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Benzodiazepine-like discriminative stimulus effects of toluene vapor

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Abstract

In vitro studies show that the abused inhalant toluene affects a number of ligand-gated ion channels. The two most consistently implicated of these are γ -aminobutyric acid type A (GABA_A) receptors which are positively modulated by toluene and *N*-methyl-D-aspartate (NMDA) receptors which are negatively modulated by toluene. Behavioral studies also suggest an interaction of toluene with GABA_A and/or NMDA receptors but it is unclear if these receptors underlie the abuse-related intoxicating effects of toluene. Seventeen B6SJLF1/J mice were trained using a two-choice operant drug discrimination procedure to discriminate 10 min of exposure to 2000 ppm toluene vapor from 10 min of exposure to air. The discrimination was acquired in a mean of 65 training sessions. The stimulus effects of 2000 ppm toluene vapor were exposure concentration-dependent but rapidly diminished following the cessation of vapor exposure. The stimulus effects of toluene generalized to the chlorinated hydrocarbon vapor perchloroethylene but not 1,1,2-trichloroethane nor the volatile anesthetic isoflurane. The competitive NMDA antagonist CGS-17955, the uncompetitive antagonist dizocilpine and the glycine-site antagonist L701,324 all failed to substitute for toluene. The classical nonselective benzodiazepines midazolam and chlordiazepoxide produced toluene-like stimulus effects but the α 1 subunit preferring positive GABA_A modulator zaleplon failed to substitute for toluene. The barbiturates pentobarbital and methohexital and the GABA_A-positive modulator neurosteroid allopregnanolone did not substitute for toluene. These data suggest that the stimulus effects of toluene may be at least partially mediated by benzodiazepine-like positive allosteric modulation of GABA_A receptors containing α 2, 3 or 5 subunits.

Keywords

drug discrimination; inhalants; toluene; GABA; NMDA

1. Introduction

Toluene is present in many products including paints, cleaners, glues and motor fuels. It is one of if not the most commonly abused inhalant (Cruz, 2012). However, the neurochemical system or systems underlying toluene's abuse-related behavioral effects are unclear (Balster, 1998; Brouette and Anton, 2001). *In vitro* data show that toluene modulates a number of ion

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channel neurotransmitter receptors. Toluene enhances recombinant γ -aminobutyric acid type A (GABA_A), glycine and serotonin type 3 (5-HT₃) receptor function while attenuating *N*-methyl-D-aspartate (NMDA) and nicotinic acetylcholine receptor function (Bale et al., 2005; Bale et al., 2002; Beckstead et al., 2001; Beckstead et al., 2000; Cruz et al., 2000; Cruz et al., 1998; Lopreato et al., 2003). Whole-cell patch clamp recording in rat cortical neurons also demonstrates that toluene increases GABA_A-mediated inhibitory post-synaptic currents while attenuating both AMPA and NMDA-mediated excitatory post-synaptic currents (Beckley and Woodward, 2011).

Toluene's behavioral effects may also be mediated by both GABA_A positive modulation and NMDA antagonism. Toluene attenuates convulsions induced by the GABA_A receptor antagonist pentylenetetrazol (Wood et al., 1984) and demonstrates locomotor cross-sensitization with diazepam (Wiley et al., 2003). Toluene has anxiolytic-like effects (Bowen et al., 1996) and increases footshock-suppressed operant responding in mice (Wood et al., 1984) comparable to GABA_A-positive modulators (Rago et al., 1988) and NMDA antagonists (Dunn et al., 1990). Toluene also reduces the severity and lethality of NMDA-induced seizures (Cruz et al., 2003) and administration of the NMDA glycine site co-agonist D-serine attenuates toluene-induced locomotor incoordination and memory impairment (Lo et al., 2009). These studies suggest that GABA_A and/or NMDA receptors may play a role in some of the behavioral effects of toluene but do not address if either system is involved in the abuse-related effects of toluene.

The drug discrimination procedure is a selective means of probing the receptor mechanisms underlying the abuse-related stimulus effects of a drug (Colpaert, 1999; Schuster and Johanson, 1988). Toluene vapor has been reported to produce partial (Rees et al., 1987a) or complete substitution (Bowen, 2009) in mice trained to discriminate ethanol from saline. Toluene produces somewhat inconsistent results when tested in animals trained to discriminate barbiturates or benzodiazepines from vehicle (Bowen et al., 1999; Rees et al., 1985) as well as uncompetitive NMDA antagonists from vehicle (Bowen et al., 1999; Shelton and Balster, 2004). In animals trained to discriminate toluene injections from vehicle, partial generalization was produced by barbiturates and benzodiazepines but not antipsychotics (Knisely et al., 1990; Rees et al., 1987b).

We have demonstrated that toluene administered by its abused inhalation route can also serve as a discriminative stimulus and that the discriminative stimulus effects of toluene vapor are mediated by its intoxicating effects rather than peripheral stimulus properties such as odor (Shelton, 2007; Shelton and Slavova-Hernandez, 2009). The present experiment used the inhaled discrimination procedure to explore the neurochemical systems underlying the stimulus effects of toluene. We also compared and contrasted the toluene vapor stimulus to that of other classes of inhalants to determine if common neurochemical mechanisms underlie their stimulus effects as well.

2. Materials and methods

2.1. Subjects

Seventeen adult male B6SJLF1/J mice (Jackson Laboratory, Bar Harbor, Maine) served as subjects. We have used this strain for several prior inhalant discrimination studies (Shelton, 2007; 2009; 2010; Shelton and Nicholson, 2010; 2012; Shelton and Slavova-Hernandez, 2009). The mice were individually housed under a 12 hr light/dark cycle (lights on 6 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".

2.2. Compounds

HPLC grade toluene, 1,1,1-trichloroethane, 1,1,2-trichloroethane, perchloroethylene, valproic acid, pentobarbital, dizocilpine maleate [(+)-MK-801] and allopregnanolone were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI). Isoflurane was purchased from Webster Veterinary Supply (Charlotte, NC). Zaleplon, chlordiazepoxide, and *cis*-4-[Phosphomethyl]-piperidine-2-carboxylic acid (CGS-19755) were purchased from Tocris Bioscience (St. Louis, MO). 7-Chloro-4-hydroxy-3-(3-phenoxyphenyl)-2(1H)quinolone (L701,324) and morphine sulfate were provided by the National Institute of Drug Abuse drug supply program. Midazolam maleate was a gift of Roche Pharmaceuticals (Nutley, NJ). Methohexital sodium was purchased as a commercially available lyophilized powder (Brevital, JHP Pharmaceuticals, Parsippany, NJ). Unless otherwise indicated injected test drugs were dissolved in 0.9% physiological saline. Zaleplon, allopregnanolone and pentobarbital were prepared in a 45% β -cyclodextrin/sterile water solution. L701,324 was suspended in a solution of 10% Cremophor in sterile water. Exposures to all vapors were 10 min in duration. All injected drugs were administered intraperitoneally (i.p.) at a volume of 10 ml/kg. A 5-min pretreatment time was used for methohexital. A 10-min pretreatment time was used for dizocilpine, midazolam, pentobarbital, morphine and allopregnanolone. Zaleplon, valproic acid, chlordiazepoxide, L701,324 and CGS-19755 were administered with a 30-min pretreatment time.

2.3. Apparatus

Drug discrimination sessions were conducted in standard 2-lever mouse operant conditioning chambers (Med-Associates, St. Albans, VT). Each chamber was equipped with two optical levers with a LED stimulus light above each lever, a houselight in the rear chamber wall and a 0.01 ml electrically-operated liquid dipper. Mice were reinforced with a milk solution consisting of 25% sugar, 25% nonfat powdered milk and 50% tap water (by volume). Training and test vapor exposures were conducted in sealed 27-liter chromatography jars. An internal fan rapidly volatilized liquid inhalants introduced into the chamber. The volume of volatile liquids necessary to generate the appropriate chamber vapor concentrations were calculated using the ideal gas law as derived for vapors at standard laboratory pressure and temperature (Nelson, 1971). Accuracy of calculated vapor concentrations were empirically verified periodically using a Miran 1A single wavelength infrared spectrometer. Vapor concentrations did not deviate by more than 10% of target values for the duration of the exposure period. The static exposure chambers and procedures for generating test vapors have been described in greater detail previously (Shelton, 2007).

2.4. 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. Responses while the dipper was elevated had no consequences. Over successive days the session length was decreased from 30 min to 5 min after which double alternation toluene vapor versus air discrimination training sessions began (e.g. toluene, toluene, air, air). During training the correct lever was based on whether the subject received a 10 min exposure to 2000 ppm toluene vapor or to air. Following the 10 min exposure the mice were rapidly removed from the chromatography jar, placed into the operant chamber and the training session immediately initiated. Over the course of training the FR1 response requirement was increased to FR12. Responding on the inactive lever reset the FR requirement on the correct lever. A mouse was determined to have

acquired the 2000 ppm toluene vapor versus air discrimination when it emitted its first FR on the correct lever in 8 of 10 consecutive training sessions at FR12.

Following acquisition, 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 accurate stimulus control during intervening training sessions. Test sessions were not recommenced until the first FR was emitted on the correct lever for three consecutive training sessions. Substitution tests with vapors were preceded by 10 min of exposure to a single concentration of the test vapor. In substitution test sessions with an injected drug, the subjects always received 10 min of air exposure prior to the start of the operant test session. For drugs with pretreatment intervals of 10 min, the injection was given and the animal immediately placed into the exposure chamber. For drugs with pretreatment intervals longer than 10 min, the injection was given and the animal returned to their homecage until 10 min remained in the pretreatment interval at which time it was placed into the exposure chamber. In the case of methohexital which required a 5 min pretreatment interval, the animal was exposed to air in the exposure chamber, removed after 5 min to receive the injection and placed back into the exposure chamber for an additional 5 min prior to testing. 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. Doses were increased until two ascending doses producing similar maximal substitution levels or one or more subject's responding was sufficiently suppressed such that it was unable to emit a complete FR within the first min of the test session. Prior to each vapor concentration-effect curve, air and 2000 ppm toluene 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 toluene or air exposure. At least 7 mice were used to generate each dose-or concentration-effect curve.

2.5. Drug discrimination data analysis

Percentage toluene-lever responding and response rates (responses/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 of very short duration which results in an underestimation of the response-rate altering effects of some inhalants when full 5-min test session response rates are calculated (Shelton 2007; Shelton and Slavova-Hernandez 2009; Shelton and Nicholson, 2010). Therefore, to insure that both substitution and operant rate suppression data represented a time period during which the maximal behavioral effects of the tested vapors were likely to be exhibited, regardless of the offset kinetics of the particular test inhalant, only the first min of each discrimination test session was examined and plotted. To maintain consistency across all test compounds, we chose to likewise restrict our analysis of the injected drug data to the first min of each test session. Group means ($\pm$ S.E.M.) were calculated for percentage first min toluene-lever selection as well as first min response rate. Any inhalant concentration or injected-drug dose that suppressed response rates to the extent that an 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 included in the substitution curve. A criterion of 75% or greater mean toluene vapor-appropriate responding was defined as full substitution, between 25% and 74% as partial substitution and less than 25% as no substitution. When possible EC_{50} or ED_{50} values and 95% confidence limits (CL) for toluene vapor-lever selection and response rate suppression were calculated based on the linear portion of each mean dose-effect curve (Tallarida and Murray, 1986). The effect of each compound on rates of operant responding

was evaluated by within subject one-way analysis of variance (ANOVA) using Geisser-Greenhouse correction for sphericity (Prism 6 for Macintosh, GraphPad Software). Significant ($P < 0.05$) main effects were followed by post hoc paired t-tests comparing each test dose or concentration to its respective air or air+vehicle control sessions.

3. Results

The mice acquired the 2000 ppm toluene vapor versus air discrimination in a mean of 65 (± 4.6) training sessions. The number of training sessions to reach acquisition ranged from 25–115 across subjects. Figure 1 shows the rate at which the discriminative stimulus effects of 2000 ppm toluene diminished as a function of delaying initiation of the operant session for increasing periods of time after removal from the inhalant exposure chamber. Full substitution was produced by beginning testing immediately following the cessation of exposure, as was done during training, as well as after delaying the start of testing for 1 min. Progressively longer delays before substitution testing resulted in diminished toluene-lever responding.

Toluene concentration-dependently substituted for the 2000 ppm training concentration with an EC_{50} of 435 ppm [CL: 304–622 ppm]. Full substitution was produced by toluene concentrations of 2000 ppm and greater. Rates of operant responding were not altered by toluene concentrations up to 8000 ppm. The chlorinated alkane 1,1,1-trichloroethane produced a maximum of 44% toluene-lever selection at a concentration of 8000 ppm. A second chlorinated alkane 1,1,2-trichloroethane produced a maximum of 37% toluene-lever selection at a concentration of 4000 ppm. 1,1,1-trichloroethane failed to affect response rates up to the highest tested concentration of 12000 ppm. However, 1,1,2-trichloroethane concentration-dependently and suppressed response rates with an EC_{50} of 3788 ppm [CL: 2784–5155 ppm]. Significant [$F_{(2.8, 16.5)} = 13.2, p < 0.01$] suppression of responding was produced by 1,1,2-trichloroethane concentrations of both 6000 and 8000 ppm. A final chlorinated hydrocarbon perchloroethylene produced 82% toluene-lever selection with an EC_{50} of 640 ppm [CL: 371–1105 ppm]. Perchloroethylene suppressed operant responding with an EC_{50} of 5855 ppm [CL: 2505–7610 ppm]. Perchloroethylene significantly [$F_{(2.5, 17.5)} = 11.4, p < 0.01$] reduced responding at 6000 ppm and nearly completely abolished responding in all mice at 8000 ppm. Isoflurane, a halogenated ether vapor anesthetic not chemically related to either toluene or the chlorinated vapors, engendered a maximum of 45% toluene-lever selection at 8000 ppm. Isoflurane concentration-dependently and significantly [$F_{(4.4, 30.7)} = 9.58, p < 0.01$] suppressed operant responding with responding abolished in all subjects at a concentration of 12,000 ppm.

Toluene substitution and response-rate altering effects produced by three positive $GABA_A$ benzodiazepine-site positive modulators are shown in figure 2. The non-selective classical benzodiazepine midazolam produced concentration-dependent partial substitution for toluene (upper panel) with an ED_{50} of 4.5 mg/kg [CL: 1.8–11.2 mg/kg]. A maximum of 66% toluene-lever responding was produced by 15.6 mg/kg midazolam which was accompanied by a 40% reduction in operant response rates compared to vehicle control levels (lower panel). Midazolam significantly [$F_{(2.4, 17)} = 5.04, P = 0.01$] suppressed operant responding with an ED_{50} of 25.4 mg/kg [CL: 11.1–58.4 mg/kg]. A second benzodiazepine, chlordiazepoxide, produced less robust substitution with a maximum of 50% toluene-lever selection at a dose of 30 mg/kg (upper panel). Chlordiazepoxide also concentration-dependently and significantly [$F_{(3.7, 25.8)} = 6.69, P < 0.01$] reduced operant response rates with an ED_{50} of 39 mg/kg [CL: 30.3–49.6 mg/kg]. (lower panel). Responding was completely abolished by 50 mg/kg chlordiazepoxide in 3 of the 8 mice tested. The $GABA_A$ receptor α -1 subunit preferring benzodiazepine-site positive modulator zaleplon engendered a maximum of 26% toluene-lever selection at the highest test dose 3 mg/kg

(upper panel). There was no significant main effect of zaleplon dose [$F_{(2, 12.5)} = 2.17$, $P = 0.15$] on operant responding but the 3 mg/kg dose reduced response rates to 44% of air control levels (lower panel) and completely suppressed responding in 3 of 7 mice tested.

Table 1 shows toluene-lever selection and response-rate effects produced by three additional GABA_A receptor allosteric modulators; pentobarbital, methohexital and allopregnanolone. The barbiturate, pentobarbital, produced a maximum of 43% toluene-lever selection at 30 mg/kg. Pentobarbital also significantly suppressed operant responding [$F_{(2.9, 17.7)} = 8.07$, $P < 0.01$] with an ED₅₀ for response-rate suppression of 25 mg/kg [CL: 19.6–30.9 mg/kg]. The ultra-short acting barbiturate methohexital exhibited a maximum of 24% toluene-lever selection at the highest test dose of 30 mg/kg. Methohexital significantly suppressed operant responding [$F_{(2.2, 13.1)} = 9.45$, $P < 0.01$] with an ED₅₀ of 18 mg/kg [CL: 13.9–23 mg/kg]. The GABA_A positive modulator neurosteroid, allopregnanolone, failed to substitute for toluene up to a dose of 50 mg/kg. There was no significant main effect of allopregnanolone dose on operant responding [$F_{(1.9, 13.3)} = 2.13$, $P = 0.16$] although mean responses rates were decreased by 50% at the highest test dose of 50 mg/kg.

Table 2 shows toluene-lever selection and operant response-rate data produced during testing of dizocilpine, CGS-19755, L701,324 and valproic acid. The noncompetitive NMDA antagonist dizocilpine failed to produce greater than 20% toluene-lever selection up to a 0.3 mg/kg dose. There was no significant main effect of dizocilpine dose on operant response rates [$F_{(2.3, 16.2)} = 3.44$, $P = 0.051$] although the 0.3 mg/kg dose abolished responding in 5 of 8 mice tested. Likewise the competitive NMDA antagonist CGS-19755 also failed to substitute for toluene up to doses which significantly [$F_{(2.4, 23.8)} = 12.88$, $P < 0.01$] reduced responding. The ED₅₀ for response-rate suppression by CGS-19755 was 8.9 mg/kg [CL 6–13.4 mg/kg]. The NMDA glycine-site antagonist L701,324 failed to elicit greater than 18% toluene-lever selection but did not suppress response rates up to the maximum test dose of 45 mg/kg which was the highest dose which could be administered due to injection volume considerations. The anticonvulsant valproic acid produced 58% toluene-lever selection at 560 mg/kg. Valproic acid significantly altered response rates [$F_{(1.9, 11.3)} = 4.78$, $P = 0.03$] and only 3 of 7 mice tested elicited any responses at the highest test dose of 560 mg/kg. Lastly, the mu opioid receptor agonist morphine produced a maximum of 26% toluene-lever selection at a dose of 10 mg/kg (data not shown). Morphine also significantly suppressed operant responding [$F_{(1.9, 9.9)} = 5.38$, $P = 0.02$] at the highest dose of 10 mg/kg with an ED₅₀ for response-rate suppression of 6.9 mg/kg [CL: 1.8–27 mg/kg].

In some cases the toluene-like discriminative stimulus effects of test drugs were specific to a single dose which varied between mice. To control for individual differences in optimal substitution dose, table 3 shows the maximal percent substitution for each test drug regardless of test dose. Data are categorized according to the percent of subjects demonstrating less than < 50%, 50%–74% or >75% toluene-lever selection at any test dose. For midazolam and the inhalants 1,1,1-trichloroethane and perchloroethylene, 87.5% of mice tested exhibited >75% toluene lever selection at one or more test dose. No mice showed full substitution of zaleplon for toluene but 87.5% of mice tested showed partial substitution for toluene at one or more zaleplon test dose. With the exception of isoflurane, for the other drugs examined almost all the mice tested emitted less than 50% toluene-lever selection at every test dose.

4. Discussion

In prior studies we demonstrated that 6000 ppm toluene vapor can be trained as a discriminative stimulus (Shelton, 2007; Shelton and Slavova-Hernandez, 2009). In the present study we extended our previous findings in this regard by training a discrimination

based on a lower concentration of 2000 ppm toluene vapor. We chose to examine a lower toluene training concentration based on data indicating concentrations in this range produce other abuse-related behaviors such as conditioned place preference and may therefore be more relevant (Funada et al., 2002; Gerasimov et al., 2003; Lee et al., 2006). The discriminative stimulus effects of 10 min of exposure to 2000 ppm toluene vapor were concentration dependent but quite short lived, declining to less than 50% toluene-lever selection within 3 min post-exposure. This is in contrast to the 10 min required before toluene-lever selection declined to 50% in mice trained to discriminate 6000 ppm toluene vapor (Shelton and Slavova-Hernandez, 2009). The rapidity with which the stimulus effects of 2000 ppm toluene vapor dissipate suggest that despite its high lipid solubility, the majority of the inhaled toluene dose is likely eliminated via exhalation or quickly metabolized. The present data confirmed those from prior drug discrimination studies demonstrating that the substitution of cross-test drugs for toluene in individual animals often only occurs at a single dose and that the optimal substitution dose is highly variable between subjects (Knisely et al., 1990; Rees et al., 1985; Rees et al., 1987a; Rees et al., 1987b). For instance, in the midazolam dose-effect curve, mean substitution for toluene never exceeded 66%, yet 7 of 8 mice demonstrated full substitution for toluene at one or more midazolam test dose. As such the most sensitive measurement of common stimulus effects may not be mean substitution dose-effect curves but rather the percentage of the test group which exhibit greater than 50% chance levels of substitution at any dose of a cross-test drug (Table 3).

We have previously shown that the discriminative stimulus effects of toluene are shared by another aromatic hydrocarbon, ethylbenzene (Shelton, 2007). In the present study we compared the stimulus effects of toluene to a volatile vapor anesthetic isoflurane and to three chlorinated hydrocarbons. Isoflurane substituted poorly for toluene, with only 3 of 8 mice tested demonstrating greater than 75% toluene-lever selection at one or more isoflurane concentration. These data are consistent with the lack of substitution of isoflurane for a higher, 6000 ppm toluene training concentration (Shelton, 2007). In contrast, at least one concentration of two chlorocarbon vapors, perchloroethylene and 1,1,1-trichloroethane, produced greater than 75% toluene-lever selection in 7 of 8 mice tested with each inhalant. A third chlorocarbon vapor, 1,1,2-trichloroethane only produced greater than 75% toluene-levers selection in 1 of 8 mice. The failure of 1,1,2-trichloroethane to substitute for toluene while 1,1,1-trichloroethane produced more robust toluene-like effects is interesting given the two compounds are structural isomers. As well as diverging in toluene substitution, 1,1,2-trichloroethane was far more potent in suppressing operant responding than 1,1,1-trichloroethane. Concentrations of 1,1,1-trichloroethane up to 12,000 ppm failed to suppress responding compared to the air control session, whereas a concentration of 6000 ppm 1,1,2-trichloroethane completely suppressed responding in 6 of the 7 mice tested. Based on these data it is possible that the greater potency of 1,1,2-trichloroethane for suppressing responding simply prevented testing sufficiently high concentrations to elicit toluene-like discriminative stimulus effects. However, more fundamental differences in receptor activity among drug isomers are common and have been demonstrated with stereoisomers of volatile anesthetics such as isoflurane (Harris et al., 1992; Lysko et al., 1994; Moody et al., 1993). The present data with 1,1,1-trichloroethane and 1,1,2-trichloroethane suggest that the same is likely the case with abused volatile inhalants.

Prior published data support the hypothesis that the behavioral effects of toluene may be mediated by GABA_A positive modulatory as well as NMDA antagonist effects (Bowen et al., 1996; Cruz et al., 2003; Lo et al., 2009; Wiley et al., 2003; Wood et al., 1984). The present data showing that neither the uncompetitive NMDA antagonist dizocilpine, the competitive NMDA antagonist CGS-19755 nor the glycine-site NMDA antagonist L701,324 substitute for toluene suggests that, despite strong *in vitro* data demonstrating an interaction

with NMDA receptors, the discriminative stimulus effects of 2000 ppm toluene vapor are probably not mediated by NMDA antagonist activity. The failure of dizocilpine to substitute for toluene is consistent with the results of a prior study in which toluene did not elicit dizocilpine-like stimulus effects in mice (Shelton and Balster, 2004). However, the present data is not in accordance with the toluene-like stimulus effects demonstrated in mice trained to discriminate PCP (Bowen et al., 1999). The reason for this inconsistency is uncertain but unlike dizocilpine, which is very selective for NMDA receptors, PCP has been shown to produce other neurochemical effects including the enhancement of dopamine release (Crosby et al., 2002; Maurice et al., 1991). It is possible that the dopaminergic effects of PCP may have been responsible for the prior substitution of toluene for PCP given that PCP failed to fully substitute in dizocilpine-trained mice (Shelton and Balster, 2004) and toluene engendered full substitution in mice trained to discriminate amphetamine from vehicle (Bowen, 2006).

The GABA_A receptor is a heteropentamer most commonly comprised of combinations of alpha, beta, and gamma subunits (Hevers and Luddens, 1998). Distinct allosteric modulatory sites have been established for neurosteroids, barbiturates and benzodiazepines (D'Hulst et al., 2009). Allopregnanolone, which is believed to positively modulate GABA_A receptors through an alpha subunit-dependent neurosteroid binding site (Hosie et al., 2009), failed to produce appreciable substitution for toluene. The positive GABA_A receptor modulator barbiturate pentobarbital also failed to substitute for 2000 ppm toluene vapor in the present study. A number of lines of evidence suggest that the positive allosteric modulatory effects of barbiturates are primarily (Cestari et al., 1996; Hanek et al., 2010; Pistis et al., 1999), although not exclusively (Drafts and Fisher, 2006), beta subunit dependent. Combined, these data suggest that toluene may not be acting through either the neurosteroid binding site nor through the barbiturate binding domain. However, in a previous experiment, toluene vapor concentrations between 2400 and 4800 ppm generalized at one or more doses in most mice trained to discriminate 5 mg/kg pentobarbital from vehicle (Rees et al., 1985) and in mice or rats trained to discriminate 100 mg/kg i.p. toluene from vehicle, pentobarbital produced partial substitution (Knisely et al., 1990; Rees et al., 1987b). Our negative pentobarbital data is difficult to reconcile with these prior reports and is what prompted our systematic replication of the lack of toluene-like stimulus effects using a second barbiturate, methohexital. There are a number of differences between the present study and the previous reports, including route of toluene administration, species, strain, test doses and pretreatment times, among others. Any one or a combination of these factors may have played a role in the discrepant results. Therefore, the present finding that barbiturates do not possess toluene-like stimulus effects cannot, without further study, be extended beyond the specific mouse strain, test conditions and 2000 ppm training concentration examined.

The benzodiazepine binding site on the GABA_A receptor, like the neurosteroid site, is believed to be alpha subunit dependent. However, unlike neurosteroids, benzodiazepine-site ligand binding is a function of GABA_A receptor alpha subunit composition (Trincavelli et al., 2012). Benzodiazepine site modulators with different alpha subunit preferences can in some cases be differentiated using drug discrimination. For instance, in animals trained to discriminate the alpha 1 preferring positive modulator zolpidem, nonselective classical benzodiazepines that modulate alpha 1, 2, 3 and 5 containing GABA_A receptors produce full substitution. However in animals trained to discriminate non-selective benzodiazepines, alpha 1 selective drugs substitute poorly (Depoortere et al., 1986; Rowlett et al., 2003; Vinkers et al., 2011). These results have been interpreted as indicating that the stimulus effects of nonselective classical benzodiazepines are not alpha 1 subunit dependent (Mirza et al., 2006; Vinkers et al., 2011). In the present study the nonselective classical benzodiazepine midazolam produced greater than 75% toluene-lever selection at one or more test dose in 7 of 8 mice tested. A second nonselective benzodiazepine,

chlordiazepoxide, produced greater than 75% toluene-lever selection in 5 of 8 mice. Although toluene vapor did not substitute in mice trained to discriminate diazepam (Bowen et al., 1999) our data is consistent with a prior study showing that another nonselective benzodiazepine, oxazepam, engenders substitution in most rats trained to discriminate 100 mg/kg i.p. toluene from vehicle (Knisely et al., 1990). While classical nonselective benzodiazepines appear to produce toluene-like stimulus effects, in the present study no mice showed greater than 75% toluene-lever selection when administered the alpha 1 subunit-preferring benzodiazepine-site positive modulator zaleplon. These data suggest that toluene may be producing its benzodiazepine-like discriminative stimulus effects through positive allosteric modulation of GABA_A receptors containing alpha 2, 3 or 5 subunits. Additional studies with drugs demonstrating greater selectivity for these individual GABA_A subunits will be necessary to confirm this hypothesis.

5. Conclusions

Overall the present results demonstrate that the discriminative stimulus effects of 2000 ppm toluene vapor are dissimilar to halogenated volatile anesthetics but similar to some chlorinated hydrocarbon vapors. However, the stimulus overlap between toluene and chlorinated hydrocarbon vapors appears to be highly dependent upon the compound's three dimensional chemical structure suggesting a specific receptor-based interaction. The discriminative stimulus effects of toluene are shared by nonselective classical benzodiazepines but not an alpha 1 subunit preferring benzodiazepine-site positive modulator, barbiturates or a GABA_A-positive neurosteroid. These data suggest that the discriminative stimulus of 2000 ppm toluene vapor may be mediated by benzodiazepine-like positive allosteric modulation of GABA_A receptors containing alpha 2, 3 or 5 subunits.

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Fig. 1.

Toluene substitution curve for tests sessions conducted after increasing post-exposure delays following 10 min of 2000 ppm toluene vapor exposure. The data points shown are based on the first min of each 5-min test session. Point above Air represents the results of an air exposure control session conducted immediately after removal from the inhalant exposure chamber. Mean ($\pm$ S.E.M.) percentages toluene-lever responding at post-exposure discrimination session test delays of 3, 5, 10, 20 and 30 min are shown in filled squares.

Fig. 2.

Dose-effect curves for midazolam (filled squares), zaleplon (open circles) and chlordiazepoxide (open triangles) in mice trained to discriminate 2000 ppm inhaled toluene vapor from air ($n=8, 7$ and 8 , respectively). The data presented are based on the first min of each 5-min test session. Points above Air (open symbols) and Tol (filled symbols) represent the results of air and 2000 ppm inhaled toluene exposure control sessions. Mean ($\pm$ S.E.M.) percentages toluene-lever responding are shown in the upper panel and mean ($\pm$ S.E.M.) 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 toluene-lever selection curve. * indicates statistically significant ($P < 0.05$) difference from air control response rate.

Table 1

Toluene lever selection and operant response rates produced by pentobarbital, methohexital and allopregnanolone.

Drug	Drug dose or vapor concentration	% toluene-lever responding ($\pm$ S.E.M.)	Response rate in responses/sec ($\pm$ S.E.M.)
Pentobarbital	Air + veh control	2 (1.3)	1 (0.2)
	Toluene control	84 (14)	1.4 (0.1)
	1 mg/kg	4 (2.6)	1.3 (0.2)
	10 mg/kg	24 (13.6)	1.2 (0.2)
	17 mg/kg	8 (5.7)	0.8 (0.2)
	30 mg/kg	43 (22.1) [3/7]	0.3 (0.1) ^a
Methohexital	Air + veh control	0 (0)	1.4 (0.1)
	Toluene control	94 (4.8)	1.6 (0.2)
	1 mg/kg	3 (2.3)	1.3 (0.2)
	3 mg/kg	1 (0.3)	1.5 (0.1)
	10 mg/kg	10 (4.8)	1.3 (0.2)
	17 mg/kg	13 (7.3) [6/7]	0.6 (0.2) ^a
Allopregnanolone	30 mg/kg	24 (24) [3/7]	0.3 (0.1) ^a
	Air + veh control	11 (5.8)	0.9 (0.1)
	Toluene control	82 (12.4)	1.2 (0.2)
	0.1 mg/kg	4 (3.3)	1.0 (0.1)
	1 mg/kg	13 (8.4)	0.9 (0.2)
	10 mg/kg	5 (2.3)	0.7 (0.2)
	30 mg/kg	14 (11.9)	0.9 (0.2)
	50 mg/kg	7 (3.3)	0.5 (0.1)

Numerals in brackets [] indicate number of animals out of the group emitting sufficient responses to be included in % toluene-lever responding determination.

^a $P < 0.05$ differences compared to air + vehicle control response rate.

Table 2

Toluene lever selection and operant response rates produced by dizocilpine, CGS-19755, L701,324 and valproic acid.

Drug	Drug dose or vapor concentration	% toluene-lever responding ($\pm$ S.E.M.)	Response rate in responses/sec ($\pm$ S.E.M.)
Dizocilpine	Air + vehicle	11 (5.6)	1.1 (0.1)
	2000 ppm toluene	94 (2.0)	1.6 (0.1)
	0.03 mg/kg	3 (2.1)	1.3 (0.1)
	0.1 mg/kg	3 (2.6)	1.1 (0.2)
	0.17 mg/kg	20 (8.8)	1.1 (0.1)
	0.3 mg/kg	6 (4.3) [3/8]	0.6 (0.3)
CGS-19755	Air + vehicle	1 (0.4)	1.2 (0.1)
	2000 ppm toluene	97 (1.7)	1.4 (0.1)
	1 mg/kg	6 (11.9)	1.2 (0.1)
	3 mg/kg	4 (2.5)	1.0 (0.1)
	10 mg/kg	5 (3.3)	0.6 (0.1) ^a
	17 mg/kg	21 (11.3) [10/11]	0.4 (0.1) ^a
L701,324	Air + vehicle	12 (6.1)	1.5 (0.2)
	2000 ppm toluene	77 (12.4)	1.5 (0.1)
	3 mg/kg	18 (12.2)	1.3 (0.2)
	10 mg/kg	15 (6.2)	1.4 (0.3)
	30 mg/kg	1 (1)	1.2 (0.1)
	45 mg/kg	1 (1)	1.2 (0.2)
Valproic Acid	Air + vehicle	1 (0.7)	0.9 (0.1)
	2000 ppm toluene	99 (0.4)	1.2 (0.2)
	30 mg/kg	9 (4.6)	1.4 (0.1) ^a
	100 mg/kg	6 (2.6)	1.1 (2.6)
	300 mg/kg	19 (6.6)	1.0 (0.2)
	560 mg/kg	58 (19.9) [3/7]	0.6 (0.2)

Numerals in brackets [] indicate number of animals out of the group emitting sufficient responses to be included in % toluene-lever responding determination.

^a $P < 0.05$ differences compared to air + vehicle control response rate.

Table 3

Summary of toluene-lever selection for all compounds tested.

Drug	Maximal substitution regardless of test dose		
	<50%	50%–74%	>75%
Midazolam	12.5	0.0	87.5
Chlordiazepoxide	12.5	25.0	62.5
Zaleplon	12.5	87.5	0.0
Pentobarbital	71.4	0.0	28.6
Methohexital	85.7	14.3	0.0
Allopregnanolone	87.5	0.0	12.5
Dizocilpine	87.5	0.0	12.5
CGS-19755	72.7	9.1	18.2
L701,324	87.5	0.0	12.5
Valproic acid	85.7	0.0	14.3
Morphine	83.3	0.0	16.7
1,1,1-trichloroethane	12.5	0.0	87.5
1,1,2-trichloroethane	85.7	0.0	14.3
Perchloroethylene	12.5	0.0	87.5
Isoflurane	25.0	37.5	37.5

Data are presented as the percentage of animals exhibiting maximum substitution of less than 50%, 50%–74% or 75% and greater irrespective of compound test dose.