competitive NMDA antagonist APV in combination with the NK-1 receptor antagonist CP96345 reduced the MAC value of isoflurane (Ishizaki et al. 1999). When administered alone, competitive, non-competitive and NMDA-receptor glycine site antagonists each decreased the MAC value of isoflurane in rats (Ishizaki et al. 1995) and the non-competitive NMDA antagonist, dizocilpine, decreased the MAC value of isoflurane in rabbits (Scheller et al. 1989). Recently it has been shown that isoflurane competes for binding with the NMDA co-agonist glycine in recombinant receptors (Dickinson et al. 2007).

These studies, as well as others, suggest that both GABA_A and NMDA receptor modulation mediates some of the effects of halogenated vapor anesthetics. However, most *in vivo* studies have focused on the high dose, clinical endpoint of anesthesia, often expressed in animal studies by loss of righting reflex. Far fewer experiments have explored the degree to

which GABA_A and NMDA receptors contribute to the sub-anesthetic behavioral effects of volatile anesthetics. One procedure that has been used extensively to examine the receptor mechanisms underlying the behavioral effects of many classes of drugs is the drug discrimination paradigm. Several experiments have been conducted in which halogenated ether anesthetics have been tested in animals trained to discriminate other injected drugs. However, no studies have previously been published in which a volatile anesthetic has been used as a discrimination training drug. The goal of the present study was first to establish the anesthetic vapor, isoflurane, as a discriminative stimulus in mice. Subsequently we sought to examine if isoflurane shares common discriminative stimulus effects with a number of site-selective drugs acting at the GABA_A and NMDA receptor complexes. Lastly, we compared the discriminative stimulus effects of isoflurane to other representative volatile anesthetics and abused vapors to gain insights on their pharmacological mechanisms of action.

Materials and methods

Subjects

Sixteen adult male B6SJLF1/J mice (Jackson Laboratory, Bar Harbor, Maine) served as subjects. We have used this strain exclusively for prior inhalant discrimination studies (Shelton 2007; 2009; 2010; Shelton and Slavova-Hernandez 2009). As a F1 cross of C57 black and SJL mice they have the advantage of an inbred strain in being genetically identical but also possess hybrid vigor making them extremely hardy subjects for long-term behavioral studies. The mice were individually housed on a 12 hr light/dark cycle (lights on 7 AM). Feeding was adjusted to maintain a healthy, stable weight of between 27–35g for the duration of the study. Studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and conducted in accordance with the Institute of Laboratory Animal Research “Guide for the Care and Use of Laboratory Animals”.

Compounds

Isoflurane and halothane were purchased from Webster Veterinary Supply (Charlotte, NC). Enflurane was obtained from Abbott Laboratories (North Chicago, IL). 99.5% anhydrous 1,1,1-trichloroethane (TCE), HPLC grade toluene, valproic acid, pentobarbital, dizocilpine maleate (MK-801), muscimol hydrobromide, and tiagabine HCl were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI). Zaleplon and CGS-19755 were purchased from Tocris Bioscience (St. Louis, MO). Midazolam maleate was a gift of Roche Pharmaceuticals (Nutley, NJ). Gamma-hydroxybutyric acid (GHB) and L-701,324 were provided by the National Institute on Drug Abuse. Nitrous oxide gas was obtained from National Welders Supply (Richmond, VA). All injected drug doses were dissolved in 0.9% physiological saline with the exception of zaleplon and L-701,324. Zaleplon was prepared in a 45% β -cyclodextrin/sterile water suspension. L-701,324 was prepared in 10% cremophor/sterile water. Exposures to isoflurane, halothane, TCE and enflurane vapors were 10 min in duration. Exposure to nitrous oxide mixed with oxygen was 20 min in duration. All injected drugs were administered at a volume of 10 ml/kg and injected intraperitoneally (i.p.). A 10-min pretreatment time was used for dizocilpine, midazolam and pentobarbital. Zaleplon, valproic acid, tiagabine, CGS19755, L-701,324, and muscimol were administered with a 30-min pretreatment time.

Apparatus

Drug discrimination sessions were conducted in standard 2-lever mouse operant conditioning chambers equipped with 0.01 ml liquid dippers (Med-Associates model ENV-307AW, St. Albans, VT). The milk solution reinforcer consisted of 25% sugar, 25% nonfat powdered milk and 50% tap water (by volume). The static vapor exposure procedures

have been previously described (Shelton 2007). Exposure to nitrous oxide gas was accomplished using an open circuit system composed of a medical grade compressed nitrous oxide gas cylinder and regulator, rotometers and Tygon tubing. Nitrous oxide was mixed with oxygen generated by an Airsep Onyx+ oxygen concentrator (Buffalo, NY) and passed through tubing into an exposure chamber constructed from a 9.9 liter Secador Mini vacuum desiccator cabinet (Bel-Art Products, Pequannock, NJ).

Discrimination training and substitution testing

Training sessions were conducted Mon-Fri. Both lever lights and the houselight were illuminated for the duration of the session. Completion of the FR requirement on the active lever resulted in 3 sec of dipper access. Responding while the dipper was elevated had no consequences. Over days the session length which began at 30 min was decreased to 5 min after which double alternation isoflurane discrimination training sessions began. During training the correct lever was determined by whether the subject received exposure to 6000 ppm isoflurane vapor or air. Following exposure the mice were rapidly placed into the operant chamber and the training session immediately initiated. The FR1 response requirement was increased over sessions to FR12. Responding on the inactive lever reset the FR requirement on the correct lever. Mice were determined to have acquired the isoflurane versus air discrimination when they met the stimulus control criteria of correct first FR and completion of greater than 80% of all responses within a session on the correct lever for 8 out of 10 consecutive training sessions.

Substitution tests were conducted on Tues and Fri with continued training sessions on Mon, Wed and Thurs. Testing was suspended if an animal did not maintain the stimulus control criteria during all training sessions since the last test session. Test sessions were not recommenced until three consecutive training sessions met stimulus control criteria. Substitution tests with vapors and nitrous oxide were preceded by exposure to a single concentration of the test compound. In substitution test sessions with an injected drug, the injection was given at the pretreatment time indicated previously and the animal was exposed to air in the exposure chamber for 10 min prior to the start of the test session. Drug discrimination test sessions were 5 min and completion of the FR requirement on either lever resulted in dipper presentation. Doses or concentrations of each compound were generally tested in ascending order until maximal substitution was produced or response rates were suppressed by greater than 50% compared to the air control session. Prior to each vapor concentration-effect curve, air and 6000 ppm isoflurane control test sessions were conducted. Control sessions prior to injected drug dose-effect curves were conducted in a similar manner with the addition of injection of the test drug's vehicle before isoflurane or air exposure.

Drug discrimination data analysis

Percentage isoflurane-lever responding and response rates (resp/sec) were recorded in 1-min bins. Prior studies from our laboratory indicated that the response-rate altering effects produced by acute vapor exposures are often extremely transient (Shelton 2007; Shelton and Slavova-Hernandez 2009). To insure that the discrimination and operant rate suppression data represented a time period during which the maximal behavioral effects of the tested vapors were exhibited, only the first min of each discrimination test session was examined. To maintain consistency only the first-min data was examined for injected compounds as well. Group means ($\pm$ SEM) were calculated for percentage first min isoflurane-lever selection as well as first min response rate. Any inhalant concentration or injected-drug dose that suppressed response rates to the extent that the animal did not complete at least one FR during the first min of the test session resulted in the exclusion of that mouse's datum from the group lever-selection analysis, although that datum was included in the response rate

analysis. Test concentrations/doses in which fewer than three mice emitted a FR in the first min of the test session were not plotted on the substitution curve. A criterion of 80% or greater mean isoflurane vapor-appropriate responding was defined as full substitution, between 20% and 79% as partial substitution and less than 20% as no substitution. When possible EC_{50} or ED_{50} values (and 95% confidence limits) for isoflurane vapor-lever selection and response rate suppression were calculated based on the linear portion of each mean dose-effect curve (Tallarida and Murray 1986). EC_{50} or ED_{50} values between curves were considered to be significantly different if their respective confidence limits did not overlap.

Results

All sixteen mice met the discrimination acquisition criteria in a mean of 51 (± 2) training sessions. Isoflurane concentration-dependently substituted for the 6000 ppm training concentration (figure 1, upper panel) with an EC_{50} value of 1750 ppm (CL: 1412–2168 ppm). Isoflurane vapor exposure also produced a concentration-dependent suppression of operant responding (lower panel) with an EC_{50} value of 9966 ppm (CL: 9224–10767 ppm). The control tests following 10 min of exposure to air and 6000 ppm isoflurane produced a mean of 8% and 97% isoflurane-lever responding, respectively.

The halogenated ether anesthetic, enflurane (figure 2, upper panel open circles), produced concentration-dependent full substitution for isoflurane with an EC_{50} value of 2118 ppm (CL: 1472–3047 ppm). Up to a concentration of 12000 ppm, enflurane failed to have any effect on response rates (lower panel, open circles). The halogenated alkane vapor anesthetic, halothane (upper panel, open squares), also produced concentration-dependent full substitution for isoflurane with an EC_{50} of 2234 ppm (CL: 1512–3301 ppm). Halothane produced a modest concentration-dependent suppression of response rates with the 10000 ppm concentration resulting in responding 75% of that during the air control session (lower panel, open squares).

The aromatic hydrocarbon, toluene (figure 3, upper panel, open circles), produced dose-dependent full substitution for isoflurane vapor with an EC_{50} of 2015 ppm (CL: 1672–2428 ppm). The chlorinated hydrocarbon, 1,1,1-trichloroethane (TCE), also produced concentration-dependent full substitution for isoflurane (upper panels, open squares) with an EC_{50} of 5890 ppm (CL: 4625–7503 ppm). Across the concentration ranges tested, neither toluene vapor nor TCE vapor produced response-rate suppressing effects (lower panel).

Pentobarbital (figure 4, upper panel open circles) produced dose-dependent partial substitution for isoflurane with an ED_{50} of 4.3 mg/kg (CL: 2.5–7.4 mg/kg). A maximum of 70% isoflurane-lever responding occurred at a dose of 30 mg/kg which also reduced mean response rates to 29% of rates following vehicle injection (lower panel, open circles). The ED_{50} for suppression of response rates by pentobarbital was 21.9 mg/kg (CL: 16.5–21.2 mg/kg). Midazolam (upper panel, open squares), dose-dependently substituted for isoflurane vapor up to a maximum of 71% isoflurane-lever selection at the 10 mg/kg dose. The ED_{50} for midazolam substitution for isoflurane was 2.6 mg/kg (CL: 0.7–10.4 mg/kg). An insufficient suppression of responding prevented calculation of a midazolam rate suppression ED_{50} value (lower panel, open squares). Zaleplon produced partial substitution for isoflurane (upper panel, filled triangles) with an ED_{50} of 1.6 mg/kg (CL: 1.3–2.0 mg/kg). A maximum of 74% isoflurane-lever selection occurred at the 3 mg/kg zaleplon dose which also resulted in complete suppression of operant responding in 2 of 8 mice. The ED_{50} for response-rate suppression by zaleplon was 5.2 mg/kg (CL: 2.7–10.5 mg/kg).

The direct GABA_A receptor agonist, muscimol, failed to substitute for isoflurane (figure 5, upper panel open circles). Muscimol, however, did dose-dependently suppress response rates (lower panel, open circles) with an ED₅₀ of 2.1 mg/kg (CL: 0.9–4.8 mg/kg). Likewise the GABA reuptake inhibitor, tiagabine, failed to substitute for isoflurane (upper panel, open triangles) but did suppress response rates (lower panel, open triangles) with an ED₅₀ of 5.7 mg/kg (CL: 4.1–7.9 mg/kg). The anticonvulsant/mood stabilizer, valproic acid (upper panel, open squares) dose-dependently and fully substituted for isoflurane vapor with an ED₅₀ of 337 mg/kg (CL: 259–439 mg/kg). The 560 mg/kg dose of valproic acid produced 85% isoflurane-lever responding but also suppressed response rates to 55% of vehicle control values (lower panel, open squares). An ED₅₀ for response-rate suppression by valproic acid could not be calculated, as the 560 mg/kg dose resulted in delayed signs of toxicity, precluding the testing of higher doses.

Figure 6 shows the isoflurane substitution (upper panel) and response-rate results (lower panel) for the competitive NMDA antagonist, CGS-19755, the noncompetitive NMDA antagonist, dizocilpine, and the NMDA glycine-site antagonist, L-701,324. Dizocilpine (open circles) produced dose-dependent partial substitution for isoflurane vapor and suppressed response rates with an ED₅₀ of 0.26 mg/kg (CL: 0.22–0.30 mg/kg). L-701,324 (open triangles) produced a maximum of 24% isoflurane-lever substitution at a dose of 17 mg/kg. Up to a dose of 56 mg/kg L-701,324 response rates were unaffected. CGS-19755 (open squares) produced dose-dependent full substitution for isoflurane vapor. A maximum of 98% isoflurane-lever responding was engendered by the 17 mg/kg dose which also resulted in pronounced rate suppressing effects. The ED₅₀'s for substitution and response rate suppression for CGS-19755 were 6.6 mg/kg (CL: 4.3–10.2 mg/kg) and 7.7 mg/kg (CL: 5.9–10.1 mg/kg), respectively.

Nitrous oxide gas produced a maximum of 31% isoflurane-lever selection at 50% nitrous oxide (Table 1). At 80% nitrous oxide, the highest that could be safely tested, only 11% isoflurane-lever responding was elicited and response rates were suppressed to 67% of the oxygen control rate. GHB produced a maximum of 30% isoflurane-lever selection at the 170 mg/kg dose (table 1). GHB dose-dependently suppressed response rates with an ED₅₀ of 157 mg/kg (CL: 102–241 mg/kg).

Discussion

The ability to train a discrimination based on isoflurane vapor is consistent with other results from our laboratory showing that the abused volatile vapors toluene and TCE can also be trained as discriminative stimuli (Shelton 2007; 2009; 2010; Shelton and Slavova-Hernandez 2009). One concern with a drug discrimination based on an inhaled volatile compound like isoflurane is the possibility that the discrimination is based upon exteroceptive effects like odor, rather than interoceptive CNS effects. The possibility that the odor of isoflurane was controlling discriminative stimulus performance is unlikely for several reasons. Firstly, in addition to the volatile vapors examined, several injected drugs with no olfactory effects substituted for isoflurane in the present study. Secondly, in animals trained to discriminate injected drugs in which odor is not a concern, isoflurane produces cross-substitution (Bowen and Balster, 1997; Bowen et al 1999). Lastly, we have demonstrated that the discriminative stimulus effects of toluene and TCE which also have pronounced odors are likely based on their CNS effects (Shelton 2007; 2009; 2010; Shelton and Slavova-Hernandez 2009). When combined, these data strongly support the hypothesis that the discriminative stimulus effects of isoflurane vapor are CNS mediated.

The related halogenated ether anesthetic enflurane fully substituted for isoflurane as did the halogenated alkane, halothane. The substitution ED₅₀'s for isoflurane, enflurane and

halothane were not significantly different from one another consistent with the relatively similar MAC values of all three anesthetic vapors (Nickalls and Mapleson 2003). These data would suggest that the reported differences in basal GABA release in synaptosomes stimulated by isoflurane, enflurane and halothane (Westphalen and Hemmings 2006) do not have a differential impact on their discriminative stimulus effects. However, isoflurane and enflurane do show different levels of substitution in mice trained to discriminate ethanol from vehicle (Bowen and Balster 1997) and in mice trained to discriminate TCE from air (Shelton 2009). The modest discrepancies in substitution results in these three discrimination studies may reflect underlying differences in neurochemical effects of volatile anesthetics or the result of variability in discrimination results across studies.

One of the most valuable practical uses of drug discrimination is in the assessment of abuse liability. Drugs with similar discriminative stimulus effects often have similar potentials for abuse due to common neurochemical mechanisms of action (Balster 1991). The abused inhalants toluene and TCE both fully substituted for isoflurane in the present study. These data suggest that toluene, TCE and isoflurane may have similar abuse-related pharmacological effects. Indeed, isolated cases of isoflurane abuse-related deaths have been reported (Pavlic et al 2002). Interestingly, isoflurane produces only partial substitution in mice trained to discriminate TCE (Shelton 2009) and does not substitute in mice trained to discriminate toluene (Shelton and Slavova-Hernandez 2009). The present asymmetrical substitution results are consistent with the hypothesis that the discriminative stimulus of isoflurane may be composed of multiple components. This hypothesis is strengthened by the positive substitution data with both GABA_A positive modulators and NMDA antagonists. In contrast, the discriminative stimulus of toluene and TCE may be more narrowly circumscribed or alternatively may be composed of multiple components that only partially overlap with isoflurane.

The positive GABA_A modulators pentobarbital, midazolam and zaleplon all showed similar high levels of partial substitution and valproic acid fully substituted for isoflurane. The overlap in discriminative stimulus effects of isoflurane and benzodiazepines mirrors previous data showing that the halogenated ether anesthetic, methoxyflurane, produces full substitution in mice trained to discriminate diazepam from vehicle (Bowen et al. 1999). However the GABA reuptake inhibitor, tiagabine, failed to substitute for isoflurane, up to doses that almost completely suppressed response rates in all of the animals tested. Likewise the direct GABA_A agonist, muscimol failed to substitute for isoflurane. These data show that isoflurane shares discriminative stimulus effects with some, but not all drugs that enhance GABA_A receptor function. Interestingly, the maximal substitution generated by zaleplon, which is selective for $\alpha 1$ subunit containing GABA_A receptors, was comparable to that of the nonselective benzodiazepine, midazolam. The importance of $\alpha 1$ containing GABA_A receptors in the discriminative stimulus effects of isoflurane are consistent with data showing a significant attenuation in isoflurane sensitivity in mice with a targeted mutation of the GABA_A $\alpha 1$ subunit (Sonner et al. 2007).

The present results with valproic acid and tiagabine are also intriguing. Valproic acid fully substituted for isoflurane vapor, albeit at doses which had pronounced effects on response rates. The pharmacological effects of valproic acid are numerous but one of the most prominent is inhibition of GABA transaminase (Terbach and Williams 2009). The resulting enhancement of GABA levels appears to be responsible for the discriminative stimulus effects of valproic acid. In pigeons trained to discriminate valproic acid from vehicle, clonazepam produced full substitution, whereas diazepam only partially substituted and pentobarbital failed to substitute (Picker et al. 1985). Valproic acid produces full substitution in rats trained to discriminate pentobarbital from saline (Grech and Balster 1994). In monkeys discriminating benzodiazepines from saline, valproic acid fails to substitute when

given alone (Ator and Griffiths 1997), however, when administered in combination with midazolam it produce a leftward shift in the midazolam dose-effect curve (McMahon and France 2003). In contrast, tiagabine which enhances endogenous GABA levels by inhibiting the GAT-1 reuptake transporter (Czapinski et al. 2005) completely failed to substitute for isoflurane. The reason for this difference is unclear but it does appear to be the case that tiagabine has discriminative stimulus effects that overlap poorly with prototypic GABA_A positive modulators. For instance, tiagabine only produces partial substitution in pentobarbital-trained rats (Grech and Balster 1994) and fails to substitute in diazepam-trained rats (Nielsen et al. 1991). Additional studies examining drugs which produce GABA_A positive modulatory effects by other mechanisms will be necessary to determine if tiagabine is unique in its ability to enhance GABA levels without demonstrating appreciable isoflurane-like discriminative stimulus effects.

We examined a competitive, noncompetitive and glycine-site NMDA antagonist in the present study. Of the three, only the competitive NMDA antagonist CGS-19755 fully substituted for isoflurane. In contrast the noncompetitive NMDA antagonist dizocipine produced 44% isoflurane-lever selection. Cross substitution studies of isoflurane in animals discriminating competitive NMDA antagonists have not been conducted. However, the poor substitution of dizocipine for isoflurane is generally consistent with experiments in which methoxyflurane failed to substitute in mice trained to discriminate dizocipine (Shelton and Balster 2004) and only partial substituted in mice trained to discriminate phencyclidine (Bowen et al. 1999).

A direct competitive interaction of isoflurane at the glycine-site on the NMDA receptor has been hypothesized (Dickinson et al. 2007) but the lack of substitution of the glycine-site antagonist L-701,324 for isoflurane does not support the conclusion that the glycine-site is involved in the discriminative stimulus effects of isoflurane. Our L-701,324 data must be interpreted with some caution as the highest dose of 56 mg/kg dose had little effect on operant responding. CGS 19755 and dizocipine did not elicit any isoflurane-lever responding until response rates were suppressed well below 50% of the air control values. Although responding was only marginally suppressed by 56 mg/kg L-701,324 a much lower dose of 10 mg/kg was sufficient to produce a greater than 50% reduction in responding in rats suggesting that our test doses were probably in a behaviorally-active range (Nicholson and Balster 2009). Similarly, in a variety of behavioral paradigms in mice doses of 10 mg/kg and lower were shown to have anticonvulsant and anxiolytic effects (Przegalinski et al. 1998; Wlaz 1998). Unfortunately higher doses of L-701,324 could not be examined due to its limited solubility. Regardless, if isoflurane was producing NMDA antagonist-like effects by specific actions at the glycine-site, it seems unlikely that it would require response-rate suppressing doses of a selective glycine-site antagonist to produce isoflurane-like discriminative stimulus effects.

The discriminative stimulus effects of GHB appear to be mediated by multiple receptor mechanisms, including positive modulation of both GABA_A and GABA_B receptors (Baker et al. 2008; Carter et al. 2009). Our negative substitution results with GHB suggest there is little overlap in its discriminative stimulus with that of isoflurane. Nitrous oxide has both GABA_A positive modulatory (Nagashima et al. 2005) as well as NMDA-antagonist effects (Grasshoff et al. 2006; Yamakura and Harris 2000) *in vitro*. However, nitrous oxide failed to produce more than low levels of partial substitution for isoflurane. This is consistent with the observation that the human subjective effects of isoflurane differ substantially from those of nitrous oxide. Humans report much more pronounced sedative-like effects from subanesthetic doses of isoflurane than nitrous oxide (Beckman et al. 2006; Zacny et al. 1994). The GABA_A $\alpha 1$ subunit is thought to be involved in the sedative effects of benzodiazepines and the $\alpha 1$ selective drug zaleplon produced isoflurane-like discriminative

stimulus effects. Recombinant GABA_A receptors containing $\alpha 1$ subunits are sensitive to nitrous oxide (Hapfelmeier et al. 2000) and nitrous oxide exposure in mice increases $\alpha 1$ receptor mRNA (Johanek et al. 2001). If nitrous oxide positively modulates GABA_A $\alpha 1$ *in vivo*, these positive modulatory effects do not appear to be sufficient to produce isoflurane-like discriminative stimulus effects. Additional studies in isoflurane-trained mice with drugs that act selectively on GABA_A receptors without $\alpha 1$ subunits as well as studies of $\alpha 1$ selective compounds in animals trained to discriminate nitrous oxide would be necessary to directly test this hypothesis.

In summary, the present data suggest that the discriminative stimulus effects of isoflurane vapor are the result of both GABA_A positive modulation as well as NMDA antagonism. This data somewhat, but not completely mirrors the findings regarding the neurochemical mechanisms underlying the discriminative stimulus effects of ethanol (Grant and Colombo 1993; Shelton and Balster 1994). Additional experiments will be necessary to refine our understanding of the specific interaction of isoflurane with these two receptors. Studies should also be undertaken to determine if, as is the case with ethanol, the relative contribution of GABA_A and NMDA receptor systems to the discriminative stimulus effects of isoflurane are influenced by training dose (Grant 1999). Lastly, while our data has implicated GABA_A and NMDA systems as playing significant roles in the discriminative stimulus of isoflurane they do not rule out the possibility that other neurotransmitter systems may also be involved.

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Fig. 1. Concentration-effect curve for inhaled isoflurane vapor in mice trained to discriminate 10 min of exposure to 6000 ppm inhaled isoflurane from air (n=15). The data presented are based on the first min of each 5-min test session. Points above Air and Iso represent the results of air (open circle) and 6000 ppm inhaled isoflurane (filled circle) exposure control sessions. Mean ($\pm$ SEM) percentages isoflurane-lever responding are shown in the upper panel. Mean ($\pm$ SEM) response rates in responses/sec are shown in the bottom panel. Numbers in parentheses indicate the number of animals out of the group that emitted sufficient responses to be included in the percentage isoflurane-lever selection curve.

Fig. 2.

Concentration-effect curves for enflurane (open circles) and halothane (open squares) in mice (n=8) trained to discriminate 6000 ppm inhaled isoflurane vapor from air. The data presented are based on the first min of each 5-min test session. Points above Air and Iso represent the results of air (open circle and square) and 6000 ppm inhaled isoflurane (filled circle and square) exposure control sessions. Mean ($\pm$ SEM) percentages isoflurane-lever responding are shown in the upper panel. Mean ($\pm$ SEM) response rates in responses/sec are shown in the bottom panel.

Fig. 3. Concentration-effect curves for toluene (open circles) and 1,1,1-trichloroethane (TCE) (open squares) in mice (n=8) trained to discriminate 6000 ppm inhaled isoflurane vapor from air. The data presented are the first min of each 5-min test session. Points above Air and Iso represent the results of air (open circle and square) and 6000 ppm inhaled isoflurane (filled circle and square) exposure control sessions. Mean ($\pm$ SEM) percentages isoflurane-lever responding are shown in the upper panel, Mean ($\pm$ SEM) response rates in responses/sec are shown in the bottom panel.

Fig. 4. Dose-effect curves for pentobarbital (open circles), midazolam (open squares) and zaleplon (filled triangles) in mice trained to discriminate 6000 ppm inhaled isoflurane vapor from air (n=8). The data presented are based on the first min of each 5-min test session. Points above Air and Iso represent the results of air (open circle, square and triangle) and 6000 ppm inhaled isoflurane (filled circle, square and triangle) exposure control sessions. Mean (± SEM) percentages isoflurane-lever responding are shown in the upper panel, Mean (± SEM) response rates in responses/sec are shown in the bottom panel. Numbers in parentheses indicate the number of animals out of the group that emitted sufficient responses to be included in the percentage isoflurane-lever selection curve.

Fig. 5.

Dose-effect curves for muscimol (open circles), valproic acid (open squares) and tiagabine (open triangles) in mice trained to discriminate 6000 ppm inhaled isoflurane vapor from air (n=6–8). The data presented are the first min of each 5-min test session. Points above Air and Iso represent the results of air (open circle, square and triangle) and 6000 ppm inhaled isoflurane (filled circle, square and triangle) exposure control sessions. Mean ($\pm$ SEM) percentages isoflurane-lever responding are shown in the upper panel, Mean ($\pm$ SEM) response rates in responses/sec are shown in the bottom panel. Numbers in parentheses indicate the number of animals out of the group that emitted sufficient responses to be included in the percentage isoflurane-lever selection curve.

Fig. 6. Dose-effect curves for dizocilpine (open circles), CGS 19755 (open squares) and L-701,324 (open triangles) in mice trained to discriminate 6000 ppm inhaled isoflurane vapor from air (n=8). The data presented are the first min of each 5-min test session. Points above Air and Iso represent the results of air (open circle, square and triangle) and 6000 ppm inhaled isoflurane (filled circle, square and triangle) exposure control sessions. Mean ($\pm$ SEM) percentages isoflurane-lever responding are shown in the upper panel, Mean ($\pm$ SEM) response rates in responses/sec are shown in the bottom panel. Numbers in parentheses indicate the number of animals out of the group that emitted sufficient responses to be included in the percentage isoflurane-lever selection curve.

Table 1

Isoflurane selection and operant response rates produced by nitrous oxide and gamma-hydroxybutyric acid (GHB)

Drug	Drug dose or concentration	% Isoflurane-lever responding ($\pm$ SEM)	Response rate in responses/sec ($\pm$ SEM)
Nitrous oxide	Oxygen control	1.7 (0.9)	1.5 (0.2)
	33%	1 (0.4)	1.4 (0.2)
	50%	31 (17.7)	1.3 (0.2)
	67%	24 (13.2)	0.7 (0.2)
	75%	11 (5.3)	1.1 (0.2)
	80%	11 (5.3)	1.0 (0.2)
GHB	Air control	8 (7.5)	1.4 (0.2)
	Isoflurane control	85 (14.2)	1.4 (0.1)
	10 mg/kg	1 (0.4)	1.4 (0.2)
	30 mg/kg	1 (0.2)	1.6 (0.3)
	100 mg/kg	17 (13.2)	0.9 (0.3)
	170 mg/kg	30 (16.7)	0.5 (0.2)