2016

Acute and Chronic Effects of Inhalants in Intracranial Self-stimulation

Matthew Tracy
Student

Acute and Chronic Effects of Inhalants in Intracranial Self-stimulation

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

By

MATTHEW E. TRACY

"Bachelor of Science in Biochemistry, University of Nebraska-Lincoln, 2009"

Director: KEITH L. SHELTON

ASSOCIATE PROFESSOR, DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY

Director: HAMID AKBARALI

PROFESSOR, DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY

Virginia Commonwealth University

Richmond, Virginia

"May 2016"

Acknowledgements

For Katya…who was always pointing up towards the sky while I point down at the ground. You're my Russian rock that keeps me going against the hardships of the world.

I wish to acknowledge the substantial contribution of the many students, post-doctoral researchers, and faculty of the Pharmacology and Toxicology department that have contributed to my education. My time here has been an incredibly humbling and enlightening experience as I've been exposed to so many different elements of the scientific process across so many different disciplines. In particular, the contributions from: Drs. Steve Negus, Joe Porter, Laura Sim-Selley, Matt Banks and John Bigbee. These projects would not be possible without your invaluable feed-back and suggestions.

I'd like to thank my friends that I've made here at VCU (Gareth, Catherine, Jason, Neil!), the camaraderie keeps me going! I'd also like to thank my friends at Richmond BJJ and the whole crew in the BJJ Revolution super family! Eric, Jarrett, Liz, Andrew, I wish I could remember 1/1000th of all the jiu-jitsu you've taught me.

Finally, I want to acknowledge my mentor Keith L. Shelton for his patience, understanding, and guidance through the trials and tribulations of the Pharmacology and Toxicology PhD program. Keith pushed me to apply myself at times when I didn't think I was ready for some of challenges that graduate school offered up. I was only able to

"cowboy up" because he was there telling me that it could be done. Thank you Dr. Shelton.

Table of Contents

List of Tables

List of Figures

Abstract

"Acute and Chronic Effects of Inhalants in Intracranial Self-stimulation"

By Matthew E. Tracy

A Dissertation submitted in partial fulfillment of the requirements for the degree of

Philosophy Doctorate at Virginia Commonwealth University.

Virginia Commonwealth University, 2016

Major Director: Keith L. Shelton

ASSOCIATE PROFESSOR, DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY

Inhalants are a loosely defined diverse group of volatile substances which people abuse. Despite widespread misuse of inhalants, there are limited preclinical methods available to study the reinforcement-like properties of inhalants. One procedure which has demonstrated substantial promise as a tool to investigate inhalant pharmacology is the intracranial self-stimulation (ICSS) procedure. ICSS utilizes pulses of electrical stimulation to the mesolimbic reward pathway to serve as a temporally defined and controlled operant reinforcer with a highly adjustable efficacy. The first aim of the project was to characterize the effects of commonly abused inhalants: including toluene, trichloroethane, nitrous oxide, isoflurane and R134a in ICSS.

The second aim was to attenuate inhalant-facilitated ICSS by utilization of compounds which would attenuate the pharmacological actions of toluene on GABAA receptors. The low efficacy benzodiazepine negative modulator Ro15-4513 significantly attenuated the ability of toluene to facilitate ICSS without itself significantly altering baseline ICSS responding. Pretreatment with Ro15-4513 also attenuated methamphetamine ICSS even though there is no evidence of methamphetamine interacting with GABAA receptors. Given these unexpected results, I employed a microdialysis procedure to examine the effect of Ro15-4513 on methamphetamine stimulated dopamine release in the nucleus accumbens. Pretreatment with Ro15-4513 significantly attenuated methamphetamine stimulated dopamine release while having a negligible effect on dopamine release when administered alone. These results suggest that a modest level of benzodiazepine-site negative modulation can reduce the reinforcement enhancing effects of abused drugs regardless of their primary mechanism of action through allosteric

modulation of GABAergic neurons within the mesolimbic pathway. Further, these results may have implications for expanding the examination of $GABA_A$ negative modulator medications beyond those trials currently being conducted with alcohol.

Finally, the effects of chronic intermittent toluene exposure on ICSS and nesting behaviors were examined. Subjects were systemically exposed to air, chronic intermittent toluene (CIT), or escalating chronic intermittent (ECIT) toluene for 15 min at 3300 PPM toluene vapor per exposure. The results show that ECIT resulted in decreased overall responding in ICSS relative to air control and showed a tolerance-like effect to facilitatory effects of 3300 ppm toluene during ICSS compared to CIT group. These results indicate that escalating use of toluene produces reductions in its reward-like effects and may contribute to escalation to other drugs of abuse.

Abbreviations

5HT – Serotonin

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDZ – Benzodiazepine

DA - Dopamine

$GABA_A$ – gamma amino butyric acid subtype A

MER – Maximum earned responses

MFB – Medial Forebrain Bundle

N_2O – Nitrous Oxide

nAChR – Nicotinic Acetylcholine receptor

NMDA – N-methyl-D-aspartate

NAC – Nucleus accumbens

VTA – Ventral tegmental area

Background and Significance

Inhalants Defined

Inhalants are a pharmacologically diverse group of compounds that have been ubiquitously defined through the exclusivity of the inhalational route by which humans abuse them. In addition, inhalants are associated with their legal, therapeutic and commercial availability in society. Broadly defined, this loosely categorical ascription presents a unique challenge in defining their place amongst the various drug classes which are all generally grouped by pharmacological action. For example, many drugs such as marijuana, crack cocaine or crystal methamphetamine are highly abused via the inhalation route and yet are not considered to fall within the scope of the definition of an inhalant. Despite the lack of a clear cut, universally accepted, definition for what constitutes an inhalant, for most purposes the amorphous definition of an abused inhalant conforms to the following taken from a inhalant debate article addressing this exact issue (Balster *et al*, 2009): "Abused inhalants contain volatile substances that are self-administered as gases or vapors to induce a psychoactive or mind-altering effect. These volatile substances are available in legal, relatively inexpensive and common household products, which can be gases, liquids, aerosols or, in some cases, solids (mothballs)" (Cheong *et al*, 2006; Kong and Schmiesing, 2005; Weintraub *et al*, 2000).

Further clouding the already socially constructed definition of inhalants is what criteria could or should be used to group them. One approach is to group inhalants by a combination of their physical and chemical properties and their functional role in either

therapeutic or commercial settings. For example, the volatile and gas anesthetics which are used clinically for their anxiolytic and anesthetic properties fall into one set of categories while commercially viable products that contain abused solvents or gases fall into another (Table 0, Modified from (Howard *et al*, 2011).

Table 0	**Intended Use**	**Volatile solvents**	**Gases**
	Anesthetics	Sevoflurane, Isoflurane, Halothane	Nitrous Oxide, Xenon
	Commercial Adhesives	Toluene, ethyl acetate, methyl chloride	—
	Vehicle fuels and accessories	n-hexane, toluene, xylene	Nitrous Oxide, R134a
	Cleaners	Toluene, acetone, trichloroethane	R134a
	Food Preparation	—	Nitrous Oxide

A more rational and useful proposal is to group inhalants based upon their similar or unique pharmacological actions (Balster *et al*, 2009). However, the promiscuity of inhalants to alter the function of a large number of receptors and their receptor subtypes has made distinct categorization of inhalants extremely difficult. For example, the prototypical and arguably one of the most well studied of the commercial inhalants, toluene, has an incredibly diverse profile of pharmacological action, acting as a positive allosteric modulator, negative allosteric modulator, antagonist or non-competitive antagonist at the following channels: AMPA , NMDA, $GABA_A$, Glycine, $5HT_3$, nAChR, ATP, sodium channels, voltage-gated calcium channels, potassium channels and gap junctions (Cruz *et al*, 2014). Substantial overlap between the effects of toluene and the gaseous anesthetic nitrous oxide exist, in that it too has effects on nAChR (Suzuki *et al*, 2003; Yamakura and Harris, 2000), calcium channels (Fan *et al*, 2007), AMPA (Mennerick *et al*, 1998), NMDA (Jevtović-Todorović *et al*, 1998; Ranft *et al*, 2007;

Yamakura and Harris, 2000) and $GABA_A$ receptor (Hapfelmeier *et al*, 2000; Mennerick *et al*, 1998).

Due to research interest in enhancing the safety profile of therapeutic anesthetics, there is a great deal of ongoing research involved in isolating their binding site(s). (Borghese *et al*, 2006; Lecker *et al*, 2013; Werner *et al*, 2011). In mutant models of $GABA_A$ receptors, it has been demonstrated that point mutations of key proteins can dramatically reduce the overall pharmacological effectiveness of anesthetics to produce anesthesia (Borghese *et al*, 2006).

In contrast to toluene, nitrous oxide and volatile anesthetics, we know relatively little regarding the neurochemical pharmacology of the many other abused inhalants that are volatile constituents in commercial products (*e.g.* trichloroethane) and almost nothing regarding other abused inhalants such as refrigerants (*e.g.* 1,1,1,2-tetrafluoroethane/ R134a) (Ritchie *et al*, 2001).

Likewise, other inhalants that have been examined appear to also have multiple receptor interactions. Given the vast diversity of receptors impacted by inhalants there is enormous utility in quantifying the importance of specific targets to the abuse-related effects of inhalants. However, at this time differences across experimental conditions and incomplete data make synthesis of complete system wide catalogues of affected receptors and receptor subtypes difficult to construct. Though substantial efforts have been made within the last decade towards identifying the primary receptor systems that contribute to the abuse related effects of inhalants (Cruz *et al*, 2014).

Inhalant Abuse Epidemiology

Initiation of use and Abuse

Inhalants are often reported as being some of the first abused drugs in a subject's lifetime, largely due to the limited regulation and application of controlled monitoring systems and their widespread availability in products accessible to youth. The monitoring the future 2016 report estimates that over one million adolescents have abused an inhalant in the past year (Johnston, L. D., O'Malley, P. M., Miech, R. A., Bachman, J. G., & Schulenberg, 2016) and these numbers are as high as 18.8% in the developing world (Akoijam *et al*, 2013). There is also growing recognition of inhalant abuse in the armed forces, due in part to their low cost, ubiquitous availability and undetectable nature in standard drug screening tests (Lacy and Ditzler, 2007). Despite the severity of the consequences following inhalant use as well as their relatively high abuse rates, inhalants represent one of the least studied areas within the field of drug addiction research.

Diversion and Abuse of Inhalants

Toluene is a volatile organic solvent found in a wide range of consumer and industrial products including paint thinner, glues, and gasoline. Due in part to its ability to dissolve hydrophobic organic compounds, toluene is almost universally included as an emulsifying agent across a diverse array of commercial products and therefore remains one of the most widely accessible of the abused inhalants (Garland *et al*, 2009; Howard *et al*, 2011). Other inhalants which continue to be abused at lesser rates, often with fatal consequences, include nitrous oxide, trichloroethane, R134a and isoflurane (Koehler and

Henninger, 2014; Meadows and Verghese, 1996; Pavlic *et al*, 2002; Wu *et al*, 2004). Some inhalants appear to be abused at much higher rates in certain demographics (Hout and Bingham, 2013; Howard *et al*, 2008; Wagner *et al*, 2012) or in developing countries (Praharaj *et al*, 2008). Abuse of other inhalants such as the solvent 1,1,1-trichloroethane and the refrigerant, chlorodifluromethane (R22/Freon) and 1,1,1-2-tetrafluoroethane (R134a) have seen decreased production and reduced use in the United States in part as a result of the Montreal Protocol, which has provisions to phase out and eliminate the harmful environmental impact of short halogenated alkyl-chain hydrocarbons (United Nations Environment Programme Environmental Effects Assessment Panel, 2016). However, R134a is still very much commercially available as a refrigerant and propellant in air duster canisters. Means of diversion of R134a primarily come from abuse of computer duster sprays and theft from air conditioning units (Caplan *et al*; Garland and Howard, 2010, 2012).

Volatile anesthetics are also subject to abuse. For instance, the volatile anesthetic isoflurane has no industrial or commercial use outside of biomedical sciences or human and veterinary medicine and the populations who abuse it are generally found to belong to these communities. Information regarding abuse rates are not well established as there are restricted mechanisms in place to prevent significant diversion to individuals outside the veterinary, biomedical sciences and medical establishments. However, several cases of volatile anesthetic abuse resulting in death have been documented in the literature (Fry and Fry, 2015; Jacob *et al*, 1989; Kuhlman *et al*, 1993; Levine *et al*, 2007; Pavlic *et al*, 2002; Rosales *et al*, 2007; Spencer *et al*, 1976). Interestingly, with increasing use of near

untraceable cryptocurrency and the rise of encrypted dark net marketplaces such as 'Silk Road 3.0', diversion of restricted anesthetics is becoming increasingly accessible to individuals outside of the medical and scientific communities (Hout and Bingham, 2013).

Among anesthetics, nitrous oxide also sees heavy commercial use as an anti-microbial food propellant in addition to its therapeutic medical use as an anesthetic adjunct in medicine and dentistry (Balster *et al*, 2009; Brouette and Anton, 2001). It is primarily through its wide use in food and in some instances automotive services that allows it to be unregulated, commercially distributed and sold through head shops or major online retail vendors.

Subjective Intoxicating Effects of Inhalants

When inhaled for recreational purposes, toluene and trichloroethane produce a rapid onset of psychoactive effects, including excitation and euphoria, while higher concentrations can cause sedation, fatigue, respiratory depression, coma and death (Cruz and Domínguez, 2011; Diana, 2011; Elkoussi and Bakheet, 2011; Winek *et al*, 1997). Nitrous oxide produces a myriad of psychoactive effects including euphoria, fatigue, numbing sensations, and detachment (Collado *et al*, 2007). When R134a in computer duster or from a refrigerant is inhaled, drug effects include euphoria, dementia, fatigue, aggression, insomnia, slurred speech, depression, and memory deficits (Caplan *et al*; Garland and Howard, 2010, 2012). While access to volatile anesthetics is limited, intentional inhalation of isoflurane produces sedation, ataxia, disruption of one's perception of time and decreased neuronal activation in a brain activation induced task

(Beckman *et al*, 2006; Cheeseman *et al*, 2012; Heinke and Schwarzbauer, 2001; Zacny *et al*, 1996).

Medical Consequences

Gaseous inhalants have the potential to result in asphyxiation due to hypoxia from their displacement of oxygen when they are continuously inhaled. The central nervous system depressant effects of inhalants also decrease self-awareness and motor function, reducing one's ability to discontinue inhalation which can lead to death from sustained hypoxic oxygen concentrations.

Exposures to inhalants have been linked to a number of severe medical conditions including arrhythmias (King *et al*, 1985), myeloneuropathies (Pema *et al*, 1998; Tatum *et al*, 2010), immune dysfunction (Fujimaki *et al*, 2011), as well as abnormal developmental conditions such as fetal solvent syndrome (Bowen and Hannigan, 2006; Bowen, 2011; Hannigan and Bowen, 2010; Jones and Balster, 1998). Inhalant exposure can also result in damage to key enzymes necessary for the production of myelin (Flippo and Holder, 1993; Wu *et al*, 2007) and neurocognitive impairment (Beckley and Woodward, 2013). Chronic nitrous oxide abuse has been associated with vitamin B12 deficiency and myeloneuropathies leading to Pseudo-Guillain-Barre syndrome (Lin *et al*, 2011; Tatum *et al*, 2010).

R134a's significant ability to absorb heat across phase changes makes it an ideal refrigerant. Unfortunately, when used as an abused inhalant from air dust canisters the compressed R134a liquid is discharged directly from the canister producing a rapid

decrease in temperature as it moves from a liquid to a gas, resulting in the possibility of very serious tissue damage. Angioedema and frostbite of respiratory tissue has been observed from repeated inhalation and rapid discharge of air dust canisters (Koehler and Henninger, 2014; Winston *et al*, 2015). Several frostbite cases have been recorded in the literature as well when the users have direct contact of their skin on an opened valve that discharges compressed nitrous oxide as the gas can reach temperatures as low as -55° C (Hwang *et al*, 1996; Svartling *et al*, 1996). Chronic toluene exposure has been shown to produce neurotoxicity as well as detrimental effects on reproductive, pulmonary, renal, cardiovascular, and hepatic organ systems (Bowen and Hannigan, 2006; Gupta *et al*, 2011; Hannigan and Bowen, 2010; Hoet and Lison, 2008; Yücel *et al*, 2008).

Inhalant Pharmacology

Physical Properties

Inhalants are characterized by their low-boiling point, high vapor pressure and high lipophilicity. The lipophilic properties of inhalants facilitate their transfer across the blood-brain barrier where they can exert their influence on protein receptor targets. The high lipophilicity of inhalants engenders their affinity to bind within pockets of transmembrane domain regions of ion channels. In general, most ion channels are highly susceptible to being influenced by inhalants/anesthetics and accumulating evidence suggests it is through interaction in transmembrane domain regions that inhalants are able to exert their neurochemical effects (Brosnan and Pham, 2014; Li *et al*, 2010; Mihic *et al*, 1997; Werner *et al*, 2011). Inhalants vary in their ability to induce conformational changes across either

specific or combinations of specific ion channel receptors and their subtypes (Brosnan and Pham, 2014). Currently, there is strong evidence that many of the neurochemical effects of inhalants are produced through specific receptors or through modulation of combinations of specific receptors with specific importance placed upon gamma amino butyric acid subtype A ($GABA_A$) and N-methyl-D-aspartate receptors (NMDA) (Bale *et al*, 2005; Beckstead *et al*, 2000; Belelli *et al*, 1999; Jurd *et al*, 2003; Lecker *et al*, 2013; Mennerick *et al*, 1998; Zhou *et al*, 2012). In addition numerous behavioral experiments *in vivo* have implicated the $GABA_A$ receptor system as being one of the more important systems that contribute to their subjective effects (Shelton and Nicholson, 2010, 2013).

Toluene

Toluene produces reward-like effects in multiple behavioral assays including conditioned place preference (Gerasimov *et al*, 2003; Lee *et al*, 2006), self-administration (Blokhina *et al*, 2004), and intracranial self-stimulation (Chan *et al*, 2012; Tracy *et al*, 2014, 2015; Yavich and Zvartau, 1994). Within the drug discrimination procedure, toluene's subjective effects can be studied across a wide variety of parameters. Depending on the specific experimental conditions and whether toluene is used as either the training drug or is the drug of substitution for another training compound, toluene has been shown to share discriminative stimulus properties with other inhalants as well as ethanol, dopaminergic, GABAergic, and NMDAergic drugs of abuse (Bowen, 2006; Bowen *et al*, 1999; Rees *et al*, 1987; Shelton and Nicholson, 2013). Neuropharmacologically, toluene has been shown to be a positive allosteric modulator of glycine receptors and to

allosterically enhance the chloride current amplitude and the frequency of inhibitory post-synaptic currents through $GABA_A$ receptors (Beckstead *et al*, 2000; MacIver, 2009). Significant focus has also been given to the non-competitive antagonist effects of toluene on NMDA receptors (Bale *et al*, 2005, 2007; Cruz *et al*, 1998, 2000). Additional ion channels have been shown to be affected by toluene exposure: $5\text{-}HT_3$ (Lopreato *et al*, 2003), nAChR (Bale *et al*, 2002), and purinergic (Woodward *et al*, 2004). When injected locally into the VTA, there is evidence that toluene either directly or indirectly activates dopaminergic projecting neurons that project to the nucleus accumbens(Apawu *et al*, 2015; Riegel *et al*, 2007). However, the toluene binding site of the receptor protein-target that may mediate this effect in the mesolimbic pathway has not been determined. In addition, there is no neuropharmacological evidence of volatile organic solvents or compounds directly activating metabotropic receptors in the absence of ionotropic receptors that are co-expressed in native cell cultures.

Nitrous Oxide

Quantifying the reward-related effects of nitrous oxide in animal models has been difficult due in part to complications of working with a purely gaseous substance. However, there is one example of nitrous oxide self-administration with squirrel monkeys. Given the option to respond for access to 15, 30 and 60 seconds of nitrous oxide, monkeys will self-administer the gas well above air rates (Wood *et al*, 1977). Conversely in rodents, nitrous oxide was poorly self-administered and produced conditioned place aversion (Ramsay *et al*, 2003). In drug discrimination, when exposure to 60% nitrous oxide is

trained as the discriminative stimulus, exposure to toluene shows near-full substitution with lower rates of partial substitution for other abused solvents and anesthetics including ethanol, isoflurane, trichloroethane and methoxyflurane, which would be consistent with the diverse and overlapping pharmacological actions of inhalants (Richardson and Shelton, 2014). Interestingly, the NMDA antagonist (+)-MK-801 significantly reduced the nitrous oxide concentration necessary to fully substitute for a training concentration of 60% nitrous oxide, suggesting that NMDA antagonism plays a substantial role in nitrous oxide's discriminative stimulus effects (Richardson and Shelton, 2015). In contrast, GABAergic specific compounds did not produce significant levels of substitution for nitrous oxide nor did they enhance the ability of lower concentrations of nitrous oxide to substitute for the training concentration of itself.

In *in vitro* expression systems, nitrous oxide exposure non-competitively inhibits NMDA receptors (Jevtović-Todorović *et al*, 1998; Mennerick *et al*, 1998). However, positive modulation of $GABA_A$ receptors is small compared to other volatile anesthetics such as isoflurane (Yamakura and Harris, 2000). Concentrations necessary to induce larger changes in $GABA_A$ receptor function require non-physiologically attainable concentrations of nitrous oxide: (100% or the maximum partial pressure concentration attainable by Henry's Law at 1 atmosphere: 29.2 mM) (Hapfelmeier *et al*, 2000). Lastly, nitrous oxide has also been shown to inhibit nicotinic acetylcholine receptors (Suzuki *et al*, 2003; Yamakura and Harris, 2000), calcium channels (Fan *et al*, 2007), and AMPA receptors (Mennerick *et al*, 1998).

1,1,1-Trichloroethane (TCE)

TCE produces reward-like effects in self-administration (Blokhina *et al*, 2004) and shares some discriminative stimulus properties with GABAergic drugs of abuse as evidenced by pentobarbital, midazolam and diazepam producing high partial substitution in mice trained to discriminate TCE (Shelton and Nicholson, 2012). However, for mice trained to discriminate the NMDA antagonist dizocilpine, TCE failed to produce any substitution (Shelton and Balster, 2004). TCE has been shown to produce positive allosteric modulatory effects on both $GABA_A$ and glycine receptors (Beckstead *et al*, 2000; MacIver, 2009). Like toluene, TCE has non-competitive antagonist effects on the NR1/2B subtype of NMDA receptors (Bale *et al*, 2005, 2007; Cruz *et al*, 1998, 2000) as well as enhancing the function of $5\text{-}HT_3$ (Lopreato *et al*, 2003).

Isoflurane

While there has been relatively little neurobehavioral research characterizing the abuse related effects of isoflurane, its discriminative stimulus properties have been characterized in mice. In mice trained to discriminate 6,000 ppm isoflurane from air, pentobarbital, midazolam and zolpidem produced high levels of substitution indicating a substantial GABAergic component of isoflurane's discriminative stimulus (Shelton and Nicholson, 2010). In substitution tests, the abused inhalants (TCE, toluene) and volatile anesthetics (enflurane, halothane) all produced full substitution for isoflurane, however nitrous oxide and the NMDA antagonist L-701,324 did not substitute at all.

Due in part to isoflurane's continued use as an anesthetic agent in clinical practices and evidence of a more substantial contribution of the $GABA_A$ complex to its mechanism of action (Yamakura and Harris, 2000), there is considerable interest in isolating the binding pockets within the hetereopentameric $GABA_A$ receptor that contribute to its effects. Point mutations in recombinant systems have isolated transmembrane domains within the $\beta_{2/3}$ subunit that confer reduced sensitivity to isoflurane induced potentiation of $GABA_A$ receptors (Rudolph and Möhler, 2004). Dual point mutations within the α_1 subunit (Borghese *et al*, 2006) and α_2 subunit (Sonner *et al*, 2007) at transmembrane domain 2 (S270H, L277A) render the $GABA_A$ receptor substantially less sensitive to isoflurane; However, compared to wild-type mice, mutants showed a lower sensitivity and faster rate of termination of GABAergic signaling. In addition, knock-in mice homozygous for the α_1 mutations showed significantly higher rates of baseline locomotor activity during the light and dark cycles, therefore the authors were unable to exclude the possibility that the mutation affected normal receptor function rather than simply blocking the allosteric site binding pocket. Knock-in mice homozygous for the α_2 mutation showed a small but significant reduction in the loss of righting reflex, suggesting a contribution from this subtype in isoflurane's motor impairing effects. More recently, isoflurane exposure has been found to competitively displace a photolabeled azietomidate (etomidate analog) which binds within the transmembrane domain of the β subunit (Li *et al*, 2010).

1,1,1,2-tetrafluoroethane (R134a)

To date, no electrophysiological experiments have been conducted to determine the functional consequences of R134a exposure at ligand gated ion channels. A search of the PubMed database using the search terms "134a abuse, HFC 134a, R134a, computer duster abuse, or R134a abuse" revealed only one basic sciences study that examined abuse-like behavioral effects of concentrations of inhaled R134a on a series of neurobehavioral measures in rats (Ritchie *et al*, 2001). In this study, Wistar rats were exposed to HFC 134a before and during testing for neurobehavioral complications in a rotawheel apparatus, operant responding for a food reinforcer, seizure induction, and for sedative and anesthetic effects. At abuse-like concentrations of 40,000 to 470,000 ppm, rats showed significantly more errors in performance on an operant task for food maintained responding and gross motor impairment on the rotawheel apparatus. When oxygen was not coadministered with R134a, subjects exhibited generalized tonic-clonic seizures (Ritchie *et al*, 2001).

Intracranial Self-stimulation: Procedure, Strategies, Neurocircuitry, Utility for Inhalant Studies, and Aspects of Test Validity

Strategies for Understanding the Reinforcing Properties of Inhalants

Despite being inexpensive, readily available and having abuse liability, the mechanism or mechanisms underlying the reinforcing effects of inhalants are poorly understood (Bowen and McDonald, 2009; Howard *et al*, 2011). One reason this may be the case is the lack of validated *in vivo* procedures for examining abuse-related effects of

inhaled substances. While the intravenous self-administration procedure is the most widely utilized paradigm to assess drug reinforcement and mechanisms of action, it is difficult to conduct self-administration studies with contingent delivery of inhaled vapors or gases. To date only one study has demonstrated intravenous self-administration of an inhalant and that experiment used a novel between-subject single day acquisition procedure in mice (Blokhina *et al*, 2004).

Intracranial Self-stimulation Procedure

The phenomenon of direct electrical stimulation of the brain producing robust reward-like behavioral effects was serendipitously first discovered in the 1950s with the seminal work of Olds and Milner (Olds and Milner, 1954). The discovery as retold by Milner, recounts that they (Olds and Milner) were interested in studying the brainstem reticular formation via direct electrical stimulation with implanted electrodes (Milner, 1991). In general, stimulation of this region produces aversive effects with locations that are associated with the stimulation being actively avoided. Olds, just having learned the surgical implantation procedure observed that one subject, rather than exhibiting the typical avoidance was showing robust appetitive behaviors and would spend more time in the location where the electrical stimulation was delivered. Further investigation revealed the electrode was incorrectly implanted, and that rather than being in the brainstem reticular formation the stimulating portion of the electrode was found to be in the septal region. Direct electrical stimulation of the septal region was subsequently found to function as a robust reinforcer that could be utilized to train new behaviors such as operant

lever pressing (Milner, 1991). In subsequent studies, electrical stimulation of many brain regions was found to support ICSS behavior including the prefrontal cortex, septal, nucleus accumbens, ventral tegmental area and the medial forebrain bundle (MFB), with the MFB supporting the highest rates of responding (Negus and Miller, 2014).

A novel feature of the brain stimulation reward phenomenon is that it is unlike natural reinforcers such as food or sex, there are no negative-feedback mechanisms in place to satiate the motivated behavior. Indeed, subjects are observed to tirelessly engage in responding for the brief electrical pulses and even endure aversive stimuli in order to maintain continued delivery of the highly efficacious reinforcer (Milner, 1991). In addition, the electrical and temporal parameters that determine the reinforcing magnitude of brain reward stimulation can be altered instantaneously to produce robust patterns of responding across a relatively small interval of time. Certain methodologies have been developed which take advantage of the strength of the behavior such as the threshold and rate-frequency procedures.

The rate-frequency procedure of ICSS (among others) has been utilized as a tool to study a wide spectrum of behavioral and neuropharmacological phenomena. These include: pharmacological mechanisms (Kelley and Hodge, 2003; Markou and Koob, 1992; Reynolds *et al*, 2012; Schaefer and Michael, 1989), abuse-liability assessment (Corbett, 1989; Schaefer and Michael, 1987; Yavich and Zvartau, 1994), state-dependent effects such as depression (Slattery *et al*, 2007), withdrawal (Lin *et al*, 1999; Manbeck *et al*, 2014; Markou and Koob, 1991; Schulteis *et al*, 1995), anxiety and post-traumatic stress disorder (Reznikov *et al*, 2016) or pain-depressed behavior (Negus *et al*, 2010).

Neurocircuitry Involved in ICSS

Critical mesolimbic reward circuits are involved in maintaining ICSS behavior. Multiple electrophysiological collision studies indicate there is strong evidence that ascending unmyelinated dopaminergic neurons in the ventral tegmental area projecting to the nucleus accumbens are the critical last stage pathway in the neurocircuitry of MFB stimulation in ICSS (Yavich and Tiihonen, 2000). Activation of VTA dopaminergic projection neurons by direct electrical stimulation of the MFB involves a multiple stage process with intermediaries that are not entirely understood, but some evidence suggests glutamatergic and cholinergic processes are involved in MFB stimulation (Kempadoo *et al*, 2013; Singh *et al*, 1997). Electrophysiological studies in which collisions between paired pulses between two electrodes that stimulate regions at varying times from the MFB and the VTA suggest that descending myelinated axons are the first stage component to activating VTA dopaminergic neurons (Bielajew and Shizgal, 1980, 1986). More recently, optogenetic studies have implicated descending glutamatergic fibers and neurotensin receptors within the ventral tegmental area as contributing to the first stage of electrical brain stimulation in the MFB at the level of the lateral hypothalamus, though multiple receptor systems may contribute to maintaining ICSS behavior (Kempadoo *et al*, 2013).

Adapting ICSS to Study Inhaled Vapors and Gases

Most classes of abused drugs facilitate ICSS-reinforced behavior (Fish *et al*, 2010; O'Neill and Todtenkopf, 2010; Straub *et al*, 2010). Likewise, the intracranial self-stimulation (ICSS) procedure has been shown to be adaptable to examining the reward-

related effects of inhalants. Two studies in rats conducted a number of years ago demonstrated that, similar to other drugs of abuse, toluene vapor facilitates ICSS responding (Bespalov *et al*, 2003; Yavich and Zvartau, 1994). In both studies toluene vapor exposure significantly enhanced responding for lower stimulation intensities as well as decreased self-stimulation thresholds.

The development of ICSS procedures to examine the reward-related effects of inhalants in mice is of particular interest given the powerful genetic tools available only in mice. Mimicking the route a drug is abused in humans has traditionally been thought to be of limited importance in preclinical studies, and it has been shown that intraperitoneal injected toluene reduces ICSS thresholds in mice in a discrete-trial current-threshold procedure (Chan *et al*, 2012). However, a recent c-Fos expression study in rats demonstrated administration route-dependent differences in neuronal activation produced by toluene (Perit *et al*, 2012). Among the important neuronal systems differentially affected by administration route were areas associated with reward and motor control, with inhaled toluene producing significant increases in c-Fos expression in the nucleus-accumbens core and caudate-putamen relative to the findings of a previous study using injected toluene (Lo and Chen, 2005). While c-Fos is only a proxy for neuronal activation, the significance of route-dependent increases in neuronal activation coupled with the fact that toluene is abused exclusively by inhalation supports studying the reward altering effects of liquid volatile inhalants through their abused inhalation route. Further, the use of inhalation exposure is the only route through which abused gaseous compounds such as nitrous oxide, R134a, R12 or xenon can be evaluated. An additional component that may

differentiate the abuse-related effects of inhalants given by the injection versus inhalation is the rapid kinetics of inhalant uptake and the immediacy of their pharmacological effects that occur following inhalation. The rate of onset of the pharmacological effects for other drugs of abuse, in particular inhalation of smoked crack-cocaine versus insufflated cocaine hydrochloride, strongly contributes to the abuse-liability of the substance (Hatsukami and Fischman, 1996).

Aspects of Test Validity in ICSS

The gold standard in abuse-liability assessment in non-human subjects has been the self-administration procedure given the obvious similarity to human drug taking behavior. In this procedure, animals are placed into an apparatus in which drug can be contingently delivered based upon completion of an operant schedule.

A number of issues arise when attempting to utilize self-administration with inhalants. One is the difficulty with establishing a controlled dose of an inhalant over a short time period. Another is that contingent vapor delivery may have transitory aversive effects that might be very tightly associated with the operant behavior that causes its delivery. For human subjects this has not been an issue and there are numerous examples in the literature where individuals will endure mild to moderate discomfort for the reward-like effects that occur from inhaling compounds that cause brief aversive effects (Hwang *et al*, 1996; Koehler and Henninger, 2014; Praharaj *et al*, 2008; Svartling *et al*, 1996). These aversive effects such as extreme cold from a compressed inhalant undergoing a phase change and vaporizing or the burning sensation of a solvent on sensitive epithelial tissue

can then enter into complex associations where they can come to function as a conditioned reinforcer. However, for most model organisms these effects serve as a strong deterrent for drug consumption and can make an effective design for self-administration very difficult to establish reliably (for an example of this difficulty, see Ramsay *et al*, 2003). Yet, there is a limited set of examples with higher order organisms such as squirrel monkeys where this has been successfully implemented (Wood *et al*, 1977).

At the expense of face validity, which may itself have limited value in preclinical animal models, a number of difficulties associated with self-administration of inhalants can be sidestepped completely in ICSS. A very useful aspect which significantly enhances the utility of ICSS for investigating the effects of inhaled vapors and gases are that their reward-like effects can be examined following non-contingent inhalant delivery. Thus any brief aversive effects can be disentangled from processes that determine reward-like effects. When comparing self-administration to ICSS, ICSS shows considerable convergent and predictive validity with the results of self-administration (SA) studies across many drug classes and types (for examples see table 1).

Table 1

Drug	SA	ICSS
Amphetamine	✓(Alderson *et al*, 2004)	✓(G J Schaefer and Michael, 1988; Todtenkopf *et al*, 2009)
Cocaine	✓(Larson *et al*, 2010)	✓(Straub *et al*, 2010)
Toluene	✓(Blokhina *et al*, 2004)	✓(Bespalov *et al*, 2003; Tracy *et al*, 2014; Yavich and Zvartau, 1994)
Diazepam	✓(Griffiths and Johnson, 2005)	✓(Sagara *et al*, 2008; Straub *et al*, 2010)
Ethanol	✓(June *et al*, 1994)	✓(Fish *et al*, 2010, 2012; Schaefer and Michael, 1987)
Morphine	✓(Gaiardi *et al*, 1985)	✓(Altarifi and Negus, 2011; O'Neill and Todtenkopf, 2010)
MK-801	✓(Beardsley *et al*, 1990)	✓(Hillhouse *et al*, 2014)

Table 1: Compounds which show reward-like effects in both self-administration (SA) and intracranial self-stimulation (ICSS).

Experimental Aims

Aim 1: The first major goal of the project was to determine if the rate-frequency variant of the intracranial self-stimulation procedure in C57BL6/J mice would be sensitive to reinforcement-enhancing effects of R134a, isoflurane, toluene, 1,1,1-trichloroethane, (TCE), and nitrous oxide.

Adult male C57BL/6J mice with bipolar ICSS electrodes chronically implanted into their medial forebrain bundle were trained to respond on a fixed-ratio schedule for stimulation. Once responding stabilized, the reward-altering effects of inhalants were examined systematically by comparing the ICSS frequency-response curves during air and inhalant exposure. Multiple concentrations of each compound were examined and order effects were minimized by using combinations of naïve and previous drug exposed animals (Carlezon and Chartoff, 2007). Inhalants which show reinforcement-enhancing effects in the rate-frequency procedure were also examined using a chained progressive-ratio (PR) schedule, a procedure whereby an exponentially growing number of responses are required for successive completions. This procedure involves generating a baseline level of responding under the chained PR schedule and then comparing baseline responding to responding during acute inhalant exposure. Cocaine and diazepam were utilized as positive controls as they have both been shown to facilitated ICSS (Fish *et al*, 2010; Straub *et al*, 2010).

Aim 2: Determine the role of $GABA_A$ receptors in inhalant-facilitated ICSS

Previous *in vitro* literature has shown that inhalants alter function of a diverse group of neurotransmitter receptors including the $GABA_A$ and NMDA receptors (Bale *et al*, 2007; Beckstead *et al*, 2000; MacIver, 2009). Gamma-aminobutyric acid A ($GABA_A$) neurons comprise ~30-35% of all neurons within the VTA and 90-95% of those in the NAc and GABAergic receptors are one of the major inhibitory contributors involved in modulating the mesolimbic system (Nieh *et al*, 2013; Taylor *et al*, 2014). Optogenetic studies have highlighted the critical role that GABAergic neurons play in modulating abuse-related phenomena. For example, optogenetic activation of GABAergic neurons in the VTA, of which a very large population function as inhibitory interneurons, produces conditioned place aversion as well as disrupts consumption of a sweetened drink reinforcer (Tan *et al*, 2012; van Zessen *et al*, 2012). Activation of GABAergic interneurons promotes release of GABA onto innervated targets producing an inhibitory effect. Similarly, pharmacological modulation of $GABA_A$ receptors has an array of effects in models of abuse-related behaviors such as self-administration, conditioned place preference and intracranial self-stimulation (ICSS) (Bossert and Franklin, 2003; Rowlett and Lelas, 2007; Di Scala *et al*, 1992). Of these behavioral endpoints, ICSS may be a particularly useful assay as it permits the study of drug effects on direct electrical stimulation of critical elements in brain reward pathways (for an extensive review of this procedure see: Negus and Miller, 2014).

Most classes of abused drugs, including those acting as positive allosteric modulators at $GABA_A$ receptors, enhance the reinforcing effects of ICSS (Steffensen *et al*, 2001;

Straub *et al*, 2010). While drugs such as benzodiazepines and barbiturates positively modulate $GABA_A$ receptors it is also possible to negatively modulate $GABA_A$ receptors pharmacologically. High efficacy benzodiazepine (BDZ)-site negative modulators such as the beta-carboline DMCM can produce pronounced behavioral effects including convulsions. Lower efficacy negative modulators, in particular Ro15-4513, are capable of producing pro-seizure effects (Miczek and Weerts, 1987). However, Ro15-4513 can reduce the behavioral effects of $GABA_A$ positive allosteric modulators such as pentobarbital, ethanol and volatile anesthetics (Miller *et al*, 1989; Schaefer and Michael, 1989; Shelton and Grant, 2001) at doses which do not promote seizure activity (Bishop and Laverty, 1989; Moody and Skolnick, 1988; Stinchcomb *et al*, 1989).

With few notable exceptions (Dixon *et al*, 2010), the general focus on the utility of negative $GABA_A$ modulators has been on reversing the behavioral effects of ethanol. For instance, Ro15-4513 reduces ethanol self-administration in both rodents (McBride *et al*, 1988) and primates (Shelton and Grant, 2001). Although use of Ro15-4513 as a therapeutic has been largely discounted, a related compound, Ro16-0154 (iomazenil) has entered clinical trials as a potential alcohol intoxication reversal agent (D'Souza, 2015). Given the key role of $GABA_A$ receptors in modulating the mesolimbic dopamine system and toluene's substantial pharmacological effects upon it, the BDZ-site negative modulators may produce profound reductions in the abuse-related behavioral effects of other drugs that affect the $GABA_A$ receptor but do not act directly at the BDZ site.

The goal of the second aim was to investigate the role of the $GABA_A$ receptor system in inhalant-facilitated ICSS utilizing drugs that either block or negatively modulate

the benzodiazepine-site on the $GABA_A$ receptor. For these studies I utilized the benzodiazepine-site negative modulator RO15-4513 and the benzodiazepine-site antagonist flumazenil. My hypothesis was that benzodiazepine-site negative GABAergic modulation but not benzodiazepine-site blockade would attenuate the ICSS-facilitating effects of toluene. In addition to examining toluene I also assessed the ability of GABAergic modulators to attenuate the effects of ICSS facilitation produced by the dopamine releaser d-methamphetamine which served as a negative control and the $GABA_A$ BDZ-site positive modulator diazepam which served as a positive control. To control for non-specific effects, I also examined the effects of RO15-4513 and flumazenil on ICSS when given alone. In addition to the behavioral tests I employed a microdialysis procedure to determine if Ro15-4513 would attenuate dopamine release in the NAc evoked by d-methamphetamine, a defining neurochemical characteristic of many drugs of abuse.

Aim 3: Determine chronic exposure effects of inhalants on ICSS and nesting

Withdrawal following repeated drug exposure and the state of the adaptations that occur in the brain's reward system following chronic drug use are one component that is critical to understanding drug addiction phenomena, especially the high compulsory relapse rate to drug seeking and continued use despite adverse consequences. Despite the importance of characterizing this phenomenon at the behavioral level, a major gap in the inhalant literature that currently exists is how the effects of chronic inhalant abuse may affect generalized reward processes. One possible reason for a gap in the literature may be

the absence of an established procedure that examines the reinforcing efficacy of a reinforcer across a broad range of discreetly defined and set intensities.

The absence of such a procedure has contributed to experiments that utilize behavioral assays that measure more indirect and tertiary effects of repeated chronic use of toluene such as how it may impact performance in behavioral assays from other drugs of abuse. For example, some studies have focused on how toluene exposure can produce locomotor sensitization effects (Bowen and Balster, 1998; Bowen *et al*, 2007); how repeated toluene exposure may affect the locomotor sensitization effects of other drugs of abuse such as amphetamine, MK-801, or diazepam (Duncan *et al*, 2014; Wiley *et al*, 2003); or how toluene may impact instrumental learning-type phenomenona such as spatial-learning (Dick *et al*, 2014).

A much subtler aspect which has been neglected in the literature is how repeated chronic exposure to toluene may produce cumulative effects on the mesolimbic reward system. For this purpose, ICSS has served as a tool for examining the cumulative production of anhedonic-like effects following repeated chronic intermittent exposure to drugs of abuse as well how cessation of continued drug exposure may manifest itself. For example, it is well established that acute exposure to cocaine will enhance the reinforcing efficacy of brain stimulation as measured by a lowering of the threshold necessary to maintain responding for brain stimulation. However, if cocaine is administered continuously through an infusion pump and is subsequently spontaneously removed, the threshold to maintain responding for brain stimulation will significantly increase to levels which are larger than that of a comparable saline control group, which would be indicative

of a withdrawal effect (Stoker and Markou, 2011). Anhedonic-like withdrawal effects following repeated chronic exposure to drugs of abuse in ICSS procedure have been observed across a number of different drug classes including ethanol (Schulteis *et al*, 1995), morphine (Holtz *et al*, 2015), nicotine (Muelken *et al*, 2015), amphetamine (Lin *et al*, 1999), phencyclidine (Spielewoy and Markou, 2003), and in at least one case THC (Gardner and Vorel, 1998).

Given the importance of understanding how repeated chronic exposure effects of inhalants impacts brain reward processes and the absence of behavioral studies that attempt to assess this at the behavioral level, my third aim examines chronic intermittent exposure effects on mouse behaviors in ICSS. Using the ICSS procedure to explore generalized effects on brain-reward systems *in vivo* across multiple parameters as a result of chronic toluene exposure had many possible avenues to explore. However, I also wanted to explore behaviors in the mouse that was more innate and non-operant. To this end, I also examined nesting behavior. Nesting is an innate behavior engaged in by mice in which they scavenge for materials to construct a nest. An aversive state such as pain has been observed to decrease the rate at which mice will gather materials to construct a nest, and this can be quantified by a procedure that has been developed by the Negus group (Negus *et al*, 2015). In this procedure nesting material is divided into six equal sized segments and placed along equal distances apart along the edge of the subject's home cage and the time to gather all these nesting materials into one place is then quantified on a logarithmic time scale. Subjects which have an induced injury are shown to decrease the rate at which they complete the task and this can be reversed with analgesics such as ketoprofen (Negus *et al*,

2015). While a withdrawal state has not been specifically examined in this procedure, it is a reasonable conjecture that this procedure would be sensitive to aversive states other than pain, such as withdrawal from chronic toluene exposure.

I have four main hypotheses to explore in this aim. My first hypothesis is that escalating the number of repeated chronic intermittent exposures to toluene will produce a cumulative decrease in overall responding for brain stimulation while in a drug free state. My second hypothesis is that gradually escalating the number of exposures to toluene will produce tolerance to the acute exposure effects in ICSS. My third hypothesis is that there would be a gradual decrement in the amount of brain stimulation that would be earned in a drug free state when subjects were exposed to an escalating chronic intermittent toluene exposure schedule. My fourth hypothesis is that repeated chronic intermittent exposure to toluene will produce gradual deficits in behaviors other than ICSS as measured in the nesting procedure.

To address the first, second and third hypotheses, I chronically exposed groups of C57BL/6J mice to either air or different repeated chronic intermittent exposure schedules of 3300 ppm toluene. The air control group was only exposed to air within the ICSS test sessions. The repeated chronic intermittent exposure group was exposed to 3300 ppm toluene every 72 hours within the test ICSS sessions. The chronic escalating exposure group was exposed every 72 hours to 3300 ppm toluene in the ICSS test session and in addition was exposed to 3300 ppm toluene on an escalating exposure schedule outside of the chambers. These three experimental groups will address these hypotheses by examining how chronic intermittent toluene exposure affects the reinforcing efficacy of

direct electrical stimulation of the mesolimbic reward pathway; by characterizing the facilitation of ICSS by toluene exposure and whether repeated exposures produce tolerance, sensitization, or produce no effect when comparing groups that are either repeatedly exposed to toluene on an escalating or fixed (72 hour separation) chronic intermittent paradigm. It will also examine whether repeated chronic intermittent exposure can produce withdrawal effects in ICSS following cessation of chronic toluene exposure. To address the fourth hypothesis, the air control and escalating chronic intermittent exposure group schedules from the ICSS experiments were replicated using the nesting procedure.

Aim 1: Acute Exposure Effects of Inhalants in ICSS

Materials and Methods

Subjects

A total of 37 adult C57BL/6J mice were used (Jackson Laboratory, Bar Harbor, Maine). Mice were individually housed under a 12h light/dark cycle (lights on a 0600). Mice were tested M-F between 0800 and 1800. Chow (Harlan, Madison, WI) and water were available *ad libitum* except during experimental sessions. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and were in accordance with the NIH "Guide for the Care and Use of Laboratory Animals: Eighth edition" (National Research Council, 2011).

Compounds.

HPLC grade toluene, isoflurane and trichloroethane (TCE) were purchased from Fisher Scientific. R134a was purchased from an office supply company in pressurized canisters sold as commercial air duster. Cocaine hydrochloride was obtained from the National Institute on Drug Abuse drug supply program and prepared in 0.9% sterile saline. Diazepam was obtained in an injectable commercial formulation and diluted in 0.9% sterile saline. Medical grade nitrous oxide and oxygen cylinders were purchased from National Welders (Richmond, VA).

Surgical Procedure

Mice were anesthetized with isoflurane vapor and implanted with 6 mm bipolar electrodes (Plastics One, Roanoke, Virginia) into the right medial forebrain bundle within the lateral hypothalamus (Paxinos and Franklin, 2001). Coordinates relative to Bregma

were: -1.5 anterior-posterior, 1.0 medial-lateral, and -5.0 dorsal-ventral. Three stainless steel screws were affixed in holes in the skull to serve as anchors for a dental acrylic headpiece. Mice were treated daily for 3 days post-surgery with 0.5 mg/kg of the analgesic carprofen. Mice were allowed six days to recover prior to beginning ICSS training sessions.

Dynamic Exposure System and Testing Apparatus

The inhalant exposure ICSS system consisted of four 20 liter acrylic cubicles each of which contained a two-lever operant conditioning chamber (Med-Associates, St. Albans, VT). A bipolar lead tether connection (Plastics One, Roanoke, VA) and mercury commutator (Mercotac 205L, Carlsbad CA) was suspended above the operant conditioning chamber by a counterbalanced arm. Toluene and TCE vapor were produced using a dynamic vapor generator composed of a filtered, pressure regulated air supply routed via tubing to two mass flow proportional valves controlled by a Matheson 8284 dynamic gas mixer (Matheson, Albuquerque, NM). The air output from one valve passed through a fritted glass bubbler submerged below the liquid inhalant, generating an inhalant-laden air stream. The inhalant-laden air was mixed with metered fresh air from the second mass control valve permitting a broad range of highly reproducible inhalant concentrations to be generated. The blended vapor stream was routed through the upper rear wall of the operant conditioning chambers via Tygon tubing. The effluent mixture was vented from the bottom rear wall of the exposure chamber through a Miran 1A single wavelength infrared spectrophotometer coupled to a computerized chart recorder (DATAQ, Akron, OH), providing real time inhalant concentration measurement relative to a previously generated

standard curve. Inhalant vapor concentrations within each ICSS chamber were also quantified by headspace gas chromatography to determine exact concentrations. To deliver nitrous oxide, the system was similar except the inhalant and mix gas sources were provided by compressed cylinders. Isoflurane was delivered via a commercially available anesthesia machine that had been previously calibrated. R134a was delivered by routing multiple computer duster cans through piping to a single source pressure regulator and then to a Matheson rotameter. ICSS stimulation and operant schedule control was provided by commercially-available components (Med-Associates, St. Albans, VT). A summary schematic of the operant conditioning chamber housed within the gas exposure system with the accompanying effluent mixture piping and delivery is shown in figure 1a.

Rate Frequency ICSS Procedure

After surgical recovery mice were trained to respond in daily 1 hr. sessions for brain stimulation under a fixed ratio 1 (FR1) schedule of reinforcement. Each active lever-press resulted in electrical stimulation consisting of a 500 ms, 158 Hz pulse train. Current amplitude for each subject was adjusted within a range of 50 to 300 uA in order to generate maximal rates of responding. When responding stabilized under these conditions the mice progressed to the three component sessions used for rate-frequency testing. Training and test sessions were 70 minutes in duration, divided into three 10-min response components separated by two 20-min timeouts. The timeout periods served as pretreatment windows prior to the second and third ICSS response components. Vehicle or air was administered prior to the second component during the first 20-min timeout. Drugs were injected or inhalant exposures initiated during the second 20-min timeout. In this manner baseline

control ICSS data (second response component) as well as test data (third response component) could be generated within the same subject on the same test day. Treatment exposures of inhalants initiated at the start of the second timeout continued through the third response component resulting in 30 minutes of total inhalant exposure. Response components were signaled by the illumination of the chamber house light. During initial training, each active lever press during the 10-min response components delivered a 500 ms pulse train of pulses at a frequency of 158 Hz. After responding under the multiple component schedule stabilized animals were transitioned to sessions in which the frequency available for self-stimulation began at 158 Hz and decreased each min thereafter by 0.05 log units ending at 56 Hz on the last min of each component. After behavior again stabilized, as defined by consistent response rates ≥30 responses per minute and lever pressing for at least four self-stimulation frequencies of the rate-frequency curve, testing began. At any point in the progression of training, animals which failed to respond reliably were removed from the study and replaced.

Toluene concentrations of 480, 1360, 2900, 3300 and 5000 parts-per-million (PPM) were examined. Nitrous oxide concentrations of 5, 20, 40, 60 and 80% combined with oxygen were also examined in an identical exposure design. Trichloroethane was tested at doses of 500, 1000 and 4000 PPM. R134a was tested at concentrations of 2.5, 5, 10 and 20%. Isoflurane was tested at 0.25, 0.5, 1 and 2%. Due to the rapid onset of pronounced effects of R134a and isoflurane, a 5-minute pretreatment window was utilized. When spectrophotometric methods were unavailable to measure inhalant concentration within the chambers, approximate concentrations of inhalant within the chambers were derived using

the equation $C = C_0 (1\text{-}e^{(-Q/V*t)})$, where C_0 is final concentration, Q is he flow rate of the mixed gas, V is the volume of the chamber, and C is the current concentration as a function of time (Figure 1b).

Sessions in which injected drugs were given were identical to inhalant test sessions with the exception that following the second response component the mice were given an IP injection of drug. Cocaine was tested at doses of 3, 10, and 18 mg/kg and diazepam was tested at doses of 0.3, 1, 3, 6 and 10 mg/kg. Inhalant concentrations and test drug doses were generally tested in an ascending order. In mice which advanced most rapidly through testing it was sometimes necessary to then test lower doses/concentrations to adequately characterize the full dose/concentration-effect curve. The ascending dose series was then updated for subsequent subjects. Upon completion of a dose/concentration-effect curve, subjects were assigned to the next planned experimental condition requiring additional subjects. Drug naïve subjects which replaced those animals lost to electrode failures or other causes were counterbalanced across test conditions with subjects with previous drug exposure to ensure that individual conditions contained similar numbers of naïve and experienced subjects.

Progressive Ratio ICSS Procedure

Following completion of rate frequency testing, drugs and inhalants that showed facilitation procedure were then transitioned to a chain PR/FR schedule (Depoortere R *et al*, 1999). Under the PR/FR schedule, the number of lever-presses required to increment the schedule was increased after each completed component. The PR step-size was increased according to an exponential step size function ($5* e^{(trial\# * 0.1)} - 5$) (Sharma *et al*,

2012). After each PR was completed the animal received five, 500 ms ICSS pulse trains over five seconds and then entered a second component in which the next ten FR1 responses were each reinforced by one 500 ms ICSS pulse train. Following the completion of the FR1 component the PR value incremented to the next higher ratio value and the PR/FR sequence repeated until a PR requirement was not completed within 6 min, terminating the session. During PR/FR training and testing the ICSS frequency was fixed at 158 Hz and the current amplitude for each mouse was the same as that used in the rate frequency procedure. Concentrations of 100, 480, 1360, and 2900 ppm toluene and 20, 40 and 80% nitrous oxide combined with oxygen were administered 20 min prior to and for the duration of the PR/FR session. Diazepam was tested at doses of 0.3, 1, 3, and 6 mg/kg administered 15 min prior to the start of the PR/FR session. Cocaine was tested at doses of 3, 10 and 18 mg/kg administered 5 min prior to the start of the PR/FR session.

Quantification of Exposure Concentrations and Inhalant Blood Levels:

Blood toluene concentrations were determined by submandibular blood sampling following 20 min of exposure to 480, 1360, 3300, and 5000 ppm toluene and after 30 minutes' exposure to 3300 ppm. The headspace gas chromatography analytical methods were similar to those previously reported (Shelton and Nicholson, 2010, 2012; Shelton, 2009). A mixture of naïve mice and mice that failed to reach criteria to be useful in the rate-frequency procedure were repurposed for the blood toluene concentration sampling from the dynamic chamber exposure system. For each subject, two to three blood sample replicates were utilized and multiple subjects were used for each concentration. Toluene

exposure conditions prior to the blood sampling were identical to the dynamic exposure ICSS session except that subjects did not engage in ICSS behavior and were confined to the operant chamber. Following the set amount of time in the dynamic chamber exposure each subject was tightly restrained and approximately 0.1 mL of blood was obtained utilizing the submandibular vascular bundle via a 5 mm lancet. Subject's blood droplets were quickly captured in a micro collection tube containing EDTA (BD lavender top Microtiter). Following capture of the blood collected sample, the subject was isolated and pressure was applied to the incision to stop further blood loss. The EDTA tube was then briefly agitated and a 20 ul blood sample was then removed and placed into a 20 ml headspace vial to which 960 ul of type 1 ultrapure water and 20 ul of o-xylene internal standard had been previously added. The blood samples were then immediately tested for toluene concentration using a Hewlett Packard model 5890A gas chromatograph (GC) equipped with a flame ionization detector, 2.5 meter 10% TCEP 100/120 Chromosorb PAW column (Restek, Bellefonte, PA) and CTC Combi-Pal headspace auto sampler. The GC parameters were: 5 min sample incubation at 90C, headspace sample volume 1.25 ml, 7 min sample run time, injector temp 200C, oven temp isothermal 110C, detector temp 200C, helium carrier gas flow rate 30 ml/min, FID hydrogen flame flow rate 25 ml/min and FID air flow rate 400 ml/min. Data were collected and analyzed by Clarity GC software (Apex data systems, Prague, CZ) using a linear regression analysis with no weighting. A 7-point calibration curve preceded the analysis of blood samples and quality control toluene standards were interspersed with each set of blood samples. Blood toluene concentrations were calculated by the internal standard method. Up to 3 replicates were

analyzed from each animal and averaged if sufficient blood was collected. Each blood concentration data point represents a mean (±SEM) toluene blood concentration (ug/ml) generated from at least 3 mice.

Data Analysis

In the three component procedure, data from the first 10-min response component was discarded as it has been shown to exhibit more variability compared to subsequent components (Negus and Miller, 2014). Stimulations earned during each 1-min trial of the second 10-min air/vehicle exposure control component were compared to the equivalent trial in the third 10-min drug test component. For each mouse, data from each trial were expressed as a percentage of the number of stimulations in the 1-min trial with the greatest number of responses, regardless of frequency, in the second baseline component. The values are reported as percentage of the maximum control responses (%MCR). This data normalization procedure allowed grouping of the data across subjects despite differences in individual maximum response rates. Data from the second control and third test component were compared individually for each drug dose or inhalant concentration by two-way within subject repeated measures analysis of variance (ANOVA). GraphPad version 7.0 for Windows (La Jolla, CA) was used for all analyses. Significant ($p < 0.05$) main effects and interactions were subsequently examined by the Two-stage step-up method of Benjamini, Krieger and Yekutieli to compare identical frequencies across the control and test components (Benjamini *et al*, 2006). This procedure for correcting for the multiple comparisons problem is more powerful than Bonferroni type corrections that provide for a

strong correction for the overall family-wise error rate (Glickman *et al*, 2014). This procedure gains power by assuming that either all comparisons that are being made either have a positive-dependence on each other (i.e. comparison of points along parallel curves) or that each comparison is independent of every other comparison. It does this by a two stage procedure that first controls for the false discovery rate of the hypotheses being tested by examining the overall P values of the comparisons being made in order to estimate the proportion of the null hypotheses that are actually true. The algorithm then uses the proportion of null hypotheses that are actually true to set the cutoff limit for when a P value is sufficiently low to be called a true discovery.

Rate-frequency data were also analyzed to calculate M50 values for the baseline and treatment conditions in the rate frequency curve to collapse data for summary statistics. If mean response rates did not differ across subjects within a dose-response curve an average was utilized to form a common reference point for statistical comparison. M50 values represent the interpolated frequency that maintains 50% maximum response rates. The estimated linear portion of the rate-frequency curve from 20%-80% of %MCR was analyzed for each subject using a least-squares regression line of best fit to estimate M50. Group treatment condition M50s were then compared by student's paired t-test to group vehicle control baseline to determine whether the %M50 shift was significant ($p < 0.05$).

To further elucidate the generality of procedures that can produce significant facilitation, compounds which showed significant facilitation in the rate frequency procedure were then also tested in the progressive ratio procedure (note: further testing with TCE was not possible due to production and importation bans in the United

States). In this procedure test condition breakpoint data were normalized by expressing them as a change in breakpoint from each subject's baseline control breakpoint. The average breakpoint of two vehicle (air or saline) test sessions was defined as the baseline breakpoint. Change in breakpoint resulting from drug exposure was calculated by subtracting the baseline breakpoint from the test breakpoint for each subject. Group mean changes in breakpoints were then compared across drug or inhalant doses by a one-way within subject repeated measures ANOVA. Significant ($p < 0.05$) main effects were examined by Holm-Sidak post-hoc tests comparing changes in breakpoint for drug and inhalant exposure conditions to either air or saline.

One of the major goals of my thesis was to develop and characterize the ICSS procedure as a method to assess the reward-related effects of inhalational delivery of abused inhalants in mice. To confirm that the ICSS procedure I adapted from rats was sensitive to assessing reward-related effect in mice I initially examined cocaine which has been established to produce robust facilitation of ICSS. I then examined the effects of toluene, nitrous oxide, isoflurane, R134a, and trichloroethane on ICSS to determine the sensitivity of the procedure to detect the reward-related effects of a diverse group of inhalants. Further I wanted to compare the effects of the inhalants to a more pharmacologically understood abused drug with some known similarities in their pharmacological effects. Given that toluene, trichloroethane, isoflurane and nitrous oxide have all been shown to act with varying specificity as positive $GABA_A$ modulators *in vitro* and have similar anxiolytic effects *in vivo* I chose to examine diazepam as a reference

compound (Bowen *et al*, 1996; Cruz *et al*, 1998; Emmanouil *et al*, 1994; Hapfelmeier *et al*, 2000). The data from each of these studies are presented in the subsequent sections.

Results

Rate Frequency Procedure

Doses of 3, 10 and 18 mg/kg cocaine significantly facilitated ICSS (Figure 2a-c). There was a significant main effect of 3 mg/kg cocaine [F (1,10) = 15.68, p = 0.0027] (Figure 2a) on ICSS responding but no drug x frequency interaction [F (1,10) = 1.121, p = 0.3561], but was significantly facilitated at 9 of 10 frequencies. There was a significant main effect [F (1, 10) = 34.05, p = 0.0002)] and a significant drug x frequency interaction [F (9, 90) = 2.480, p = 0.0141] of 10 mg/kg cocaine on %MCR (Figure 2b) with responding at 8 frequencies significantly increased over control. There was a significant main effect [F (1, 10) = 28.09, p = 0.0003] and a significant drug x frequency interaction [F (9, 90) = 4.278, p = 0.0001] of 18 mg/kg cocaine on %MCR (Figure 2c) with the 7 lowest frequencies showing significantly higher rates of responding compared to control. Cocaine doses of 3 mg/kg [t (10) = 2.961, p = 0.0143] and 10 mg/kg [t (10) = 3.101, p = 0.0112] significantly lowered the M50 (table 2). A M50 value for 18 mg/kg cocaine could not be calculated as responding was too robustly increased across the ICSS frequency curve and responding never dropped below 50% of MCR to permit the analysis.

In the rate frequency procedure 0.3 mg/kg diazepam failed to significantly alter ICSS (Figure 3a). 1 and 3 mg/kg diazepam significantly facilitated ICSS (Figure 3b-c). 6 and 10 mg/kg significantly depressed ICSS responding (Figure 3d-e). There was a

significant main effect of 1 mg/kg diazepam [F (1, 8) = 5.478, p = 0.0474] (Figure 3b) but no drug x frequency interaction [F (9, 72) = 1.374, p = 0.2160] with 6 frequencies significantly elevated over baseline. There was a significant drug x frequency interaction [F (9, 72) = 3.473, p = 0.0013] of 3 mg/kg diazepam on %MCR (Figure 3c) with responding at 6 of the lowest frequencies significantly increased over control. There was a significant drug x frequency interaction of 6 mg/kg diazepam on %MCR [F (9, 72) = 4.139, P = 0.0003] (Figure 3d) with the highest frequency showing a significant decrease of ICSS responding compared to control. There was a significant drug x frequency interaction of 10 mg/kg diazepam on %MCR [F (9, 72) = 2.624, P = 0.0111] (Figure 3e) with the 2 highest frequencies showing a significant decrease of ICSS responding compared to control. Diazepam doses of 1 mg/kg [t (8) = 4.314, p = 0.0026] and 3 mg/kg [t (8) = 3.406, p = 0.0093] also significantly lowered M50 values (table 2).

480 ppm toluene (Figure 4a) failed to significantly facilitate ICSS while concentrations of 1360-5000 ppm toluene (Figure 4b-f) all produced significant effects on ICSS. There was a significant main effect (F (1, 10) = 16.51, p = 0.0023) and significant interaction [F (9, 90) = 7.134, p < 0.0001] of 1360 ppm toluene on ICSS with 8 of 10 frequencies showing significantly greater %MCR (Figure 4b) compared to control. There was significant interaction [F (9, 90) = 2.455, p = 0.0151] of 2000 ppm toluene on ICSS with 9 of 10 frequencies showing significantly greater %MCR (Figure 4c) compared to control.

There was a significant interaction of 2900 ppm toluene [F (9, 90) = 5.200, p < 0.0001] on %MCR (Figure 4d) with 3 of the lowest frequencies showing a significantly

enhancement of ICSS responding compared to control. There was a significant interaction of 3300 ppm toluene [F (9, 90) = 7.136, $p < 0.0001$] on %MCR (Figure 4e) on ICSS with the two highest frequencies demonstrating a significant depression and the two lowest frequencies a significant enhancement of ICSS responding compared to control. The 5000 ppm toluene concentration produced a significant interaction [F (9, 90) = 8.499, $p < 0.0001$] on %MCR (Figure 4f) with 9 of 10 frequencies exhibiting a significant suppression of ICSS responding compared to control. Toluene concentrations of 1360 ppm [t (10) = 4.946, p = 0.0006] (table 2) and 2000 ppm significantly decreased the M50 [t (10) = 3.652, p = 0.0045] (table 2).

Concentrations of 5 and 20% nitrous oxide failed to significantly alter responding for ICSS (Figures 5a-b). Nitrous oxide concentrations of 40, 60 and 80% all significantly altered ICSS responding (Figures 5c-e). There was a significant interaction [F (9, 54) = 2.400, p = 0.0228] of 40% nitrous oxide on ICSS with 2 of 10 frequencies showing significantly greater %MCR than control (Figure 5c). The 60% nitrous oxide concentration produced a significant interaction [F (9, 54) = 3.101, p = 0.0045] with the highest frequency showing a significantly lower %MCR than control (Figure 5d). The highest, 80% nitrous oxide concentration produced a significant interaction [F (9, 54) = 9.944, $p < 0.0001$] on %MCR (Figure 5e) with 5 of 10 frequencies showing suppressed rates of ICSS responding compared to control. The 40% nitrous oxide also significantly lowered the M50 [t (6) = 3.771, p = 0.0093] (table 2).

Isoflurane concentrations of 0.25, 0.5, and 1% failed to significantly alter ICSS responding (Figures 6a-c). Isoflurane at 2% significantly decreased responding for ICSS

(Figure 6d). There was a significant interaction [F (9, 36) = 4.277, p = 0.0008] of 2% isoflurane on ICSS with 6 of 10 frequencies showing significantly depressed %MCR than control (Figure 6d). Isoflurane did not shift M50s (table 2).

A R134a concentration of 2.5% failed to alter ICSS responding (Figure 7a). Concentrations of 5, 10 and 20% R134a significantly and concentration-dependently depressed ICSS responding (Figures 7b-d). There was a significant main effect [F (1, 6) = 17.74, p = 0.0056] of 5% R134a on ICSS with 2 of 10 frequencies showing significantly depressed %MCR compared to control (Figure 7b). There was a significant main effect [F (1, 6) = 13.3, p = 0.0107] of 10% R134a on ICSS with 1 of 10 frequencies showing significantly depressed %MCR compared to control (Figure 7c). The 20% R134a concentration produced a significant interaction [F (9, 54) = 12.06, p < 0.0001] on %MCR (Figure 7d) with 7 of 10 frequencies showing suppressed rates of ICSS responding compared to control. R134a did not shift M50s (table 2).

TCE concentrations of 500 and 1000 PPM failed to alter ICSS responding (Figures 8a-b). A concentration of 4000 PPM TCE produced a significant interaction [F (9, 27) = 3.171, p < 0.0001] on %MCR (Figure 8c) with 5 of 10 frequencies showing facilitation of ICSS responding compared to control. The 4000 PPM TCE also significantly lowered the M50 [t (6) = 2.691, p = 0.0360] (table 2).

Progressive Ratio Procedure

Cocaine significantly increased progressive-ratio breakpoint [F (1.280, 7.681) = 9.534, p= *0.0126*] (Figure 9a). Doses of 10 and 18 mg/kg cocaine significantly (p < 0.05) increased breakpoint compared to saline control. Diazepam significantly increased [F

(2.657, 18.60) = 4.447, p = 0.0189] progressive-ratio breakpoint (Figure 9b). The 3 mg/kg diazepam dose significantly increased breakpoint compared to saline control. Toluene (Figure 9c) also significantly increased breakpoint [F (2.462, 17.23) = 7.964, p = 0.0023]. The 1360 ppm toluene concentration significantly increased breakpoint compared to air control. Nitrous oxide (Figure 10d) significantly decreased ICSS breakpoints [F (1.719, 10.32) = 5.400, p = 0.0282]. Breakpoint at both the 40% and 80% nitrous oxide concentrations significantly ($p < 0.05$) were significantly lower than air control.

Inhalant Blood Levels of Toluene

There was a linear relationship between toluene vapor exposure concentration and toluene blood concentration (Figure 10). The lowest exposure concentration of 480 ppm toluene produced a mean blood concentration of 3.7 ug/ml after 20 min of exposure increasing to 61.2 ug/ml at the highest, 5000 ppm toluene exposure concentration. To determine the extent to which toluene blood concentration may have increased during the third ICSS response component I also examined toluene blood levels after 30 min of exposure to the intermediate concentration of 3300 ppm. Exposure to 3300 ppm toluene for 30 min resulted in about ~15% increase in blood toluene concentration compared to exposure for 20 min (Figure 10a).

Effect of duration of toluene exposure on magnitude of ICSS facilitation

In the prior studies, a toluene pretreatment exposure time of 20 min prior to the start of ICSS testing was utilized to maintain some consistency with prior toluene drug discrimination studies in the laboratory (Shelton and Nicholson, 2013; Shelton, 2007). However, it was possible that pretreatment time may have influenced the magnitude of the

facilitation produced by toluene. Therefore, I conducted an additional study utilizing pretreatment durations of 5 and 15 min to explore the relationship between toluene pretreatment time and the start of the ICSS test session to explore the relationship on toluene's ability to facilitate ICSS relative to the longer duration exposures of 20 min. Five minutes of exposure to 1000 ppm toluene (Figure 10b a) failed to significantly facilitate ICSS when it was administered 5 min prior to and throughout the ICSS test session. The main effect of treatment was [F (1, 6) = 1.166, P=0.3217] and the interaction was [F (9, 54) = 1.367, P=0.2261]. Fifteen minutes of exposure to 1000 ppm toluene (Figure 10b b) also failed to significantly facilitate ICSS when it was administered 15 min prior to and throughout the ICSS test session. The main effect of treatment was [F (1, 6) = 0.4595, P=0.5231] and the interaction was [F (9, 54) = 1.339, P=0.2391].

There was a significant main effect (F (1, 6) = 32.02, p = 0.0013) and interaction [F (9, 54) = 6.904, $p < 0.0001$] of five minutes of exposure to 3300 ppm toluene on ICSS with 8 of 10 frequencies showing significantly greater %MCR (Figure 10b c) compared to control. There was a significant main effect (F (1, 6) = 7.532, p = 0.0335) and interaction [F (9, 54) = 4.933, $p < 0.0001$] of 15 minutes of exposure 3300 ppm toluene on ICSS with 7 of 10 frequencies showing significantly greater %MCR (Figure 10b d) compared to control and the highest frequency of 156 Hz showing significantly lower %MCR than control.

Five minutes of exposure to 5600 ppm toluene (Figure 10b e) failed to significantly facilitate or depress ICSS. There was no main effect [F (1, 6) = 2.338, P=0.1771] or interaction [F (9, 54) = 0.6231, P=0.7721]. Fifteen minutes of exposure to 5600 ppm

toluene significantly affected responding for ICSS. There was a significant main effect (F (1, 6) = 12.64, p = 0.0120) and significant interaction [F (9, 90) = 3.519, p = 0.0017] of 5600 ppm on %MCR (Figure 4d) with the 4 highest frequencies demonstrating a significant depression compared to control.

Table 2

Drug	Dose/Conc.	%M50 Shift (±SEM)			%Max Rate (±SEM)		
Diazepam	0.3 mg/kg		6.0	7.5		88.9	9.2
	1 mg/kg	↓	-18.9	4.0		117.9	8.8
	3 mg/kg	↓	-19.9	5.4		110.8	8.6
	6 mg/kg		-9.3	7.6		93.9	12.5
	10 mg/kg		NC			47.0	20.9
Cocaine	3 mg/kg	↓	-10.2	3.4	↑	110.9	4.2
	10 mg/kg	↓	-19.3	5.3		106.0	5.9
	18 mg/kg		NC		↑	122.3	9.0
Nitrous Oxide	5%		2.7	9.4		102.8	7.3
	20%		1.6	4.3		101.2	5.8
	40%	↓	-16.0	4.2		94.7	4.6
	60%		-4.4	6.9	↓	82.4	3.4
	80%		NC		↓	71.7	4.1
Toluene	480 PPM		-4.6	5.2	↑	107.3	5.2
	1360 PPM	↓	-26.7	6.0		99.6	5.3
	2000 PPM	↓	-21.6	5.7		97.5	5.7
	2900 PPM		-11.9	8.6		88.4	4.9
	3300 PPM		NC		↓	79.9	7.2
	5000 PPM		NC		↓	42.4	7.2
R134a	2.5%		3.2	1.3		99	5.3
	5%		7.6	1.4		84	4.4
	10%		8.6	1.5		92.1	2.5
	20%		NC		↓	18.8	12.6
Isoflurane	0.25%		4.2	4.6		100.3	5.4
	0.5%		-0.2	4.3		100.7	4.1
	1.0%		-0.6	8.6		100.2	9.8
	2.0%		NC		↓	49.0	13.9
Trichloroethane	500 PPM		1.8	5.9		104.3	11.8
	1000 PPM		-7.1	5.7	↑	125.3	9.4
	4000 PPM	↓	-26.4	12.6		100.5	6.0

Table 2: Percentage M50 shift and percentage maximum rate of baseline for Diazepam (n=9), Cocaine (n=11), Nitrous Oxide (n=7), Toluene (n=11), R13a (n=7). Isoflurane (n=5) and

Trichloroethane (n=4). ↑↓indicate direction of significance from vehicle baseline ($p < 0.05$). NC, not calculated.

Figure 1a

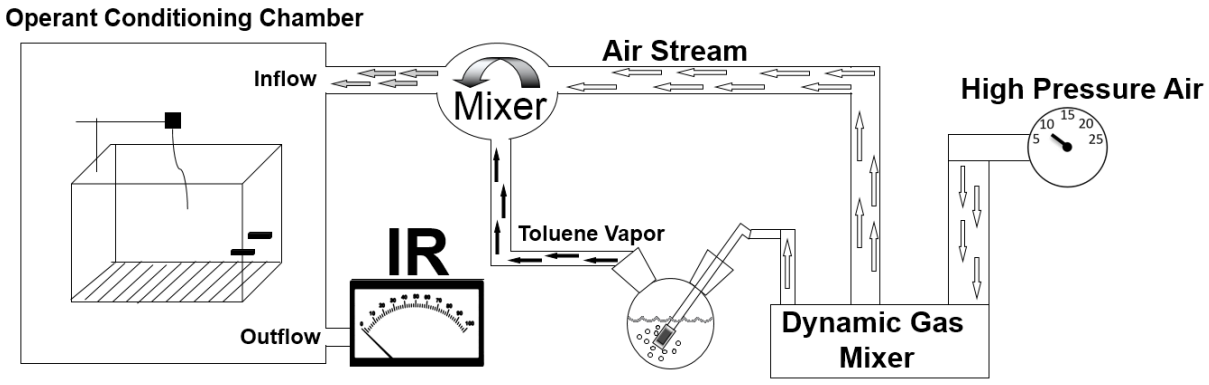

Figure 1a: Schematic representation of the experimental apparatus constructed to expose mice to toluene vapor. A dynamic gas mixer controlled the flow rate of compressed air directed through a 1L glass bubbler container partially filled with liquid toluene. The toluene vapor laden air stream thus generated was then proportionally mixed with a clean air stream before being directed into a 20L exposure cubicle housing an operant conditioning chamber. Effluent from the exposure chamber was routed through an IR spectrometer connected to a computerized chart recorder to monitor real-time exposure chamber concentrations. N_2O and R134a delivery were identical except that nitrous oxide gas or R134a and medical oxygen were delivered from compressed cylinders and the bubbler was not utilized. Isoflurane was delivered via an aesthetic gas vapor machine.

Figure 1b

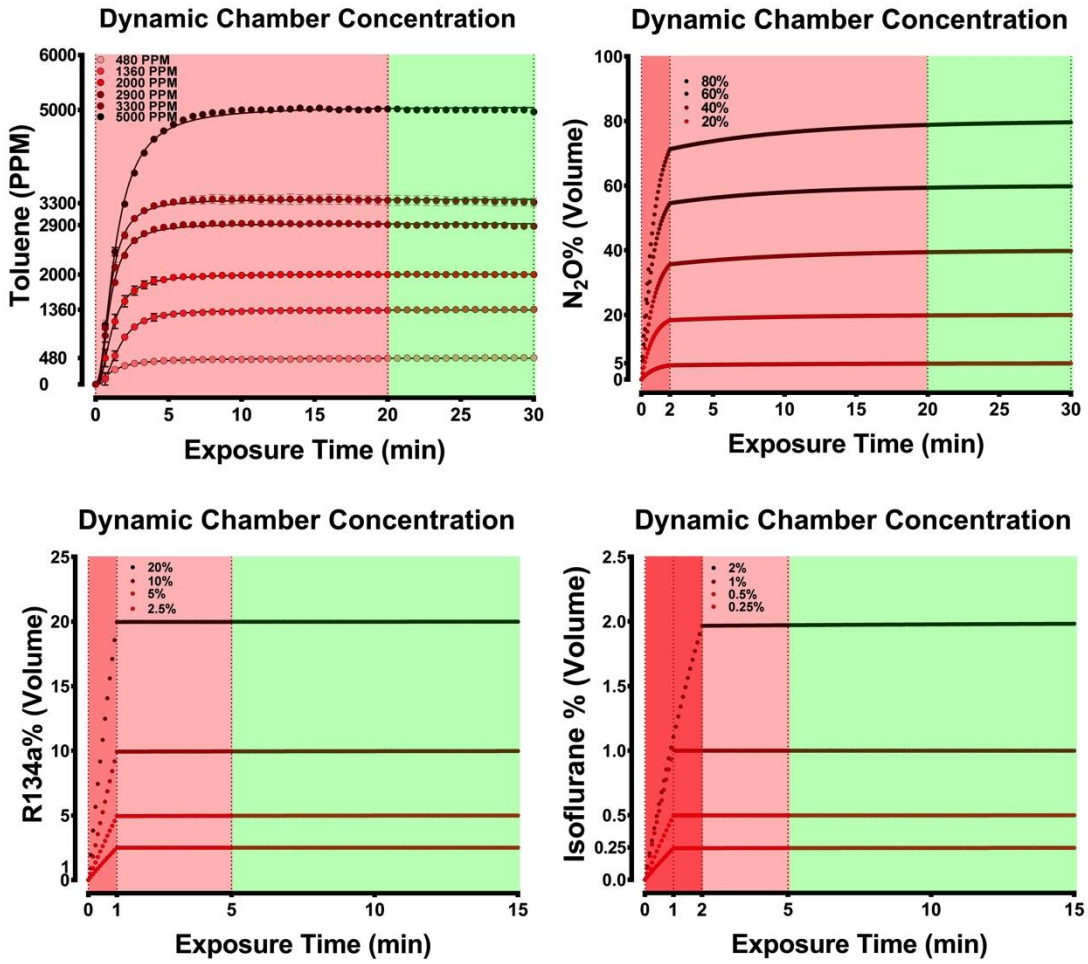

Figure 1b: Measure (Toluene) and derived concentrations of inhalants (N_2O, Isoflurane, R134a). Red represents the rising concentration to steady state levels and green represents time when subjects were tested in ICSS. In the case of nitrous oxide, R134a, and isoflurane, burst kinetic concentrations were used to rapidly speed up the delivery of inhalants in that for a brief period in the beginning a larger concentration was pumped into the chambers before switching to the steady-state concentration being examined.

Figure 2

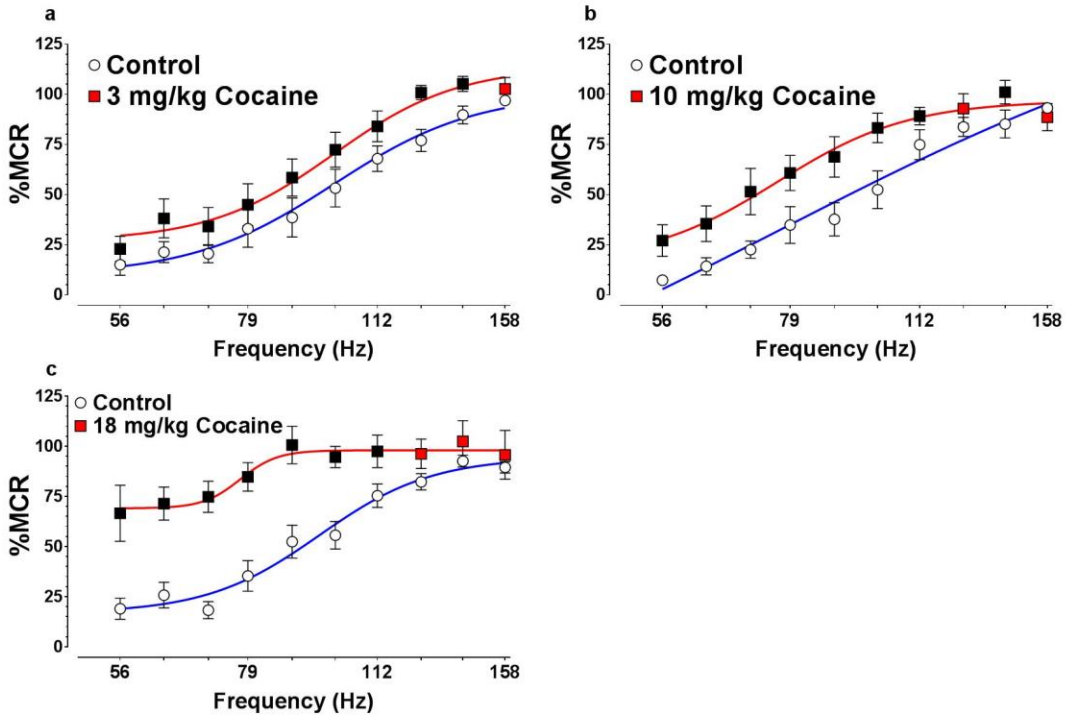

Figure 2: Mean (±SEM) percent maximal control ICSS response rate following 20-minute pretreatment with I.P. cocaine. Red square symbols represent the cocaine treatment condition and white circle symbols the saline control condition. (a) 3 mg/kg cocaine (b) 10 mg/kg cocaine (c) 18 mg/kg cocaine. Filled black squares denote significant differences compared to saline control ($p < 0.05$).

Figure 3

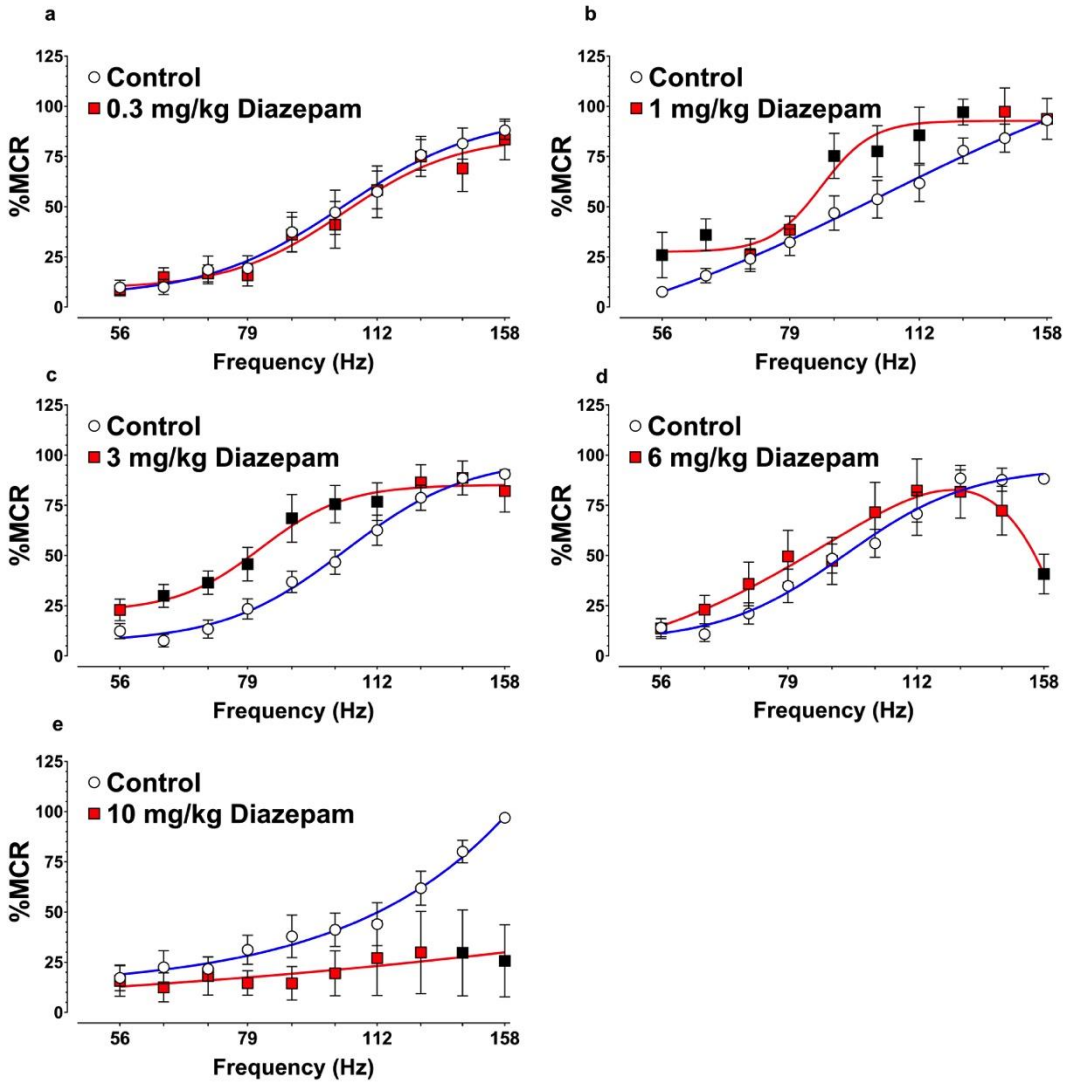

Figure 3: Mean (±SEM) percent maximal control ICSS response-rate following pretreatment with I.P. diazepam. Red square symbols represent the diazepam treatment condition and white circle symbols the saline control condition. (a) 0.3 mg/kg diazepam (b) 1 mg/kg diazepam (c) 3 mg/kg diazepam (d) 6 mg/kg diazepam (e) 10 mg/kg diazepam. Filled black square denotes significant differences compared to saline control ($p < 0.05$).

Figure 4

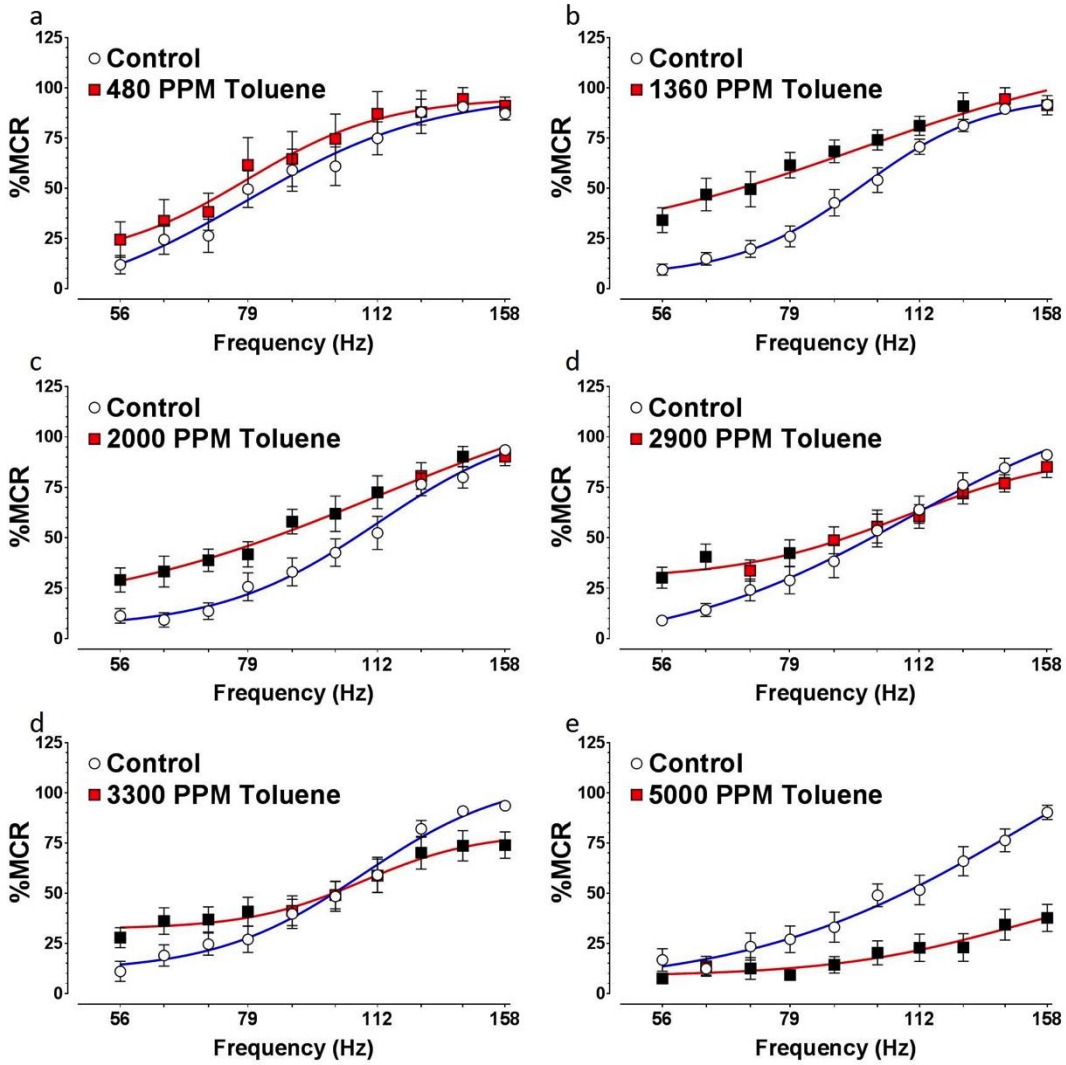

Figure 4: Mean (±SEM) percent maximal control ICSS response rate following 20 minute of exposure to toluene vapor. Red square symbols represent the toluene vapor treatment condition and white circle symbols the air control condition. (a) 480 ppm toluene (b) 1360 ppm toluene (c) 2900 ppm toluene (d) 3300 ppm toluene (e) 5000 ppm toluene. Filled black square denotes significant differences compared to saline control ($p < 0.05$).

Figure 5

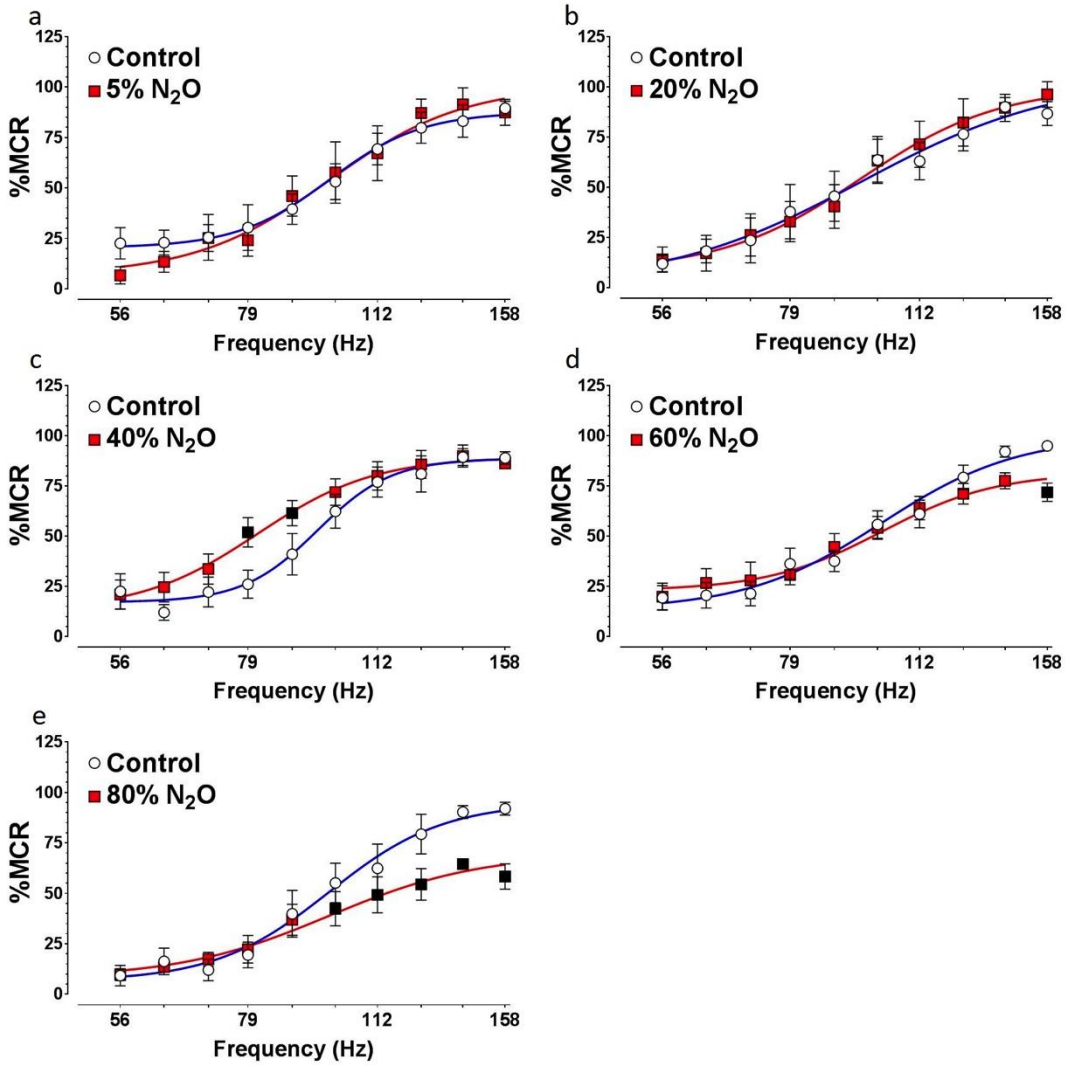

Figure 5: Mean (±SEM) percent maximal control ICSS response following exposure to nitrous oxide gas combined with 100% oxygen. Red square symbols represent the nitrous oxide treatment condition and white circle symbols the air control condition. (a) 20% nitrous oxide (b) 40% nitrous oxide (c) 60% nitrous oxide (d) 80% nitrous oxide. Filled black squares denote significant differences compared to control ($p < 0.05$).

Figure 6

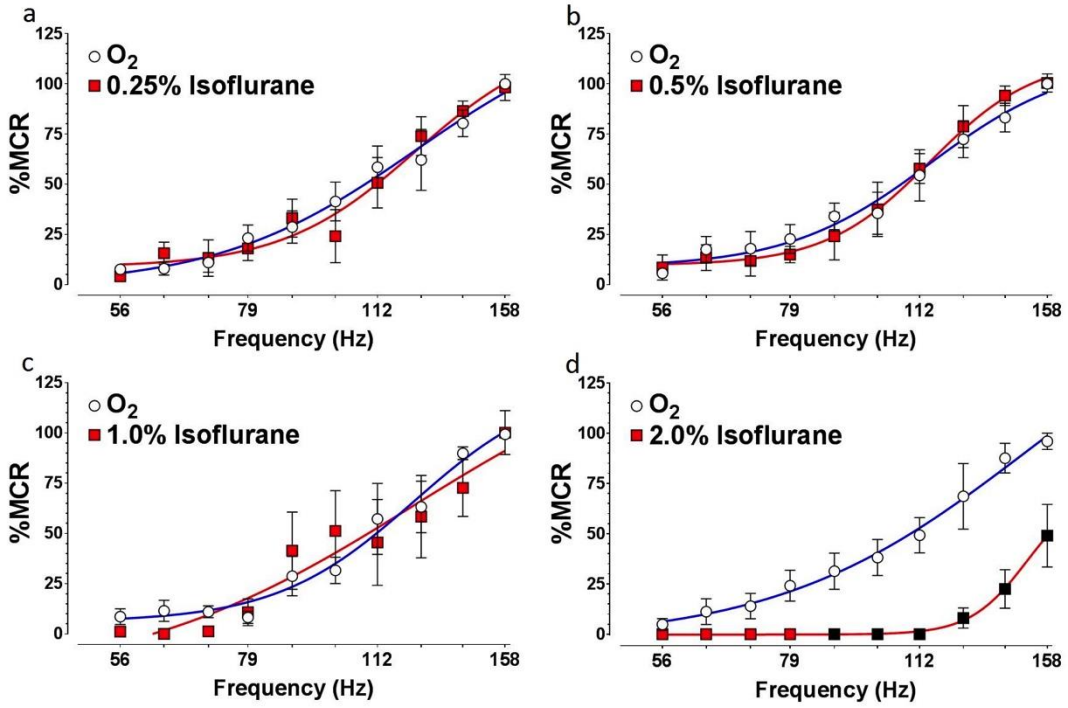

Figure 6: Mean (±SEM) percent maximal control ICSS response rate following pretreatment with exposure to isoflurane combined with 100% oxygen. Red square symbols represent the oxygen control treatment condition and white circle symbols the air control condition. (a) 0.25% isoflurane (b) 5% isoflurane (c) 10% isoflurane (d) 20% isoflurane. Filled black squares denote significant differences compared to control ($p < 0.05$).

Figure 7

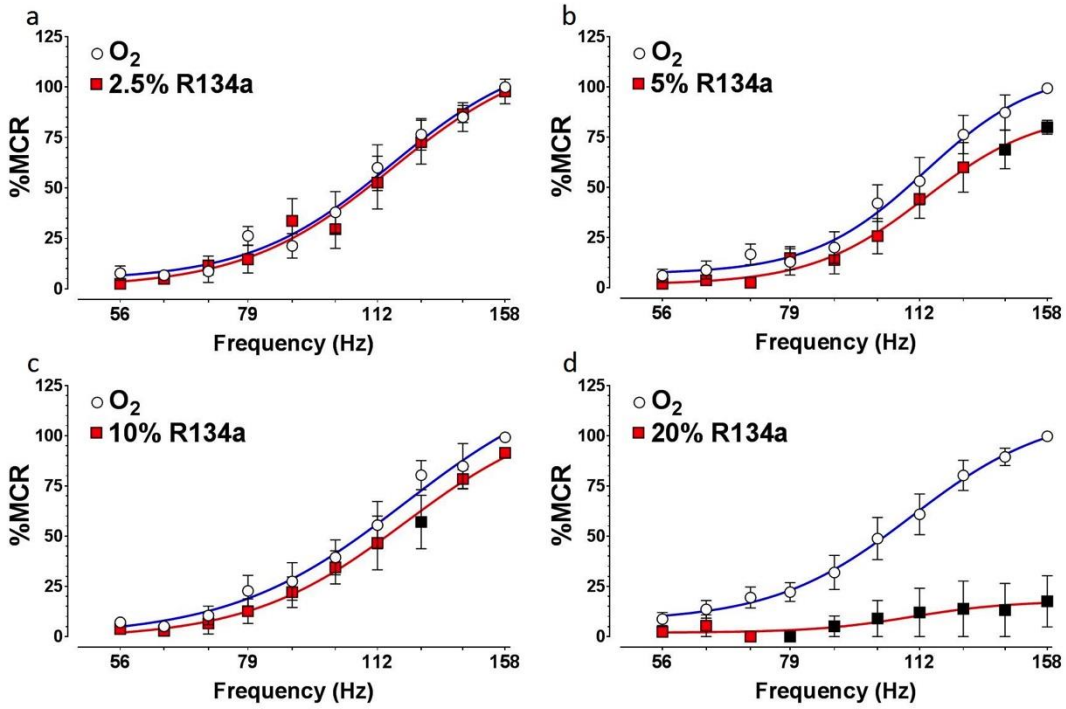

Figure 7: Mean (±SEM) percent maximal control ICSS response rate following pretreatment with exposure to R134a combined with 100% oxygen. Red square symbols represent the R134a treatment condition and white circle symbols the air control condition. (a) 2.5% R134a (b) 5% R134a (c) 10% R134a (d) 20% R134a. Filled black squares denote significant differences compared to control ($p < 0.05$).

Figure 8

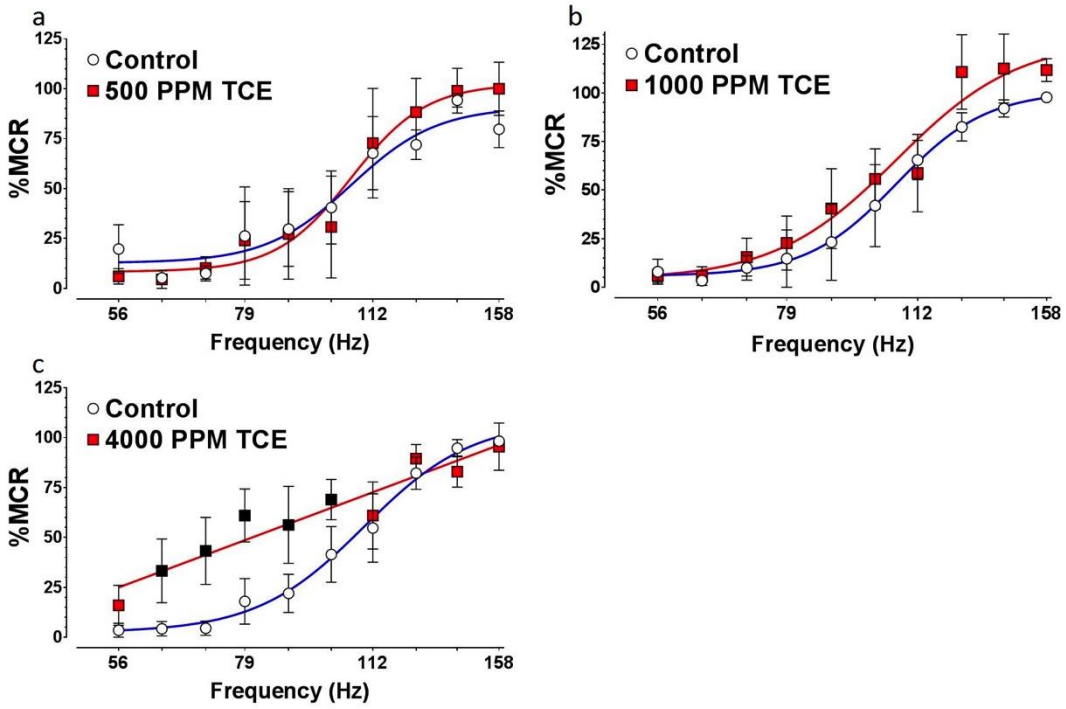

Figure 8: Mean (±SEM) percent maximal control ICSS response rate following pretreatment with exposure to TCE. Red square symbols represent the TCE treatment condition and white circle symbols the air control condition. (a) 500 PPM TCE (b) 1000 PPM TCE (c) 4000 PPM TCE. Filled black squares denote significant differences compared to control ($p < 0.05$).

Figure 9

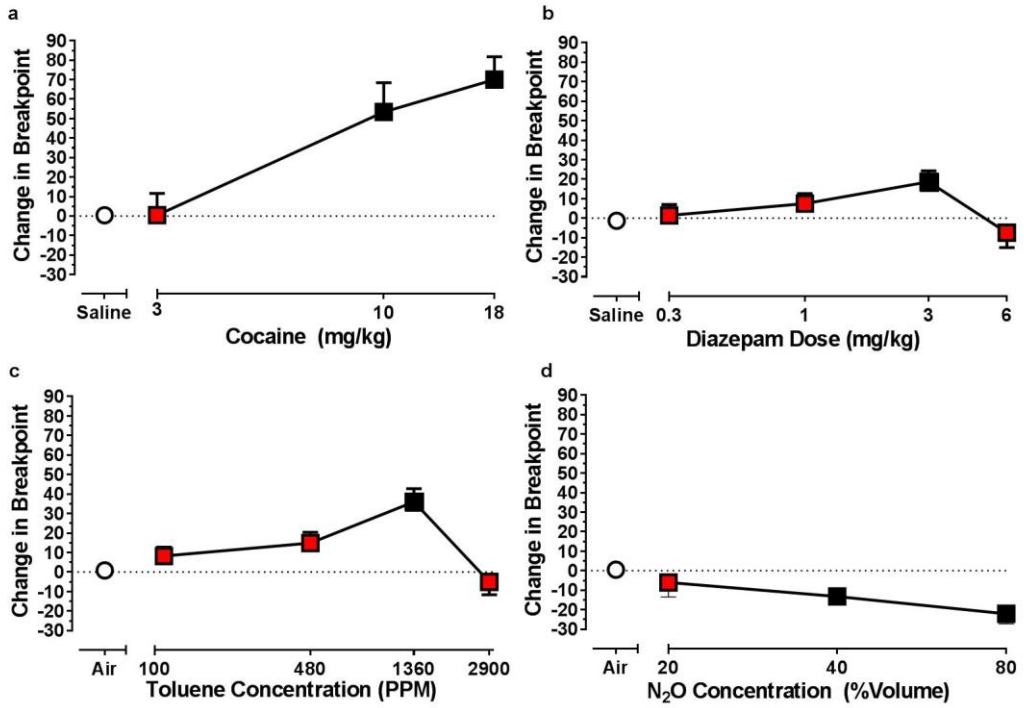

Figure 9: (a) Mean (±SEM) change in progressive-ratio breakpoint relative to saline control for 3, 10 and 18 mg/kg I.P. injected cocaine (b) Mean (±SEM) change in progressive-ratio breakpoint relative to saline control for 0.3, 1, 3 and 6 mg/kg I.P. injected diazepam (c) Mean change in progressive-ratio breakpoint relative to air control for exposure to 110, 480, 1360, and 2900 ppm toluene (d) Mean change in progressive-ratio breakpoint relative to air control for exposure to 20, 40 and 80% nitrous oxide. Filled black squares denote significant differences compared to vehicle control ($p < 0.05$).

Figure 10a

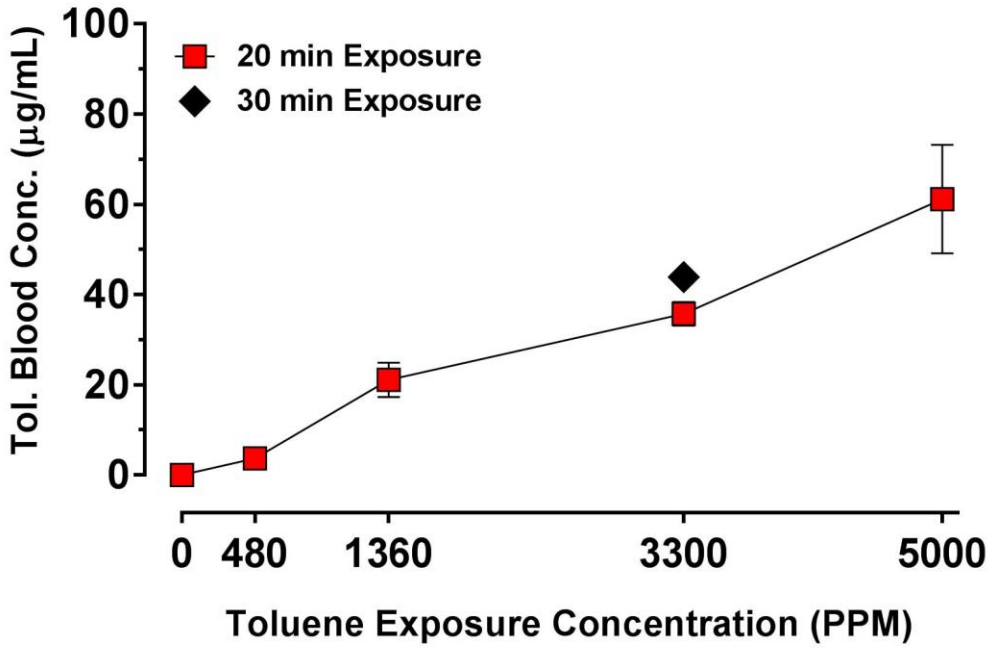

Figure 10a: Mean (±SEM) C57BL/6J mice toluene blood concentrations measured after 20 minutes of exposure (red square) in the dynamic chamber per exposure concentration and 30 minutes after exposure (black diamond, 3300ppm only) (n=3-4/concentration).

Figure 10b

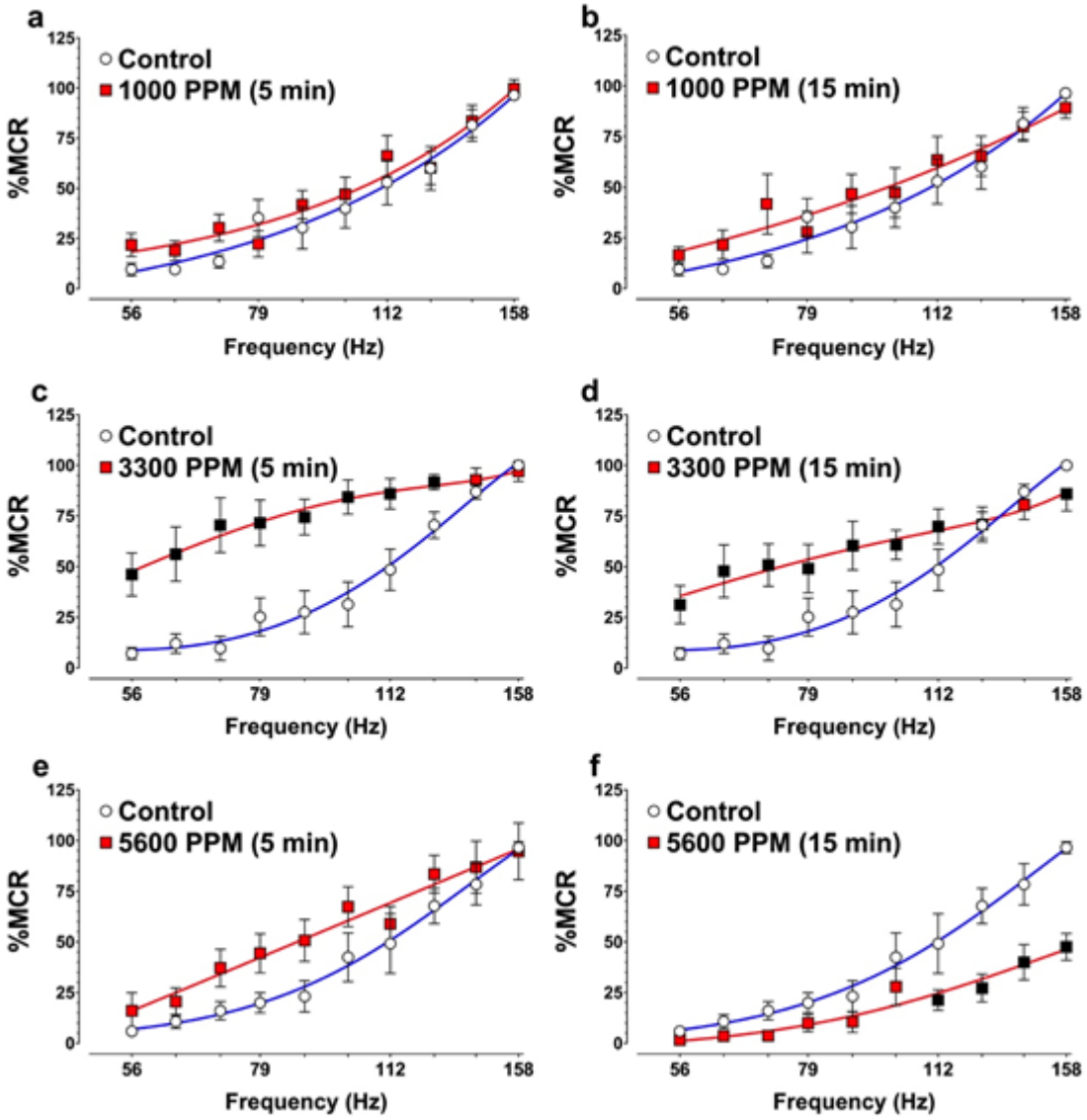

Figure 10b: Mean (±SEM) % MCR following 5 or 15 min of exposure to toluene vapor. Red square symbols represent the toluene vapor treatment condition and white circle symbols the air condition. (a) 1000 ppm toluene (5 min) (b) 1000 ppm toluene (15 min) (c) 3300 ppm toluene (5

min) (d) 3300 ppm toluene (15 min) (e) 5600 ppm toluene (5 min) (f) 5600 ppm toluene (15 min). Filled black square denotes significant differences compared to control ($p < 0.05$).

Discussion Aim 1

As expected, cocaine produced a robust dose-dependent facilitation across ICSS responding at all three tested doses. These data are in agreement with previous literature examining cocaine treated C57BL/6J mice in the rate frequency ICSS procedure (Fish *et al*, 2010; Straub *et al*, 2010). The positive $GABA_A$ allosteric modulator diazepam (Figure 3a-d) produced a less robust effect on ICSS in the rate-frequency procedure than has previously been reported. The 0.3 mg/kg diazepam dose did not alter ICSS, 1 and 3 mg/kg doses facilitated ICSS and 6 and 10 mg/kg dose suppresses ICSS. Similarly, the 3 mg/kg dose of diazepam also significantly increased ICSS breakpoint in the progressive ratio procedure (Figure 9a). Although less robust, the data are consistent with previous ICSS studies in mice that utilized a different curve-shift ICSS procedure. That study in C57BL/6J mice showed no effect of 0.3 mg/kg diazepam and a significant facilitation of ICSS by 1-4 mg/kg diazepam (Reynolds *et al*, 2012; Straub *et al*, 2010). Taken together these data confirm that diazepam reliably facilitates ICSS in mice across multiple ICSS procedural variants. Overall the data with cocaine and diazepam demonstrate that the three component rate-frequency and progressive ratio ICSS test conditions I employed are sensitive to the reward-facilitating effects of abused drugs.

The present data substantially extend the conditions under which it can be demonstrated that toluene will facilitate ICSS to include both the rate-frequency and

progressive-ratio ICSS procedure variants. While more limited in scope, the data also describe the effects of TCE in the rate frequency procedure. In contrast to cocaine which facilitated ICSS across the entire tested dose range, toluene produced a complex pattern of rate increasing and decreasing effects more robust than, but generally similar to the second positive control diazepam. Specifically, intermediate toluene concentrations facilitated ICSS in both the rate frequency and the PR procedure. Moderately high toluene concentrations produced a biphasic effect, facilitating ICSS at low frequencies and suppressing ICSS at high frequencies while having no effect on PR responding. The highest toluene concentration suppressed ICSS regardless of stimulation frequency in the rate-frequency procedure.

Interestingly, the duration of toluene pretreatment prior to testing appeared to strongly influence the degree of ICSS facilitation it engendered. The trend for this appeared to favor a higher abuse-like concentration delivered over a shorter amount of time. It was found that 5 minutes of exposure to 3300 ppm toluene produced the most robust increases in ICSS facilitation (Figure 10b c) and parallels the results of ethanol in ICSS where the most reinforcement enhancing effects are observed when responding for ICSS is in close temporal proximity to the initial delivery of the ethanol (Fish *et al*, 2010).

This robust facilitation was a necessity for attempting to utilize ICSS to examine ligands to probe contributions from particular receptor systems and subtypes.

Similar to past studies in which volatilized TCE was given to subjects in alternative ICSS reinforcement-enhancement models (Bespalov *et al*, 2003; Yavich and Zvartau, 1994), TCE also produced reward-like effects in the rate-frequency procedure at 4000

ppm. Unfortunately, while TCE remains a major inhalant of abuse in some countries, the United States Environmental Protection Agency has banned import and production in United States. Therefore, the quantity of TCE available did not permit further investigation at higher concentrations in the rate frequency procedure testing in the chain PR/FR10 procedure. However, like toluene, higher concentrations of TCE are associated with production of sedative-like effects in locomotor activity (Bowen and Balster, 1998) and producing pronounced rate deceasing effects in operant behavior (Moser and Balster, 1986; Shelton, 2009).

In ICSS, an enhancement of responding for stimulation following drug pretreatment is usually interpreted as a facilitation of brain reward circuitry (Wise *et al*, 1992). In contrast, a depression of ICSS responding by a test drug has multiple interpretations (Carlezon and Chartoff, 2007). The first is that the pretreatment drug is producing anhedonic-like effects, reducing the subject's sensitivity to the reinforcing effects of brain stimulation. For example, it has been shown that the kappa-opioid receptor agonist U69,593 produces a characteristic anhedonic-like effect when administered alone as well as blocking the reinforcement enhancing effects of ICSS produced by cocaine (Tomasiewicz *et al*, 2008). It is unclear if toluene is producing anhedonic-like effects in the present study although it is possible given previous data demonstrating that extended exposure to 1650-3300 ppm toluene vapor for 4 hours can produce a conditioned taste aversion (Miyagawa *et al*, 1984). However, in a conditioned place preference study, a significant preference for an environment paired with a shorter 30 minutes of exposure to 2000 and 3000 ppm toluene was observed while exposure to 5000 ppm toluene failed to

produce a place preference, possibly indicating the recruitment of anhedonic-like effects at this higher concentration (Lee *et al*, 2006). Interestingly, at a concentration of 5600 ppm toluene, I observed a significant depression in responding in the ICSS rate-frequency procedure which could be an indicator to anhedonic effects.

A second interpretation of depression of ICSS responding is that the test drug interferes with the ability of the subjects to physically perform the operant. For example, the sedative zolpidem has been shown to decrease operant performance in ICSS in the rate frequency procedure (Reynolds *et al*, 2012). It is certainly possible that this could be responsible for the suppression of ICSS at 5600 ppm toluene given that exposure to 15 min of 8000 ppm toluene vapor suppresses locomotor activity in C57BL/6J mice (Bowen *et al*, 2010). Exposure to concentrations of toluene vapor greater than 2000-3000 ppm suppress operant responding under fixed ratio as well as fixed interval schedules in mice (Moser and Balster, 1985; Bowen and Balster, 1998) suggesting that operant responding is even more sensitive to motor incoordination effect of toluene than locomotor activity. However, in the present study the effect of toluene at 3300 ppm was ICSS frequency dependent with responding at low ICSS frequencies increased by toluene whereas responding was suppressed at high ICSS frequencies. This effect could have been due to motor incoordination manifesting itself primarily at the high frequencies which supported the greatest rates of responding but it may also suggest a classic rate-dependent effect (Dews, 1977). This latter interpretation is possible, although under fixed interval schedules for food maintained responding response-rate increasing effects of toluene in mice are either not apparent (Bowen and Balster, 1998) or extremely modest (Moser and Balster, 1986).

Additional studies will be necessary to further explore these possibilities as they relate to ICSS performance.

The facilitation of ICSS by nitrous oxide was much less pronounced than that produced by toluene, diazepam or cocaine. In the rate-frequency procedure 40% nitrous oxide facilitated ICSS, although only significantly so at two intermediate stimulation frequencies (Figure 5b). The 60% and 80% nitrous oxide concentrations suppressed ICSS in the rate-frequency procedure and both 40% and 80% nitrous oxide suppressed PR responding. Prior preclinical studies examining the rewarding effects of nitrous oxide have also have generated conflicting results. Squirrel monkeys self-administered a mixture of 60% N_2O / 40% O_2 (Wood *et al*, 1977). However, in a rat place conditioning and self-administration study, repeated pairings of an environment with 40 minute of exposures to 30% and 60% nitrous oxide produced a conditioned place aversion and inconsistent results in self-administration (Ramsay *et al*, 2003). Human self-administration and subjective tests of N_2O exposure have been equally divergent among healthy subjects. In human subjects given the option to self-administer 30% N_2O, oxygen vehicle or air, but blinded to the identity of their choice, 5 of 12 subjects consistently chose N_2O, 4 consistently avoided N_2O, and 3 subjects were divided between N_2O and the oxygen/placebo alternatives (Walker and Zacny, 2001). Taken together the present results and previous studies suggest that the rewarding effects of nitrous oxide at least as measured by self-administration, ICSS and conditioned place preference are at best fairly modest compared to other drugs of abuse including other inhalants such as toluene.

In contrast to toluene, both isoflurane and R134a produced no facilitation of ICSS at lower concentrations, coupled with a strong decrease in ICSS responding at higher concentrations. These findings suggest these compounds may have limited abuse-liability. This finding is, however, at odds with demonstrated evidence of abuse of both compounds in humans (Koehler and Henninger, 2014; Kuhlman *et al*, 1993; Pavlic *et al*, 2002; Ritchie *et al*, 2001). It is possible that like nitrous oxide, there is a limited window where facilitation of ICSS by isoflurane and R134a would be most pronounced. It may be the case that the concentration necessary to produce facilitation was missed as the rate-decreasing effects for isoflurane and R134a occurred over a very narrow range in the curve, particularly in the case of isoflurane. Indeed, the observation with 1% isoflurane at the end of the session was the appearance of sedative-like effects in some subjects. The onset of sedation in some subjects may have masked facilitation produced by isoflurane for other subjects effectively producing a null group effect as is seen in the 1% isoflurane exposure (a mean of facilitation and sedative effects across subjects). The variability of individual subjects to sedative effects that interfere with operant responding has also been observed in drug discrimination with diazepam (Shelton and Nicholson, 2012). It may therefore be useful to examine ICSS dependent measures utilizing an alternative concentration-response curves where subjects are excluded that show strong signs of sedation.

In summary, the findings indicate that toluene robustly facilitates progressive ratio ICSS breakpoint and enhances the reinforcing efficacy of low to mid-range frequencies of stimulation in the rate-frequency procedure. TCE also enhances the reinforcing efficacy of

low to mid-range frequencies of stimulation in the rate-frequency procedure. In contrast N_2O only weakly facilitates ICSS responding in the rate-frequency procedure and produced a concentration dependent suppression of ICSS breakpoint in the chain PR/FR10 procedure. These results suggest that while demonstrating some reinforcement enhancing effects, N_2O may also produce effects which more strongly compete with behaviors necessary to progress in the PR procedure relative to rate-frequency schedule. In the rate-frequency procedure every lever-press results in some degree of stimulation, the production of interfering behaviors may have a much more limited effect on response output as compared to the progressive ratio procedure which requires successively greater numbers of responses in the absence of reinforcer presentation. Lastly, isoflurane and R134a produced only a concentration dependent depression of ICSS in the rate frequency procedure.

Given the relative difficulty of quantifying the reinforcing effects of inhaled substances using standard measures of drug reinforcement, developing alternative procedures is important for advancing knowledge in the area. The data with toluene, TCE and nitrous oxide in mice suggest that ICSS is a viable method to probe the neurobiological mechanisms underlying the rewarding effects of abused inhalants. However, further examination of additional ICSS procedural parameters that might generate data entirely consistent with the known abuse liability of individual inhalants should be further investigated.

Aim 2: Determine the role of $GABA_A$ receptors in inhalant facilitated ICSS

Materials and Methods

Subjects

A total of 17 and 16 adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were used for the ICSS and microdialysis experiments, respectively. Mice were individually housed under a 12h light/dark cycle and tested during the light phase of the cycle. Laboratory chow (Harlan, Madison, WI) and water were available *ad libitum* except during experimental sessions. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and were in accordance with NIH guidelines (National Research Council, 2011). For the ICSS studies, not all subjects were tested in all conditions. Order effects were controlled by separating test sessions by at least 36 hours and counterbalancing naive subjects with drug experienced subjects across experiments. Dose response curves and antagonism studies were tested in ascending dose order to minimize the effects of repeated exposure.

Compounds

Toluene was purchased from Fisher Scientific. D-methamphetamine hydrochloride was obtained from the National Institute on Drug Abuse Drug Supply Program and prepared in 0.9% sterile saline. Diazepam was obtained in an injectable commercial formulation. Flumazenil was obtained from Sigma-Aldrich (St. Louis, MO). Ro15-4513 was obtained from Tocris Bioscience (Minneapolis, MN). Flumazenil, Ro15-4513 and diazepam were prepared in a 45% hydroxypropyl-β-cyclodextrin (HP-BCD) vehicle (Sigma-Aldrich, St. Louis, MO).

Dynamic Vapor Exposure System and Testing Apparatus

The dynamic inhalant vapor exposure/ICSS system utilized has been described previously (Tracy *et al*, 2014). Briefly, toluene vapor was produced using a microprocessor controlled vapor generator and routed into acrylic cubicles containing two-lever operant conditioning chambers. Toluene concentrations within each chamber were verified by headspace gas chromatography. ICSS stimulation and schedule controlled delivery were provided by commercially-available components (Med-Associates, St. Albans, VT). Concentrations of toluene produced the chamber are shown in Figure 11.

ICSS Surgical Procedure

Mice were implanted under isoflurane and morphine (0.2 mg/kg s.c.) anesthesia with bipolar electrodes (Plastics One, Roanoke, VA) directed at the right medial forebrain bundle (Paxinos and Franklin, 2001). Coordinates relative to bregma were: -1.5 anterior-posterior, 1.0 medial-lateral, and -5.0 dorsal-ventral. Mice were treated 4 days' post-surgery with 0.5 mg/kg/day of the analgesic carprofen and allowed 6 days' recovery prior to beginning training.

ICSS Rate Frequency Procedure

Mice were trained to respond on an FR1 at 141 Hz frequency at 100 uA. If responding did not equal or exceed 30 stimulation deliveries per minute the amperage was increase 25% and tested again. Following stable responding at ≥ 30 responses/min, mice were transitioned to a repeated 10-minute fixed ratio 1 with a 10-minute timeout at the set amperage from the FR1 and at 158 Hz. Following reliable responding during the active component (ratio > 8) mice were transitioned to a four component rate-frequency

procedure. Rate-frequency sessions were approximately 80 min, divided into four 10-min response components separated by time outs. The use of timeouts allowed for adequate time for treatment conditions to be applied between control and subsequent drug treatment curves within a single test session. Each response component consisted of ten 1-min trials beginning at a stimulation frequency of 158 Hz and descending by 0.05 log units in each subsequent trial. After the first response component and following a timeout, vehicle or air was administered five minutes prior to running two control response components which served as the baseline. After an additional timeout following the end of the 3rd component, treatment conditions were administered five minutes before the start of the 4th component.

Toluene vapor concentrations of 1000, 3300 and 5600 parts-per-million (PPM) were examined. Test sessions with injected drugs were identical to toluene test sessions except that following the baseline response components, the mice were administered a S.C. injection of drug. D-methamphetamine was injected 5 min prior to the 4th component at doses of 0.3, 1, and 3 mg/kg. Ro15-4513 was tested at doses of 0.3, 1, and 10 mg/kg. Flumazenil was tested at doses of 1, 3, and 10 mg/kg. Flumazenil and Ro15-4513 were administered 6 min prior to the 4th component, a time interval chosen based upon previous pharmacokinetic studies in male C57 mice (Kida *et al*, 2003; Potier *et al*, 1988).

Antagonism of Drug-facilitated ICSS

A single concentration of toluene vapor (3300 PPM) and dose of d-methamphetamine (3 mg/kg), which produced roughly equivalent facilitation of ICSS, were utilized. Antagonism tests employed the same procedure previously described, with the exception that Ro15-4513 and flumazenil were administered 1 min prior to d-

methamphetamine, diazepam or toluene treatment. In all cases Ro15-4513 and flumazenil were tested alone prior to being combined with toluene, d-methamphetamine or diazepam.

Data Analysis

Data from the first 10-min response component were discarded as they have been shown to exhibit greater variability than data from subsequent components (Carlezon and Chartoff, 2007). Stimulations earned during each 1-min trial of the 2nd and 3rd air/vehicle exposure control components were averaged and used as the baseline for comparison with the 4th treatment component. For each mouse, data from each trial were expressed as a percentage of earned stimulations in the 1-min trial with the greatest number of earned responses, regardless of frequency, in the average baseline component. These values are reported as a percentage of the maximum control responses (%MCR). Data from the baseline and treatment component were compared individually for each drug dose or inhalant concentration by 2-way within subject repeated measures analysis of variance (ANOVA). To allow for comparisons between treatment conditions, for each experimental group (i.e. vehicle baseline control vs. vehicle + methamphetamine vs. Ro15-4513 + methamphetamine) respective control curves were compared to determine if they were significantly different from each other by two-way repeated measures ANOVA. As no control curves within a given study were significantly different the repeated control curves were averaged to a single baseline curve for comparison across experimental treatment conditions and analyzed by 2-way repeated measures ANOVA. Significant ANOVA effects were followed by Holm-Sidak multiple comparisons test to determine significant

differences at individual frequencies between the baseline and treatment conditions (Aickin and Gensler, 1996; Jason C. Hsu, 1996; Stanton A. Glantz, 2006).

Microdialysis

Surgical Procedure

Mice were pretreated with morphine (0.2 mg/kg) and implanted under isoflurane anesthesia with a 5 mm guide cannula (CXG-6, Eicom, San Diego, CA) just above the nucleus accumbens at 1.34 mm anterior-posterior, 1 mm medial-lateral, and -4.0 mm dorsal-ventral. Mice were treated for 3 days' post-surgery with 0.5 mg/kg/day of the analgesic carprofen and allowed 6 days of surgical recovery.

Microdialysis Test Procedure

Mice were fitted in an elastic swivel jacket/tether assembly connected to a suspended fluid swivel. A microdialysis probe with a 1 mm regenerated cellulose membrane (CX-I-6-01, Eicom) was inserted through the guide cannula with the membrane extending into the NAc. The mice were then returned to the microdialysis chamber. Artificial cerebrospinal fluid (ACF) was perfused through the probe at a rate of 1.2 uL/min. Prior to beginning sample collection, probes equilibrated for at least two hours or until stable dopamine dialysate levels were achieved. Samples were collected every 7 min for up to 3.5 hours and protected from degradation by the addition of 2.5 uL 0.01M HCl. Dopamine concentrations were considered stable after three consecutive samples that varied by no more than 0.05 pg or 20% of the running mean. Sampling delay for the analyte traveling between the probe membrane tip and sample collection output was calculated to be roughly 8 minutes at a flow rate of 1.2 uL/min. After establishing a stable

baseline, saline was administered to examine the effects of injection on dopamine levels. After 5 additional samples, 45% HP-BCD vehicle or 1 mg/kg Ro15-4513 was administered 1 min prior to a dose of 3 mg/kg d-methamphetamine or saline. An additional comparison group of 1 mg/kg Ro15-4513 + saline was used to determine the effects of Ro15-4513 alone on dopamine levels. Samples were then collected for an additional 2 hrs. Each group's pretreatment baselines were averaged for each respective group and then directly compared to each post injection time-point using multiple comparisons to determine significance. To determine significance between independent treatment groups, a 2-way mixed measures ANOVA was performed using Fisher's LSD post-hoc tests to determine significance. Measurements are reported without subtracting the lag time between probe membrane tip and sample collector.

HPLC analysis

Dialysate samples were analyzed using HPLC-EC (HTEC-500, Eicom) with a C18-column (PP-ODS II, Eicom), graphite working electrode, and an Ag vs. AgCl reference electrode with an applied potential of +450 mV. Dopamine retention time and concentration were determined by comparison to a standard curve (0.01-100 pg/5 µL) established prior to analysis of each subject's experimental samples. The lower limits of quantification were set at 0.1 pg.

Data Analysis

HPLC data were analyzed by eDAQ PowerChrom software (eDAQ, Colorado Springs, CO). The mean dopamine concentration of the last set of consecutive stable samples before injections were defined as the baseline for each subject. Subsequent data

were then expressed as a percentage of this value. Data were analyzed by a 2-way (treatment x time) mixed ANOVA. Significant ($p < 0.05$) main effects and interactions were examined by Fisher's LSD multiple comparisons post-hoc tests. 19 missing data points out of 432 total points were substituted using individual group mean data. Missing data was roughly equally distributed between vehicle + d-methamphetamine, Ro15-4513 + saline, and Ro15-4513+ d-methamphetamine groups.

Histology

Microdialysis mice were euthanized with Euthasol (pentobarbital/phenotoin), their brains removed and grossly dissected to isolate the area containing the microdialysis guide cannula and probe tract. This tissue block was then immersed in 4% formalin for at least 10 days. The block was then rinsed, placed in phosphate buffered saline and later sectioned using a Leica VT1000S vibratome (Buffalo Grove, IL). Sections were transferred to slides and stained with 0.1% cresyl-violet. Probe tract locations were identified using a stereotaxic mouse brain atlas (Paxinos and Franklin, 2001).

Results

Effects of abused drugs on ICSS

Five minutes of exposure to toluene vapor facilitated ICSS over air control (Figure 12a). The ANOVA found a significant treatment x frequency interaction [F (27, 162) = 2.518, $p = 0.0002$]. Concentrations of 1000, 3300 and 5600 PPM toluene significantly increased %MCR over baseline at 1, 8 and 3 stimulation frequencies, respectively. D-methamphetamine significantly facilitated ICSS over vehicle control (Figure 12b) producing a significant treatment x frequency interaction [F (27, 189) = 2.664, $p < 0.0001$].

Doses of 0.3, 1 and 3 mg/kg d-methamphetamine significantly increased %MCR over baseline at 1, 8 and 8 stimulation frequencies, respectively.

Effects of Flumazenil and Ro15-4513 administered alone on ICSS

Flumazenil when administered alone did not alter ICSS compared to vehicle (Figure 12c). There was neither a significant main effect of flumazenil treatment [F (3, 18) = 1.810, p = 0.1815] nor treatment x frequency interaction [F (27, 162) = 1.252, p = 0.1968]. Ro15-4515 when administered alone significantly reduced responding for ICSS (Figure 1d) resulting in a main effect of Ro15-4513 treatment [F (3, 21) = 5.171, p = 0.0078]. The 0.3 and 1 mg/kg doses of Ro15-4513 failed to alter ICSS relative to vehicle, whereas the 10 mg/kg dose significantly depressed %MCR relative to control at 3 stimulation frequencies.

Effects of flumazenil on drug-facilitated ICSS

Pretreatment with 1 or 3 mg/kg flumazenil failed to block ICSS facilitated by 3300 PPM toluene (Figure 2a). The ANOVA indicated a significant treatment x frequency interaction [F (27, 162) = 2.040, p = 0.0035] with vehicle + 3300 PPM toluene facilitating ICSS at 8 frequencies compared to control. The 1 and 3 mg/kg flumazenil + 3300 PPM toluene conditions significantly increased %MCR over control at 7 and 6 frequencies, respectively. Further %MCR in the 1 and 3 mg/kg flumazenil + 3300 PPM toluene conditions was not significantly lower than the vehicle + 3300 PPM toluene condition at any frequency.

Pretreatment with 3 mg/kg flumazenil also failed to block ICSS facilitation produced by 3 mg/kg d-methamphetamine (Fig 13b). The ANOVA indicated a significant

main effect of treatment [F (2, 12) = 7.949, p = 0.0063] but no treatment x frequency interaction [F (18, 108) = 1.620, p = 0.0675]. Vehicle + 3 mg/kg d-methamphetamine significantly elevated responding at 6 frequencies over baseline control. The 3 mg/kg flumazenil + 3 mg/kg d-methamphetamine condition did not significantly suppress %MCR at any frequencies compared to the vehicle + 3 mg/kg d-methamphetamine condition resulting in the 3 mg/kg flumazenil + 3 mg/kg d-methamphetamine condition being significantly elevated over baseline control at 6 frequencies.

Pretreatment with 3 mg/kg flumazenil completely blocked facilitation of ICSS produced by 3 mg/kg diazepam (Figure 13c). The ANOVA found a significant treatment x frequency interaction [F (18, 108) = 3.238, p < 0.0001]. The vehicle + 3 mg/kg diazepam condition was significantly elevated over baseline control at 3 frequencies. The 3 mg/kg flumazenil + 3 mg/kg diazepam treatment condition was significantly suppressed compared to the vehicle + 3mg/kg diazepam condition at 4 frequencies resulting in it not being significantly different from the baseline control at any frequency.

Effects of Ro15-4513 on drug facilitated ICSS

Pretreatment with 0.3 Ro15-4513 attenuated and 1 mg/kg Ro15-4513 completely blocked facilitation of ICSS produced by 3300 PPM toluene (Figure 13d). The ANOVA indicated a significant treatment x frequency interaction [F (27, 189) = 2.125, p = 0.0018]. The vehicle + 3300 PPM toluene condition was significantly elevated over baseline at 8 frequencies. The 0.3 mg/kg Ro15-4513 + 3300 PPM toluene condition significantly suppressed %MCR at 5 frequencies when compared to the vehicle + 3300 PPM toluene condition resulting in it only being significantly different from baseline control at two

frequencies (Figure 13d). Responding in the 1 mg/kg Ro15-4513 + 3300 PPM condition was significantly suppressed at 6 frequencies compared to the vehicle + 3300 PPM toluene condition resulting in it not being significantly different from baseline at any frequency.

Pretreatment with 0.3 and 1 mg/kg Ro15-4513 completely blocked facilitation of ICSS produced by 3 mg/kg d-methamphetamine (Figure 13e). The ANOVA found a significant main effect of treatment [F (3, 18) = 8.296, p = 0.0011] but no treatment x frequency interaction [F (27, 162) = 1.396, p = 0.1066]. The vehicle + 3 mg/kg d-methamphetamine condition was significantly elevated over the baseline control at 4 frequencies. Responding in the 0.3 mg/kg Ro15-4513 + 3 mg/kg d-methamphetamine condition was significantly suppressed at 4 frequencies compared to the vehicle + 3 mg/kg d-methamphetamine condition resulting in it not being significantly different from the baseline control condition at any frequency (Figure 13e). Responding in the 1 mg/kg Ro15-4513 + 3 mg/kg d-methamphetamine condition was significantly suppressed at 4 frequencies compared to the vehicle + 3 mg/kg d-methamphetamine condition resulting in it not being significantly different from the baseline control condition at any frequency (Figure 13e).

Pretreatment with 0.3 mg/kg Ro15-4513 also completely blocked facilitation of ICSS produced by 3 mg/kg diazepam (Figure 13f). The ANOVA found a significant treatment x frequency interaction [F (18, 108) = 1.767, p = 0.0388]. Vehicle + 3 mg/kg diazepam condition significantly facilitated responding at 2 frequencies compared to the baseline control (Figure 13f) whereas the 0.3 mg/kg Ro15-4513 + 3 mg/kg diazepam

treatment condition was not significantly different from the baseline control at any frequency.

Maximum rates of responding across experimental conditions

Maximum rates of responding in each test condition which were used to compute %MCR are reported in table 3. For each experimental condition, the maximum rate of responding in the baseline condition was not different from the treatment condition with the exception of 10 mg/kg Ro15-4513 alone and 1 mg/kg Ro15-4513 + 3 mg/kg d-methamphetamine which were both significantly lower than baseline.

NAc microdialysis

Mean baseline dopamine levels (±SEM) across all subjects prior to drug treatment was 1.29 ± 0.50 pg/5uL. Administration of vehicle 1-min prior to injection of 3 mg/kg d-methamphetamine resulted in extracellular dopamine levels peaking at 903% of baseline 21 min after d-methamphetamine injection (Figure 14). Administration of 1 mg/kg of Ro15-4513 1-min prior to d-methamphetamine attenuated maximal extracellular dopamine levels in the NAc which peaked at 434% of baseline. A mixed 2-way ANOVA demonstrated a treatment x time interaction [F (52, 338) = 4.978, $p < 0.0001$]. Post hoc analysis indicated that dopamine levels in the dialysate samples from 14-70 min post-injection were significantly lower in the 1 mg/kg Ro15-4513 + 3 mg/kg d-methamphetamine treatment group compared to the vehicle + 3 mg/kg d-methamphetamine group. The vehicle + 3 mg/kg d-methamphetamine and 1 mg/kg Ro15-4513 + 3 mg/kg d-methamphetamine conditions were significantly elevated over the mean pretreatment baseline at 14-77 and 14-91 time points post injection, respectively.

Dopamine in the 1 mg/kg Ro15-4513 + saline group was slightly elevated and was significantly greater at the 7-28, 42 and 56 min time points compared to pretreatment baseline. Two out of 18 subjects were excluded from the study due to inaccurate probe placement from the intended accumbens target. Approximate probe locations are plotted on a coronal histological brain atlas from Paxinos and Franklin and a representative micrograph are shown in figure 15 (Paxinos and Franklin, 2001).

Table 3

Drug	Dose	Baseline±SEM	Treatment±SEM
Toluene (n=7)	1000	51.0±3.4	51.7±4.1
	3300	51.5±4.1	52.0±4.0
	5600	48.5±3.3	47.9±4.7
Meth (n=8)	0.3	50.5±1.7	50.4±1.4
	1	49.3±1.4	50.9±1.6
	3	50.6±2.0	48.5±2.4
Ro15-4513 (n=8)	0.3	51.8±3.7	47.3±4.0
	1	54.1±3.8	49.3±4.9
	10	52.1±3.5	42.3±4.4*
Flumazenil (n=7)	1	43.1±2.0	48.3±2.9
	3	51.5±2.9	52.4±2.9
	10	47.2±3.5	51.4±4.5
Ro15-4513 + 3300 PPM Toluene (n=7)	0.3	48.8±4.0	44.6±4.4
	1	52.9±4.4	45.6±6.5
Ro15-4513 + 3 mg/kg d-methamphetamine (n=7)	0.3	50.7±4.4	43.0±4.6
	1	53.6±4.6	43.3±4.2*
Ro15-4513 + Diazepam (n=7)	0.3	53.4±1.8	53.7±2.2
Flumazenil + 3300 PPM Toluene (n=7)	1	43.9±2.3	49.3±3.3
	3	47.6±3.3	47.1±3.4
Flumazenil + 3 mg/kg d-methamphetamine (n=7)	3	49.6±2.1	52.4±1.9
Flumazenil + 3 mg/kg Diazepam (n=7)	3	54.2±2.0	53.0±1.7

Mean maximum response rates for each experimental condition. Differences in maximum response rates between baseline and treatment conditions were compared with a two-way mixed measures ANOVA with Holm Sidak's multiple comparisons test. * Indicates significant difference.

Figure 11

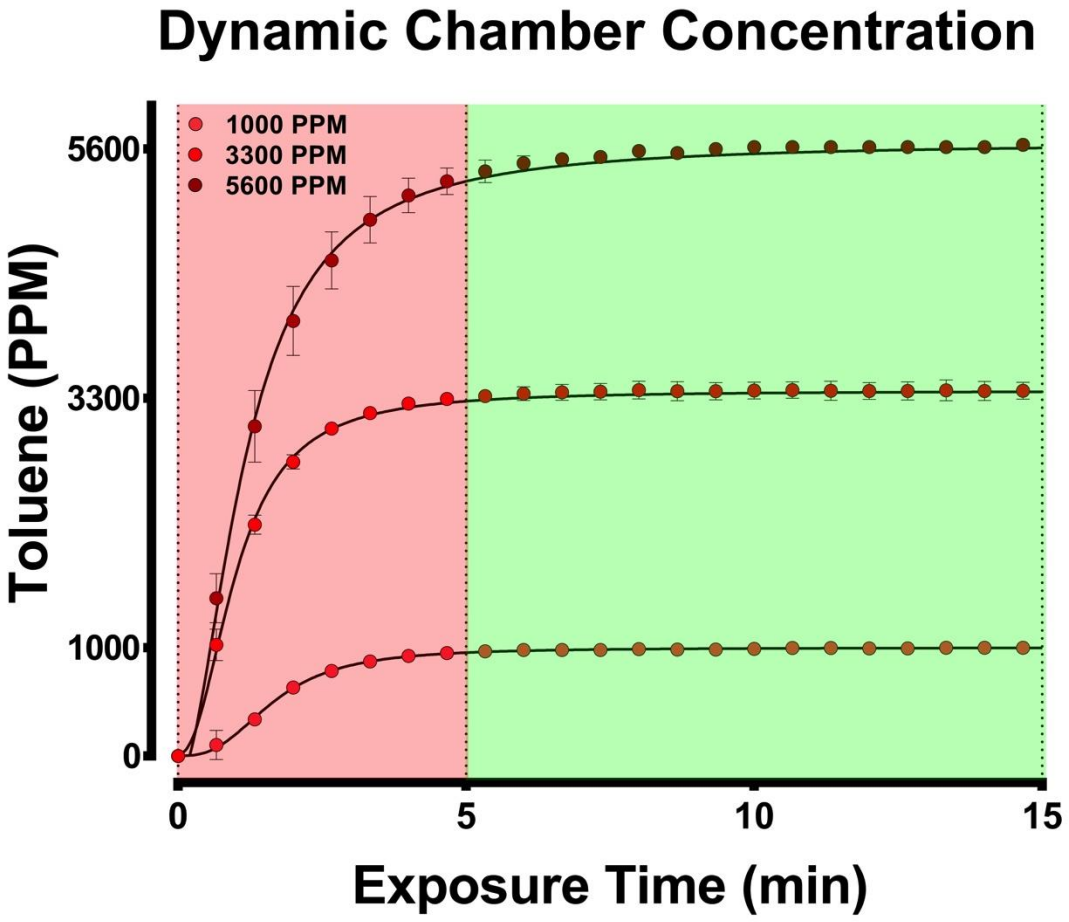

Figure 11 Toluene concentration in the chamber as a function of time for 1000 to 5600 ppm. Green area represents when the ICSS test session begins.

Figure 12

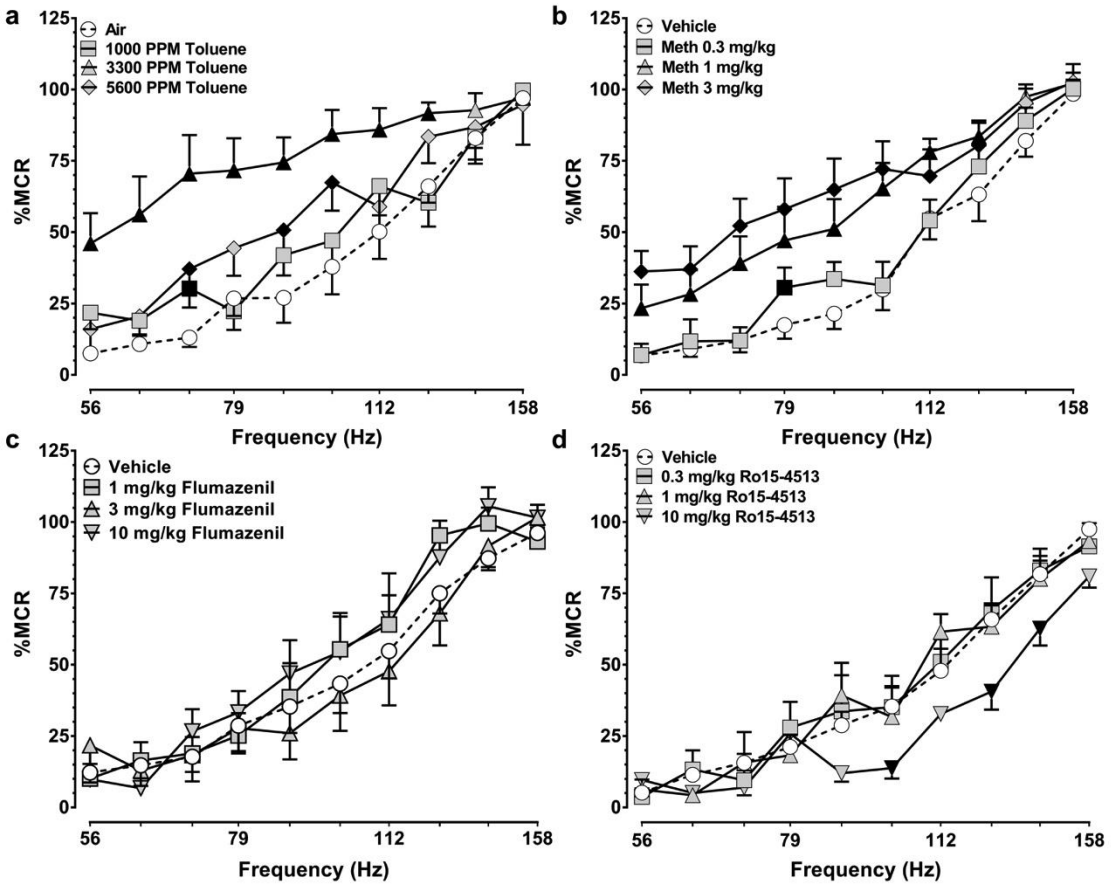

Figure 12 Mean (±SEM) percent maximal control ICSS response rate (% MCR) following a 5 min (a) exposure to 1000-5600 PPM toluene vapor (b) pretreatment with 0.3-3 mg/kg d-methamphetamine (c) Pretreatment with 1-10mg/kg flumazenil (d) pretreatment with 0.3-10 mg/kg Ro15-4513. Filled symbols denote significant differences compared to control ($p < 0.05$).

Figure 13

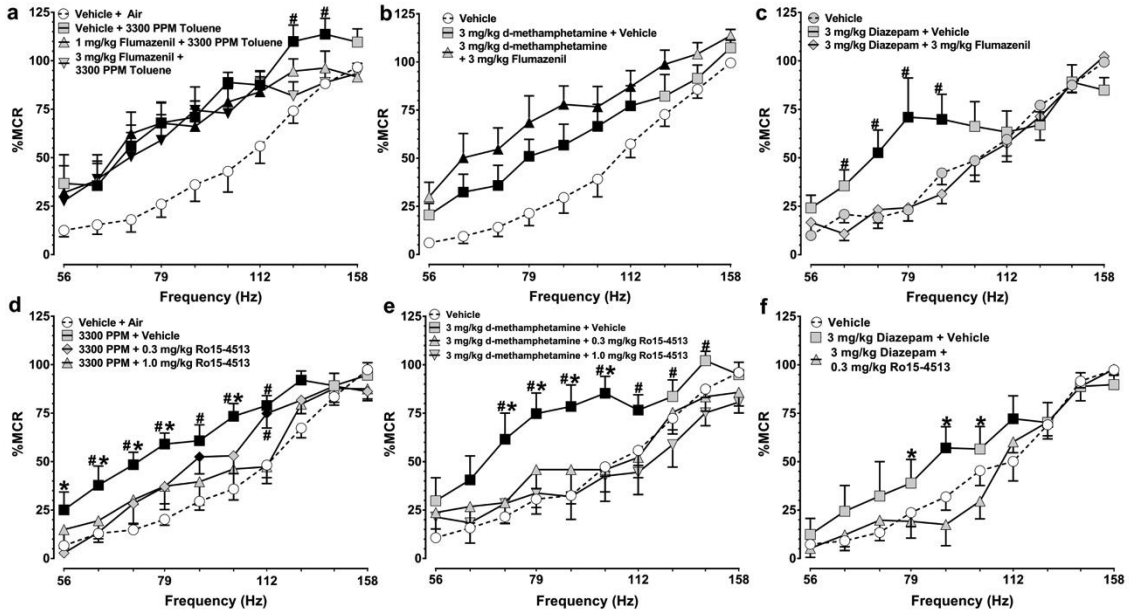

Figure 13 Mean (±SEM) percent maximal control ICSS response rate (% MCR) following a pretreatment with vehicle, flumazenil or Ro15-4513 prior to 5-minute exposure to 3300 PPM toluene vapor or to injection of 3 mg/kg d-methamphetamine or 3 mg/kg diazepam. (a) 3300 PPM toluene + vehicle, 1 or 3 mg/kg flumazenil. (b) 3300 PPM toluene + vehicle, 0.3 or 1 mg/kg Ro15-4513 (c) 3 mg/kg d-methamphetamine + vehicle or 3 mg/kg flumazenil (d) 3 mg/kg d-methamphetamine + vehicle, 0.3 or 1 mg/kg Ro15-4513 (e) 3 mg/kg diazepam + vehicle or 3 mg/kg flumazenil (f) 3 mg/kg diazepam + vehicle or 0.3 mg/kg Ro15-4513. Filled symbols denote significant difference vs. control. * denote significant difference for labeled point vs pretreatment with 1 mg/kg flumazenil or 0.3 mg/kg Ro15-4513, # significant difference for labeled point vs pretreatment with 3 mg/kg flumazenil or 1 mg/kg Ro15-4513.

Figure 14

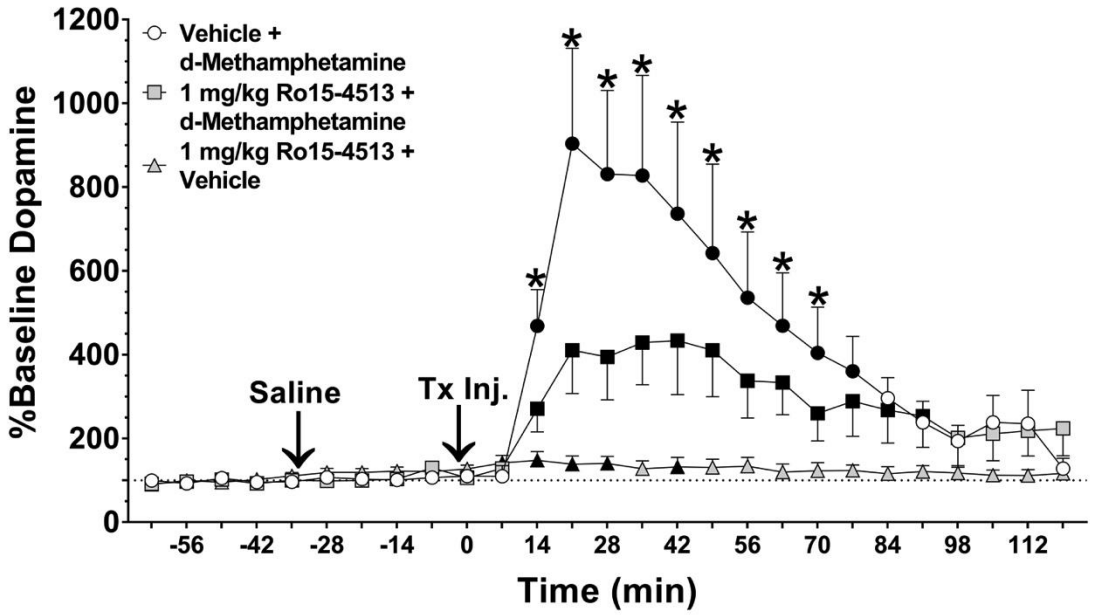

Figure 14: Mean baseline (±SEM) percent maximal dopamine levels as a percentage of control baseline for each respective group: Ro15-4513 + vehicle, vehicle + d-methamphetamine, and Ro15-4513 + d-methamphetamine. Points prior to first downward arrow (Saline) indicate equilibration baseline dopamine levels. Second downward arrow (TX Inj.) indicates injection time of vehicle + 3 mg/kg d-methamphetamine (open circles), 1 mg/kg Ro15-4513 + 3 mg/kg d-methamphetamine (grey squares), or 1 mg/kg Ro15-4513 + vehicle (grey triangle). Filled symbols denote significant difference compared to each treatment groups respective pretreatment mean (±SEM) dopamine baseline ($p < 0.05$). * denotes significant differences between vehicle + d-methamphetamine, 1 mg/kg Ro15-4513 + 3 mg/kg d-methamphetamine ($p < 0.05$).

Figure 15

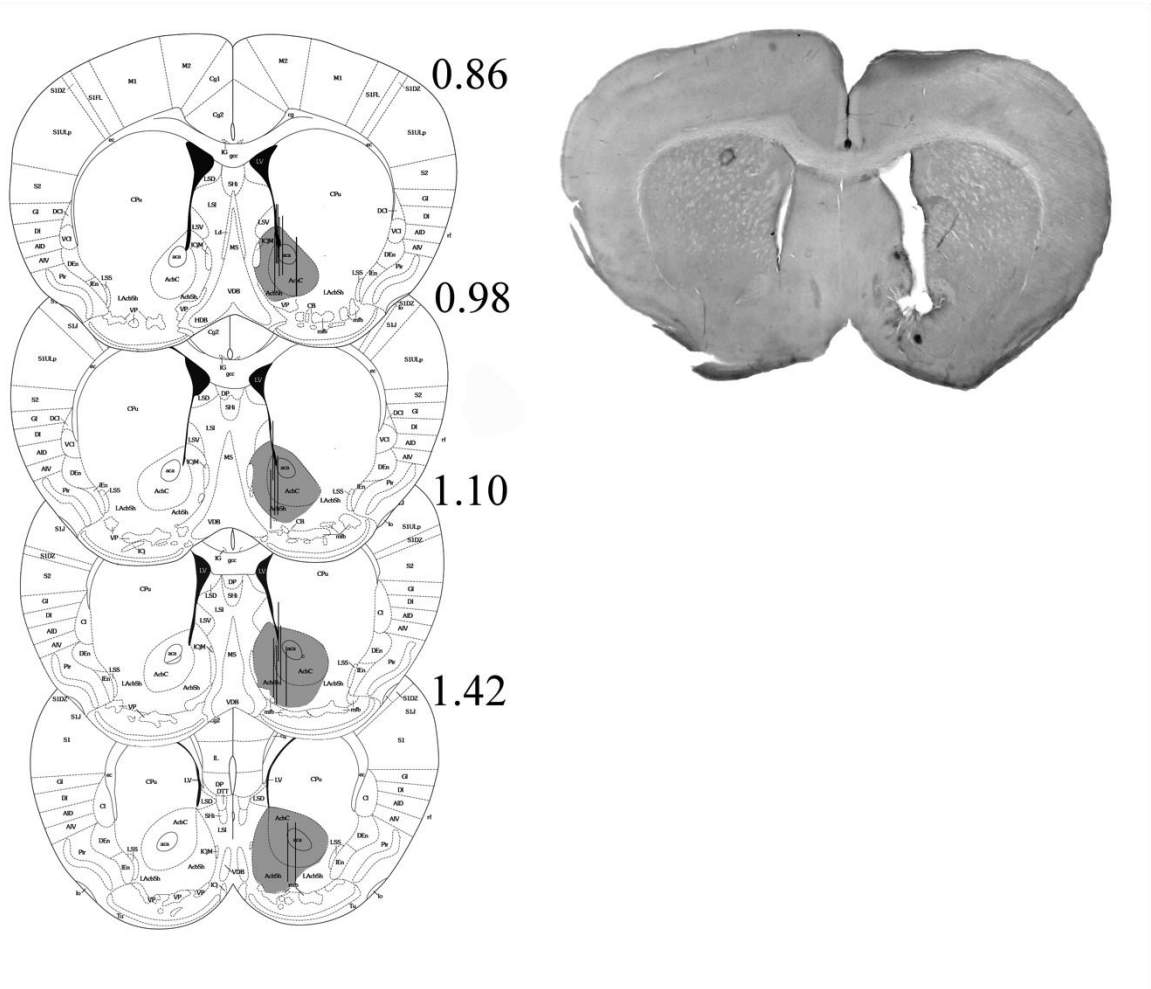

Figure 15: Serial coronal sections along anterior-posterior plane from 0.86 to 1.42 mm relative to bregma and representative photomicrograph. Black lines indicate 1mm long microdialysis probe membrane tracts. Shaded region indicates approximate location and size of nucleus accumbens (Paxinos and Franklin, 2001).

Discussion Aim 2

As expected based on prior studies, methamphetamine, toluene and diazepam all significantly facilitated ICSS responding (Bauer *et al*, 2013; Chan *et al*, 2012, 2015; Robinson *et al*, 2012; Tracy *et al*, 2014). Methamphetamine and toluene vapor produced comparably robust ICSS facilitation whereas the effect of diazepam on ICSS was modest relative to previous studies (Straub *et al*, 2010; Tracy *et al*, 2014). In the present study it was noted that increasing the toluene exposure concentration and shortening the pre-exposure duration prior to ICSS testing resulted in a much greater facilitation of ICSS than that reported following a longer exposure to a lower toluene concentration (Tracy *et al*, 2014). Although I did not explore this interaction systematically this finding parallels similar results with ethanol which also noted the greatest degree of ICSS facilitation was found immediately after alcohol administration (Fish *et al*, 2010). A shorter duration of exposure at higher toluene concentrations resulting in increased ICSS facilitation may be a result of conducting ICSS testing while drug levels are rapidly rising in the CNS. This interpretation is consistent with the observation in humans that the euphoric/hedonic effects of amphetamine and ethanol are most prominent when the drug plasma levels are on the ascending portion of the pharmacokinetic curve (Brauer *et al*, 1996; Lukas *et al*, 1986a, 1986b).

It was my goal to test the hypothesis that negative allosteric modulation of $GABA_A$ receptors via the benzodiazepine-site, rather than simply a blockade of the benzodiazepine-site, would attenuate the reward-related effects of the inhalant toluene as it has been demonstrated and shown to produce substantial neuropharmacological effects at $GABA_A$

receptors (Beckstead *et al*, 2000; MacIver, 2009). It was therefore important to differentiate the negative allosteric effects of Ro15-4513 from those produced by a BDZ-site antagonist, which itself in limited cases has been shown to reduce some of the behavioral effects of some volatile anesthetics (Dias Cicarelli *et al*, 2016; Liang *et al*, 2014). When the BDZ-site antagonist flumazenil was pre-administered before each drug, the ICSS facilitating effects of the BDZ site positive $GABA_A$ allosteric modulator diazepam were completely blocked by flumazenil. However, a dose of flumazenil sufficient to block a high efficacy benzodiazepine-site agonist had no effect on ICSS facilitation produced by either the monoamine releaser methamphetamine or the inhalant toluene. These data are consistent with flumazenil's clinical profile as a BDZ antagonist (Votey *et al*, 1991) and supports previous literature demonstrating that neither toluene nor methamphetamine interact directly with the BDZ binding site of $GABA_A$ receptors (Páez-Martínez *et al*, 2008). However, given toluene's interaction with $GABA_A$ receptors it remains plausible that other behaviors produced through toluene's positive allosteric modulation of $GABA_A$ receptors could be affected by flumazenil binding to the BDZ site such that the behavior could be altered.

With respect to preadministration of Ro15-4513, it was able to completely block methamphetamine, toluene and diazepam-facilitated ICSS. While toluene has positive $GABA_A$ modulatory effects (Beckley and Woodward, 2013; Beckstead *et al*, 2000; Shelton and Nicholson, 2013; Williams *et al*, 2005), d-methamphetamine does not (Hondebrink *et al*, 2011). Therefore, the hypothesis that toluene's reinforcement enhancing effects would be sensitive to Ro15-4513 negative allosteric $GABA_A$ modulation via the BDZ site is not

supported as Ro15-4513 blocked reward-like effects independently of the drugs pharmacological mechanism of action. Most surprisingly, the effect of Ro15-4513 on d-methamphetamine facilitation trended towards being even more suppressed than toluene. The observation thus leads to supporting the possibility that negative allosteric modulation of $GABA_A$ receptors can strongly affect the reward-related effects of drugs of abuse in general, regardless of their specific pharmacological mechanism of action.

It is important to note that Ro15-4513 has been previously shown to produce pronounced effects on operant behavior. For example, Ro15-4513 decreases operant behavior on a fixed ratio:15 and fixed interval:15 schedule of reinforcement in ICSS in rats (Schaefer and Michael, 1989). In self-administration studies in monkeys, an intramuscular injection at similar dosages as those used in this study produces generalized decreases in operant responding for both alcohol and a sweetened drink reinforcer (Shelton and Grant, 2001). It is therefore possible that any suppression of ICSS produced by Ro15-4513 may have been the result of a non-selective attenuation of operant performance. To address this concern, I assessed the effects of both Ro15-4513 and flumazenil alone on ICSS responding. In the subsequent interaction study, I only tested the 0.3 and 1 mg/kg doses of Ro15-4513 and the 3 mg/kg dose of flumazenil which were all well below doses which produced measurable effects on ICSS. I therefore am relatively confident that the effects observed when flumazenil or Ro15-4513 were pre-administered before drug treatment were not a result of non-selective rate-altering effects. However, it is possible that a higher dose of flumazenil may have been able to produce a measurable effect in ICSS. Although at higher doses flumazenil may produce quantifiable effects as a weak negative modulator

at select subtypes of the $GABA_A$ receptor (File *et al*, 1986; De Vry and Slangen, 1985) or even produce weak positive modulatory effects at $\alpha_{1\text{-}2}$-containing $GABA_A$ receptors (Smith *et al*, 2001), the latter of which are believed to be involved in the reinforcing and reinforcement enhancing effects of benzodiazepines (Engin *et al*, 2014; Rudolph and Knoflach, 2011; Tan *et al*, 2010).

A critical constituent in maintaining a subject's responding in intracranial self-stimulation is the indirect end stage component of mesolimbic dopamine release in the nucleus accumbens resulting from the direct phasic activation of neuronal substrates in the medial forebrain bundle. Therefore, it was plausible that Ro15-4513 would have little effect on mesolimbic dopamine release in the nucleus accumbens resulting from ICSS. However, it may substantially inhibit the ability of compounds to enhance mesolimbic dopamine release. To test whether Ro15-4513 was having a preferential effect on mesolimbic dopamine enhancement, I extended my behavioral results to a direct measure of mesolimbic dopaminergic activity utilizing a microdialysis procedure.

I therefore examined whether Ro15-4513 would attenuate d-methamphetamine stimulated dopamine release in the NAc. The choice of only examining d-methamphetamine was a pragmatic one, as amphetamine type stimulants are well established to produce much more robust dopamine release than either toluene or diazepam. It had been previously established that 3 mg/kg d-methamphetamine provided a sufficient experimental dynamic to detect changes in dopamine levels across multiple treatment conditions (Apawu *et al*, 2015; Invernizzi *et al*, 1991; Riegel *et al*, 2007). To allow direct comparison with the ICSS data, I utilized the same dose combination of 1

mg/kg Ro15-4513 with 3 mg/kg d-methamphetamine that was examined in the ICSS procedure and found to produce robust suppression of facilitation without producing a generalized suppression of ICSS responding when administered alone. Administration of 1 mg/kg Ro15-4513 with 3 mg/kg d-methamphetamine produced a ~58% reduction in peak dopamine release compared to that produced by d-methamphetamine alone. In contrast, administration of 1 mg/kg Ro15-4513 alone resulted in only a small increase in baseline dopamine levels, the magnitude of which was quite small relative to baseline. Time course differences between testing in ICSS and the observed peak dopaminergic activity were minimally separated when accounting for the lag time between microdialysis probe tip, the length of the connecting tubing and the collection apparatus as well as previously observed temporal differences in ICSS performance and changes in mesolimbic dopamine (Leitl *et al*, 2013; Miller *et al*, 2015). Under the limited set of experimental conditions examined, these data substantiate that exogenously induced negative allosteric modulation of $GABA_A$ receptors can attenuate drug-stimulated dopamine release within the NAc.

The critical modulatory role of $GABA_A$ receptors in the mesolimbic reward pathway is further supported by data showing that not only will systemically administered Ro15-4513 reduce drug reward-related behavior, but that Ro15-4513 microinjected into the posterior VTA of mice will reduce ethanol consumption without affecting non-drug reinforcers such as a 5% sucrose solution or water intake (Melón and Boehm, 2011). Additional evidence of the importance of $GABA_A$ receptors in modulating dopamine activity comes from non-pharmacological direct activation of VTA GABAergic neurons through optogenetics, which showed a profound effect on reducing the excitability on

downstream NAc dopamine release (van Zessen *et al*, 2012). A large population of these VTA GABAergic neurons provide local inhibitory GABA release onto dopaminergic neurons and in the presence of a negative allosteric modulator such as Ro15-4513 may significantly increase the interneuron's release of GABA. An interesting observation within the VTA is the differential expression of α_1 subunits in $GABA_A$ receptors located on VTA GABAergic neurons as compared to the presence of α_3 subunits in $GABA_A$ receptors located on VTA dopaminergic projecting neurons (Tan *et al*, 2010). It has been reported that Ro15-4513 shows higher efficacy at α_1-containing $GABA_A$ receptors for reducing chloride influx relative to α_3-containing $GABA_A$ receptors (Smith *et al*, 2001). These data support a differential action of Ro15-4513 on VTA interneurons while having little to no effect on α_3-containing $GABA_A$ receptors found on dopaminergic neurons themselves.

These results, when combined with previous findings, suggest that Ro15-4513 may enhance the function of inhibitory VTA GABAergic interneurons, which may substantially contribute to a reduction of exogenously stimulated dopamine release in the NAc. The observed complete blockade of methamphetamine facilitated ICSS by Ro15-4513 may partially depend upon the observed decrease in NAc dopamine release. However, given I administered Ro15-4513 systemically there are a substantial number of additional GABAergic pathways that could contribute to the present behavioral and neurochemical results, especially given the microdialysis data showing that methamphetamine-stimulated dopamine release is attenuated but not entirely blocked by Ro15-4513. The actions of Ro15-4513 in the NAc may result from disinhibition of multiple subtypes of GABAergic

neurons that influence the activity of dopaminergic medium spiny neurons (Russo and Nestler, 2013) or through effects on $GABA_A$ receptors found in the NAc on neurons which provide GABAergic feedback to the dopaminergic neurons within the VTA (Xia *et al*, 2011). Within the NAc, D1-containing medium spiny neurons project inhibitory feedback to the VTA which has been shown to preferentially release GABA on GABAergic neurons, some of which are known to provide inhibitory control of VTA dopaminergic projecting neurons. Repeated administration of drugs such as cocaine have been shown to strengthen the D1-containing medium spiny neuron ability to release GABA within the VTA, potentially enhancing the activity of dopaminergic neurons via disinhibition through inhibition of local VTA inhibitory GABAergic interneurons (Bocklisch *et al*, 2013).

Other examples of GABAergic connection that can influence mesolimbic dopaminergic activity are GABAergic connections from the rostromedial tegmental nucleus (RMTg) that project to the VTA. Behavioral and neuroanatomical data suggest that activation of RMTg neurons inhibits the locomotor and reward-like functions found in midbrain dopaminergic cells and is important in mediating and encoding signals of aversive stimuli (Jhou *et al*, 2009). The RMTg has been described as functional 'master braking system' for the reward processing system and the presence of Ro15-4513 could serve to disinhibit RMTg inhibition of dopaminergic projecting neurons within the VTA (Barrot *et al*, 2012). Further, the RMTg receives excitatory glutamatergic input from the lateral habenula which itself has strong GABAergic innervation from the medial globus pallidus (Araki *et al*, 1984; Shabel *et al*, 2012). Ro15-4513 could therefore function to

enhance excitatory connections from the lateral habenula, which would further the drive the activation of the inhibitory RMTg on dopaminergic projecting neurons.

Ultimately, given the diversity of $GABA_A$ receptors on neurons that feed into or are a part of the mesolimbic reward pathway, it is not possible to neuroanatomically isolate any one particular mechanism based on the present study. However, given the profound behavioral effects observed, determining whether the present results are the consequence of a specific regionally localized mechanism related to one $GABA_A$ receptor subtype or the summation of multiple $GABA_A$ receptor subtypes in several brain regions is an important future direction.

In summary the current data set shows that GABAergic negative allosteric modulation attenuates drug facilitated ICSS in mice across three abused drugs with differing underlying mechanisms of action. Given the *in vivo* microdialysis data these behavioral results may be at least partially mediated by attenuation of drug-stimulated dopaminergic output within the mesolimbic reward pathway. However, Ro15-4513 is a fairly nonselective tool, producing negative allosteric action at all $\alpha_{1\text{-}3,5}$-containing $GABA_A$ receptors (BDZ-sensitive) which are ubiquitously expressed through mammalian brains, therefore additional work is needed to clarify the role of specific $GABA_A$ subunits in this effect. Future experiments involving more selective pharmacological tools may help identify the specific brain regions that contribute to depression and facilitation of ICSS produced by drugs of abuse. From a clinical standpoint, selective allosteric modulation of $GABA_A$ receptor subtypes may be a promising avenue for developing medications which reduce the abuse-related effects of drugs without the potential adverse side effects

associated with nonselective $GABA_A$ negative modulators (Pokk and Zharkovsky, 1997; Venault and Chapouthier, 2007). Lastly, these results in conjunction with the outcome of the clinical trial currently underway with the Ro15-4513 analog iomazenil for treatment of ethanol intoxication, may suggest implications for the use of $GABA_A$ negative modulators as therapeutics for attenuating the effects of other drugs of abuse (D'Souza, 2015).

Aim 3: Effects of Chronic Intermittent Toluene on ICSS and Nesting Behavior

Materials and Methods

Subjects

A total of 24 and 12 adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine) were used for the ICSS and nesting procedures, respectively. Mice were individually housed under a 12h light/dark cycle and tested during the light phase of the cycle. Laboratory chow (Harlan, Madison, WI) and water were available *ad libitum* except during experimental sessions. All procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and were in accordance with NIH guidelines (National Research Council, 2011).

Compounds

Toluene was purchased from Fisher Scientific and stored in 500 mL Pyrex gas bubblers.

Dynamic Vapor Exposure System and Testing Apparatus

The inhalant exposure ICSS system consisted of four 20 liter acrylic cubicles each of which contained a two-lever operant conditioning chamber (Med-Associates, St. Albans, VT). A bipolar lead tether connection (Plastics One, Roanoke, VA) and mercury commutator (Mercotac 205L, Carlsbad CA) was suspended above the operant conditioning chamber by a counterbalanced arm. Toluene and TCE vapor were produced using a dynamic vapor generator composed of a filtered, pressure regulated air supply routed via tubing to two mass flow proportional valves controlled by a Matheson 8284 dynamic gas mixer (Matheson, Albuquerque, NM). The air output from one valve passed through a

fritted glass bubbler submerged below the liquid inhalant, generating an inhalant-laden air stream. The inhalant-laden air was mixed with metered fresh air from the second mass control valve permitting a broad range of highly reproducible inhalant concentrations to be generated. The blended vapor stream was routed through the upper rear wall of the operant conditioning chambers via Tygon tubing. The effluent mixture was tested from the front of the exposure chamber with a MiniRAE 2000 Volatile Organic Gas Detector (San Jose, CA). ICSS stimulation and operant schedule control was provided by commercially-available components (Med-Associates, St. Albans, VT). A summary schematic of the operant conditioning chamber housed within the gas exposure system with the accompanying effluent mixture piping and delivery is shown in figure 1a.

Chronic Dynamic Vapor Exposure System

Six sealable polycarbonate food storage containers (325x265x200mm) were utilized to construct the chronic exposure system. A high pressure air source was connected to a regulator lowering the pressure to 5 PSI. The low pressure air was then split into two individual airline circuits each connected to an electrically-operated solenoid valve (Open-frame/8003G/H), the output of each solenoid valve was then connected to a Matheson 604 rotameter to control air and toluene vapor flow rates. Air from the toluene circuit rotameter was then routed through a gas stone submerged in a 500 mL gas wash flask filled with toluene, creating a toluene vapor-laden air stream. The toluene vapor-laded air stream was then mixed with the independently regulated fresh air stream to produce a mixed stream at the appropriate toluene vapor exposure concentration. The mixed stream then flowed through a six channel divider to deliver toluene vapor to each of

the individual exposure chambers. Waste gas was expelled through a separate outflow circuit into a fume hood where it was evacuated from the building.

Testing indicated that following two minutes of continuous toluene vapor delivery into the chamber, the toluene concentration approached the 3300 ppm target (Figure 16a). Figaro 2620 volatile organic gas sensors were utilized to monitor real time changes in toluene concentration within the environmental chambers. Near steady-state concentrations of toluene were maintained at 3300 ppm by an Arduino Mega 2560 R3 microcontroller which switched the toluene valve off and on according to a binary harmonic sine function shown in figure 16b.

Gas Delivery Schedule

The gas delivery dosing schedule for the three experimental groups is shown in figure 17 (air control, chronic intermittent toluene and escalating chronic intermittent toluene). Subjects in the air control group received no toluene exposures but were otherwise treated identically to the toluene-exposed groups. Subjects in the chronic intermittent toluene (CIT) delivery and escalating chronic intermittent toluene (ECIT) delivery group were exposed to 3300 ppm toluene for 15 min every 72 hours beginning five minutes before the fourth component of the ICSS procedure. The escalating chronic intermittent group also received additional 15 min exposures to 3300 ppm toluene in their home cages. The daily number of additional exposures escalated every three days according to the following pattern: 1, 3, 6, 10 and 15 exposures (e.g. 1 exposure on days 1-3, 3 exposures on days 4-6, etc.). The subjects in the chronic intermittent and escalating exposure group were tested in ICSS at least 6 hours after the most recent toluene exposure

in order to reduce the likelihood of residual blood toluene impacting the ICSS or nesting test results.

IR/Vis Nesting and Monitoring Camera System

Twelve 2.1mm Wide Angle ELP Mjpeg Camera for Industrial Machine Vision (Ailipu Technology Co., Shenzhen, Guangdong, China) connected to three 4-Port independent USB Root Hub controller cards to allow for continuous video and image recording. The camera control script for the nesting procedure was developed in house on a Linux Mint 17.3 system using the Python 2.7 programming language and FFmpeg video and image library encoding software. These control programs have been made available for free download for academic or non-commercial purposes (https://github.com/metracy/METcamtimer).

ICSS Surgical Procedure

Mice were implanted under isoflurane and morphine (0.2 mg/kg s.c.) anesthesia with bipolar electrodes (Plastics One, Roanoke, VA) directed at the right medial forebrain bundle (Paxinos and Franklin, 2001). Coordinates relative to bregma were: -1.5 anterior-posterior, 0.9 medial-lateral, and -4.9 dorsal-ventral. Mice were treated 4 days' post-surgery with 0.5 mg/kg/day of the analgesic carprofen and allowed 6 days' recovery prior to beginning training.

ICSS Rate Frequency Procedure

Mice were trained to respond on a FR1 at 141 Hz frequency at 100 uA. If responding did not equal or exceed 30 stimulation deliveries per minute the amperage was increase 25% and training continued. If responding was still below criteria level, this

process was repeated. Any animal that failed to respond at criteria level at a stimulation amperage of 350 was removed from the study. Following stable responding at ≥ 30 responses/min, mice were transitioned to a repeated 10-minute fixed ratio 1 with a 10-minute timeout at the set amperage from the FR1 and a frequency of 158 Hz. Following reliable responding during the active component with a ratio greater than eight for the lever presses in the active 10-minute component over the inactive one, mice were transitioned to a four component rate-frequency procedure. Rate-frequency sessions were approximately 65 min, divided into four 10-min response components separated by time outs. The use of timeouts between response components allowed for adequate time for vapor levels of toluene to reach near steady-state levels within the chamber to a single test session. Each response component consisted of ten 1-min trials beginning at a stimulation frequency of 158 Hz and descending by 0.05 log units in each subsequent trial. On toluene test days, toluene exposure began five minutes before the start of the 4th ICSS component and continued for the duration of the 4th component (15 minutes of total toluene exposure per session).

Nesting Procedure

Pressed cotton nesting sheets (6 cm x 6 cm x 0.1 cm) were cut into six equal sized rectangular pieces and placed at each of the four corners and at adjacent edges at the midpoint of the polycarbonate home cage of the chamber, dividing the cage in six approximate equal rectangles. The time required for a subject to clear zones of the nesting material was measured over the course of 100 minutes. An overhead camera captured an image of the subject's progress along a logarithmic time scale beginning at 34 seconds and

proceeding by 0.25 log units over the course of the 100 minute (6000 seconds) test session. A representative image of a mouse engaging in the nesting behavior is shown in figure 18. Due to a known effect of clean cages affecting behavioral performance (Bouchatta *et al*, 2016; Negus *et al*, 2015), mice were only tested in their own home cage and cages were kept clean by gradual removal of old bedding and addition of new bedding to keep the integrity of olfactory cues.

Data Analysis

In order to examine effects over time, the maximum number of earned stimulations from the second and third components during the first four days of the experiment were averaged for each subject to compute that subject's baseline maximum earned responses (MER). Starting on day five, all earned stimulations for every component at each frequency were normalized by dividing it by each subject's MER to allow for tracking changes in earned stimulations over time across the different experimental conditions. These percent values are reported as a percentage of the maximum control responses (%MCR). For each ICSS session, the baseline was taken as an average of the computed %MCR from the second and third components at each individual frequency. This allowed for the baseline of 10 values across the rate-frequency curve to be subsequently compared to the 10 values obtained following delivery of toluene exposure which began five minutes prior to the 4th ICSS component.

For each individual day within the experiment, data from the baseline and 4th test component were compared individually within each group by 2-way within subject repeated measures analysis of variance (ANOVA) (Negus and Miller, 2014). GraphPad

version 7.0 for Windows (La Jolla, CA) was used for all analyses. For within session curves, significant ($p < 0.05$) main effects and interactions were subsequently examined by the two-stage step-up method of Benjamini, Krieger and Yekutieli to compare identical frequencies across the control and test components (Benjamini *et al*, 2006). This procedure for correcting for the multiple comparisons problem is more powerful than Bonferroni type corrections that provide for a strong correction for the overall the family-wise error rate (Glickman *et al*, 2014). This procedure gains power by assuming that either all comparisons that are being made either have a positive-dependence on each other (i.e. comparison of points along parallel curves) or that each comparison is independent of every other comparison. It does this by a two stage procedure that first controls for the false discovery rate of the hypotheses being tested by examining the overall P values of the comparisons being made in order to estimate the proportion of the null hypotheses that are actually true. The algorithm then uses the proportion of null hypotheses that are actually true to set the lower limit for when a P value is sufficiently low to be called a true discovery.

In order to facilitate analyzing between group variables in an ICSS procedure, each baseline and treatment components had the area under the curve (AUC) calculated from the log transformed rate-frequency curve. Each AUC value was then turned into a percentage of the average of each groups first four pre-toluene or pre-air exposure days by dividing each day's AUC by their group average AUC. All references to %pre-exposure baselines or AUC are referring to this data having being normalized in this manner.

These values were then examined in a two-way mixed measures ANOVA comparing differences in baseline brain stimulation across all three test groups. In addition, a linear regression model was fitted to the baseline AUC group data to determine relationships between a continuous predictor variable (repeated toluene exposures) and the area under the curve (the overall amount subjects respond for varying intensities of brain stimulation across a rate frequency entire curve). An advantage of using linear regression in addition to the ANOVA is that ANOVAs ignore the order of cumulative repeated exposures and treat each test exposure as a factor independent of any directional trend (Motulsky and Christopoulos, 2004). Significance was determined by comparing the y-intercept (the baseline starting point) ± SEM and the slope (rate of change for each group) ± SEM between regression lines and whether the 95% confidence intervals for the two regression lines overlapped across the modeled data sets.

Chronic Intermittent Exposure Effects on Baseline and Drug Facilitated Brain Stimulation Reward

To assess the effects of repeated toluene exposure on responding for brain-stimulation following acute toluene exposure, ECIT subjects were assessed in ICSS after six hours of air exposure since their last 15-minute exposure to toluene. This period of air exposure was designed to reduce any residual toluene blood levels in the ECIT exposed group to trace levels. To examine whether CIT exposure would produce changes in its reinforcement enhancing effects of brain stimulation, toluene was assessed in ICSS every 72 hours for the CIT and ECIT exposure groups following a baseline period of 5 days.

Area under the curve was computed by transforming the frequency to a log scale and summing the area under the baseline and 4th test component curves.

Results

Effect of Repeated Exposures of Toluene on Acute Toluene-facilitated ICSS

The results for the individual within-subject two-way repeated measures ANOVAs are shown in table 4. Each group's daily baseline ICSS rate frequency curve was compared to their respective 4th component ICSS curve. For the control group, only air was administered across all 24 days during the 4th component. For the chronic intermittent toluene (CIT) and escalating chronic intermittent toluene (ECIT) exposure groups, on days 5, 8, 11, 14, 17, 20 and day 24 a concentration of 3300 PPM toluene was administered 5 minutes before the 4th component. For the CIT and ECIT groups that were exposed to toluene, at no point in the days in which they were only exposed to air did their within-session baseline ICSS curve differ from their air treatment condition (4th component). A detailed summary of significant 2-way within-subject ANOVA data is shown in Table 4. In summary, for the chronic intermittent exposure group on days in which 3300 ppm toluene was administered prior to the 4th component, the number of significantly facilitated frequencies are as follows: test day 5 – 7 frequencies, test day 8 – 7 frequencies, test day 11 – 7 frequencies, test day 14 – 10 frequencies, test day 17 – 7 frequencies, test day 20 – 7 frequencies, test day 24 – 7 frequencies. For the escalating chronic intermittent exposure group on days in which 3300 ppm toluene was administered prior to the 4th component, the number of significantly facilitated frequencies are as follows: test day 5 – 6 frequencies, test day 8 – 5 frequencies, test day 11 through 17 – NS, test day 20 – 3 frequencies, test day 24 – NS frequencies.

Area under the Curve of Repeated Exposures of Toluene in ICSS

The percentage of pre-exposure baselines for the CIT and the ECIT experimental conditions (toluene exposure) were compared to allow for a direct comparison of toluene's facilitatory effect as a function of repeated cumulative exposures of toluene. A two-way between groups ANOVA was conducted on the two groups (Figure 19). There was a significant main effect of toluene exposure schedule on the area under the curve [F (1, 77) = 62.78 P < 0.0001], no effect of exposure day [F (6, 70) = 0.8834, P=0.5113], but a significant interaction [F (6, 70) = 2.432, P=0.0450]. For the ECIT group, the AUC on the 3, 4, 5, 6 and 7th acute exposure tests on were significantly below the facilitation produced by the CIT group, suggesting tolerance to the acute reinforcing effects between group types. When the ECIT toluene tests were compared to their first toluene test, the 3, 4, 5 and 6^{th} tests were significantly depressed, indicating a tolerance compared to their first reinforcement enhancing.

ICSS test day comparison of within-group baseline to its test condition

When a linear regression model was applied to the air control group's baseline and air treatment test days, the null hypothesis that one regression model would fit both data sets was tested and accepted [F (2, 68) = 0.109, p = 0.8969]. The model fit both data sets with an adjusted r^2 of 0.8433 (figure 20a). The best fit value for the Y-intercept for both data sets was 95.02±6.563. The best fit value for the slope for the regression for both data sets was 0.7867±1.685.

When a linear regression model was applied to the CIT group's baseline and acute toluene exposure test days, the null hypothesis that one regression model would fit both

data sets was tested and rejected [F (2, 68) = 57.98, p < 0.0001]. The models fit each data sets with an adjusted r^2 of 0.853 for the baseline and a r^2 of 0.8607 (figure 20b). The best fit values for the Y-intercept for the baseline regression line was 111.1±10.77 and for the test baseline was 165.9±8.154. The best fit values for the slope for the baseline regression line was 0.3295±2.765 and for the test baseline was 2.872±2.094.

When a linear regression model was applied to the ECIT group's baseline and acute toluene exposure test days, the null hypothesis that one regression model would fit both data sets was tested and rejected [F (2, 68) = 6.935, p = 0.0018]. The models fit each data set with an adjusted r^2 of 0.8544 for the baseline and an r^2 of 0.8756 (figure 20c). The best fit values for the Y-intercept for the baseline regression line were 95.34±14.23 and for the test baseline was 152.8±15.03. The best fit values for the slope for the baseline regression line was -2.133±3.654 and for the test baseline was -9.596±3.86.

Effects of Escalating Chronic Intermittent Toluene Exposure on ICSS Baseline Responding

To compare how each group's baseline changed across the experimental sessions, the three groups were compared with a two-way mixed groups ANOVA. There was a significant main effect of treatment condition [F (2, 300) = 11.95, P<0.0001] but no effect of exposure day [F (19, 300) = 0.306, P=0.9981] nor a significant interaction [F (38, 300) = 0.5017, P=0.9942].

When a linear regression line was applied to the AUC of the baseline stimulations across the CIT and ECIT groups the null hypothesis that one regression line would fit both data sets was tested and rejected [F = 3.459 (2, 354), P = 0.0325]. The regression models

fit the data with an adjusted r^2 of 0.841 and 0.84447 for CIT and ECIT groups respectively. The two lines with 95% confidence intervals are plotted in figure 21. The best fit values for the Y-intercept for the CIT regression line were 101.8±5.161 and for the ECIT regression line was 100.6.8±6.543. The best fit values for the slope for the CIT regression line was 0.5285±0.4308 and for the ECIT was -1.125±0.5387.

Rate-frequency curves at first exposure and 5th Exposure for Air, CIT and ECIT Groups

A comparison of the rate-frequency curves across the three groups at the first test day (day 5) and the 5th test day (day 17) are shown in figure 22. A two-way within-subject ANOVA was performed on the %MCR for baseline and exposure test for each group on day 5 and 17. For the air control group on test day 5, the baseline curve did not significantly differ from the air exposure condition (Figure 22a). On day 17, again, the baseline curve did not significantly differ from the air exposure condition (Figure 22b).

For the CIT group on day 5 (Figure 22c), there was a significant main effect [F (1, 5) = 7.416, P = 0.0416)] but no significant drug x frequency interaction [F (9, 45) = 1.258, P=0.2860] with responding at 7 frequencies significantly increased over control. For the CIT group on day 17 (Figure 22d), there was a significant main effect [F (1, 5) = 21.96, P=0.0054] and a significant drug x frequency interaction [F (9, 45) = 3.763, P =0.0014] with responding at 7 frequencies significantly increased over control.

For the ECIT group on day 5 (Figure 22e), there was a significant main effect [F (1, 5) = 31.8, P=0.0024] and a significant drug x frequency interaction [F (9, 45) = 2.836, P = 0.0099] with responding at 6 frequencies significantly increased over control. For the

ECIT group on day 17 (Figure 22f), there was no significant main effect [*F* (1, 5) = 0.005352, P=0.9445] and no significant drug x frequency interaction [*F* (9, 45) = 1.616, P=0.1395] with responding at no frequencies different than control.

Effects of Chronic Intermittent Toluene Exposure on Nesting Behavior

The area under the curve for the air control and escalating chronic intermittent exposure condition were computed to allow for direct comparison of repeated toluene exposure on performance in the nesting procedure while in a drug free state. A two-way between groups ANOVA was conducted between the two groups (Figure 23). There was a significant main effect of exposure day on the area under the curve [F (19, 200) = 5.864, P <0.0001], no main effect of exposure day [F (1, 200) = 3.375, P=0.0677] but there was a significant interaction [F (19, 200) = 1.949, P=0.0126]. Post-hoc analysis revealed nesting performance on day 13 following 6 exposures/day, day 14 following 10 exposures/day, and day 15 following 10 exposures per day was significantly decreased for the chronic escalating group compared to air control.

Acute Exposure of Toluene on Nesting Behavior

Acute exposure to 15 minutes of 3300 ppm toluene resulted in a significant right and downward shift in the nesting completion time curve (Figure 24). A two-way within-subject repeated measures ANOVA was conducted on mice that were first exposed to air and subsequently to toluene. There was a significant main effect of time on zone [F (10, 50) = 111.7, P<0.0001], a significant main effect of toluene exposure [F (1, 5) = 147.7, P<0.0001], and a significant interaction [F (10, 50) = 3.188, P=0.0031].

Table 4

Exposure Day	Control	CIT	ECIT
1 Air	NS	NS	NS
2 Air	NS	NS	NS
3 Air	NS	NS	NS
4 Air	NS	NS	NS
5 Air/Tol	NS	7↑ ME: [F (1, 5) = 7.416 , P=0.0416]	6↑ ME: [F (1, 5) = 31.8 , P=0.0024] INT: [F (9, 45) = 2.836 , P=0.0099]
6 Air	NS	NS	NS
7 Air	NS	NS	NS
8 Air/Tol	NS	7↑ ME: [F (1, 5) = 2.334 , P=0.0297]	5↑ ME: [F (1, 5) = 21.56 , P=0.0056] INT: [F (9, 45) = 5.445 , P<0.0001]
9 Air	NS	NS	NS
10 Air	NS	NS	NS
11 Air/Tol	NS	7↑ ME: [F (1, 5) = 27.55 , P=0.0033] INT: [F (9, 45) = 3.804 , P<0.0012]	NS
12 Air	NS	NS	NS
13 Air	NS	NS	NS
14 Air/Tol	NS	10↑ ME: [F (1, 5) = 45.16 , P=0.0011] INT: [F (9, 45) = 3.286 , P<0.0037]	NS
15 Air	NS	NS	NS
16 Air	NS	NS	NS
17 Air/Tol	NS	7↑ ME: [F (1, 5) = 21.96 , P=0.0054] INT: [F (9, 45) = 3.763 , P<0.0014]	NS
18 Air	NS	NS	NS
19 Air	NS	NS	NS
20 Air/Tol	NS	7↑ ME: [F (1, 5) = 12.43 , P=0.0168] INT: [F (9, 45) = 5.348 , P<0.0001]	1↑2↓ INT: [F (9, 45) = 3.521 , P= 0.0023]
21 Air	NS	NS	NS
22 Air	NS	NS	NS
23 Air	NS	NS	NS
24 Air/Tol	NS	7↑ ME: [F (1, 5) = 28.21 , P=0.0032] INT: [F (9, 45) = 3.208 , P<0.0044]	NS

Table 4: Statistical difference in ICSS using within-session responding during the baseline component (Air) and testing component (Air/Tol) for control, chronic intermittent toluene (CIT), and Escalating Chronic Intermittent Control (ECIT). Red denotes toluene test days for CIT and ECIT. ME – Main Effect, INT – Interaction, NS represents not significant, # and the arrow ↓↑ represents number of statistically significant facilitated points on the curve and the direction of significance for the frequency in the testing component.

Figure 16a

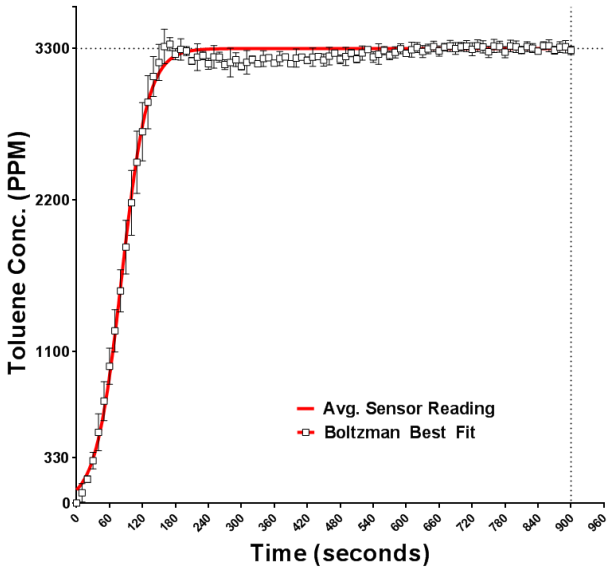

Figure 16a: Toluene concentration as measured with volatile organic compound sensors. The red line is the best fit non-linear regression line to an average (±SEM) of the sensor data collected within three of the polycarbonate chambers during toluene exposure.

Figure 16b

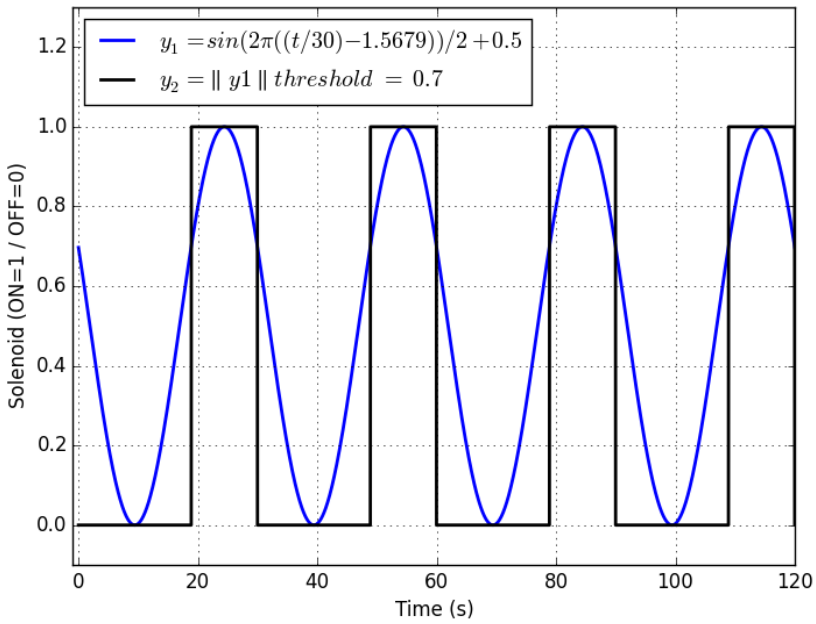

Figure 16b: Blue line y_1 represents oscillating function that varies with time that provides the necessary temporal separation for maintaining a relatively continuous concentration of toluene within the chamber. When the binary function shown by the black line y_2 is 1, the toluene solenoid valve is set to on and air allowed to bubble through the 500 ml bubbler. When set to 0 the valve remains shut off.

Figure 17

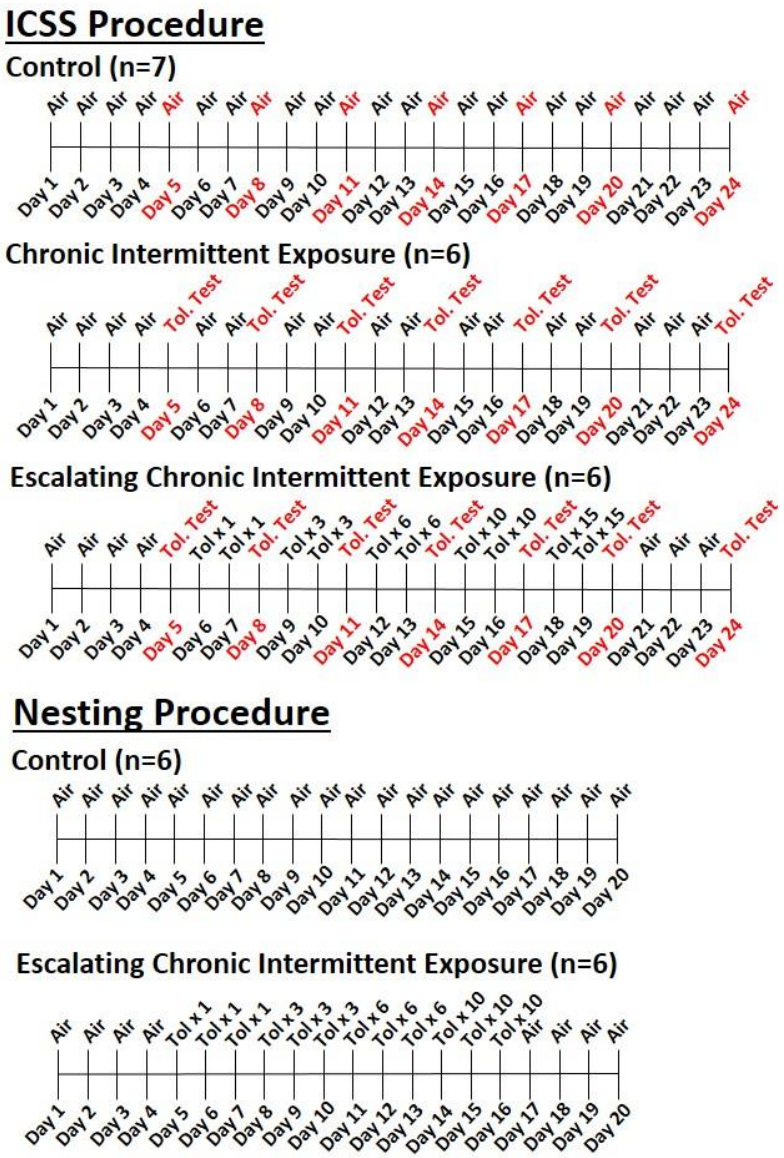

Figure 17: Order and schedule of the three ICSS and two nesting experimental groups. Red represents test days where Chronic Intermittent and Escalating Chronic Intermittent exposure groups were administered toluene five minutes before the start of the fourth component.

Figure 18

Figure 18: Still frame from the video feed at the start of the nesting procedure. Six individually placed nestlets are placed at the six positions and throughout the course of the 100 min session the mice will consolidate the nestlets into a corner.

Figure 19

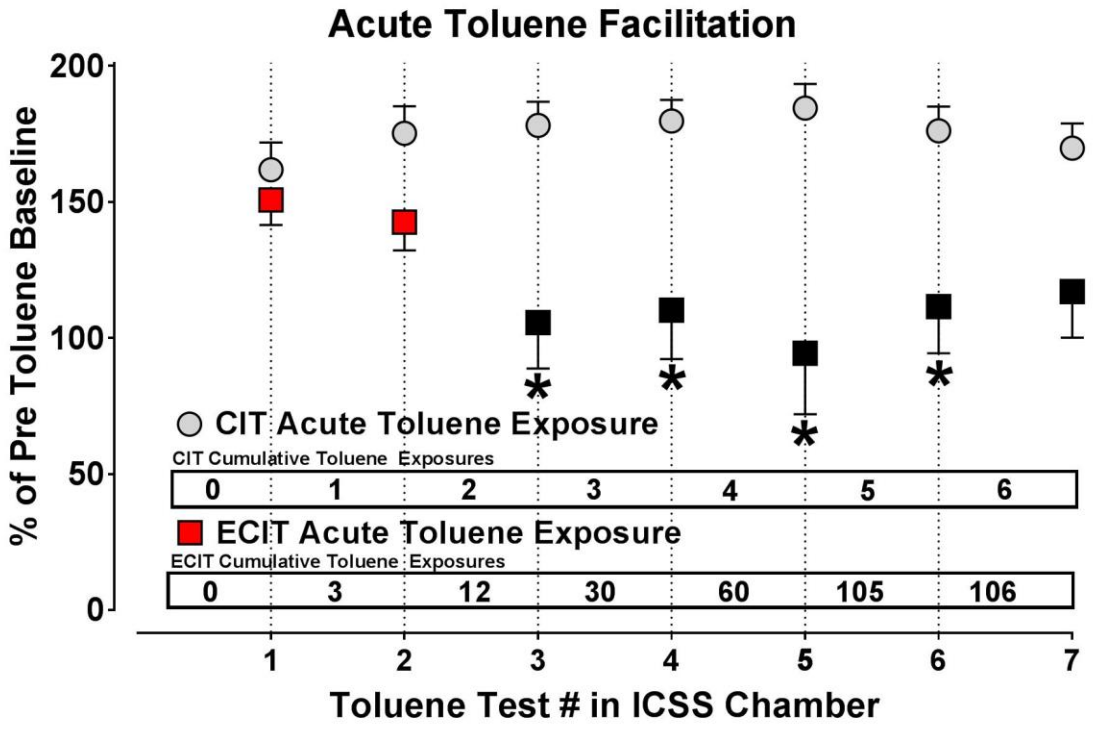

Figure 19: Comparison of acute toluene exposure on facilitation of ICSS relative to toluene pre-exposure baselines. Statistical significance between CIT and ECIT test day is denoted by filled black squares. * denotes statistical significance from ECIT's first toluene test. Number of cumulative exposures prior to each test for each group are bound in the black boxes.

Figure 20

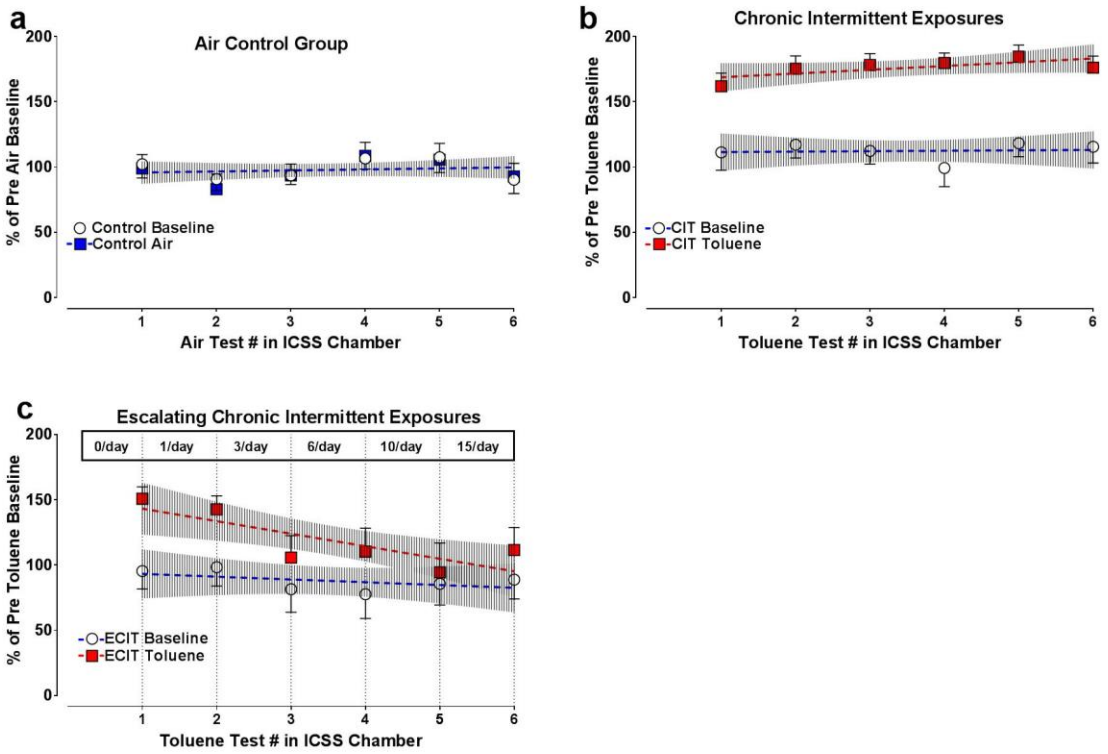

Figure 20: ICSS test day data showing the area under the curve for baseline and test components that were performed on day 5, 8, 11, 14, 17, and 20. All data are shown as a percentage of their pre-exposure baselines from day 1-4 with regression lines drawn for the baseline in dashed blue and test component in dashed red a) Air control baseline data (white circle) with air test exposure data (blue square) b) Chronic intermittent baseline data (white circle) and acute 3300 ppm toluene exposure (red square). c) Escalating chronic intermittent baseline data (white circle) and acute 3300 ppm toluene exposure (red square).

Figure 21

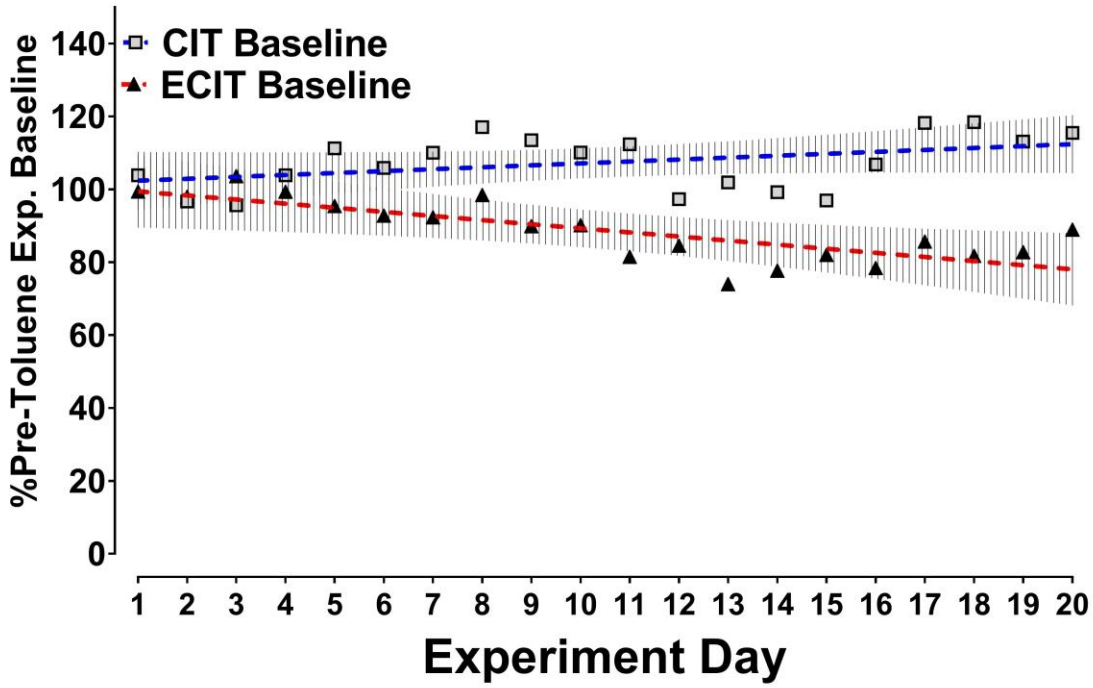

Figure 21: Linear regression lines with 95% confidence intervals (shaded areas) for trend lines fitting the comparison of the %Pre-Toluene Exp. Baseline for the area under the curve for baseline stimulations earned within ICSS for the chronic intermittent toluene (CIT) exposure and the escalating chronic (ECIT) exposure groups.

Figure 22

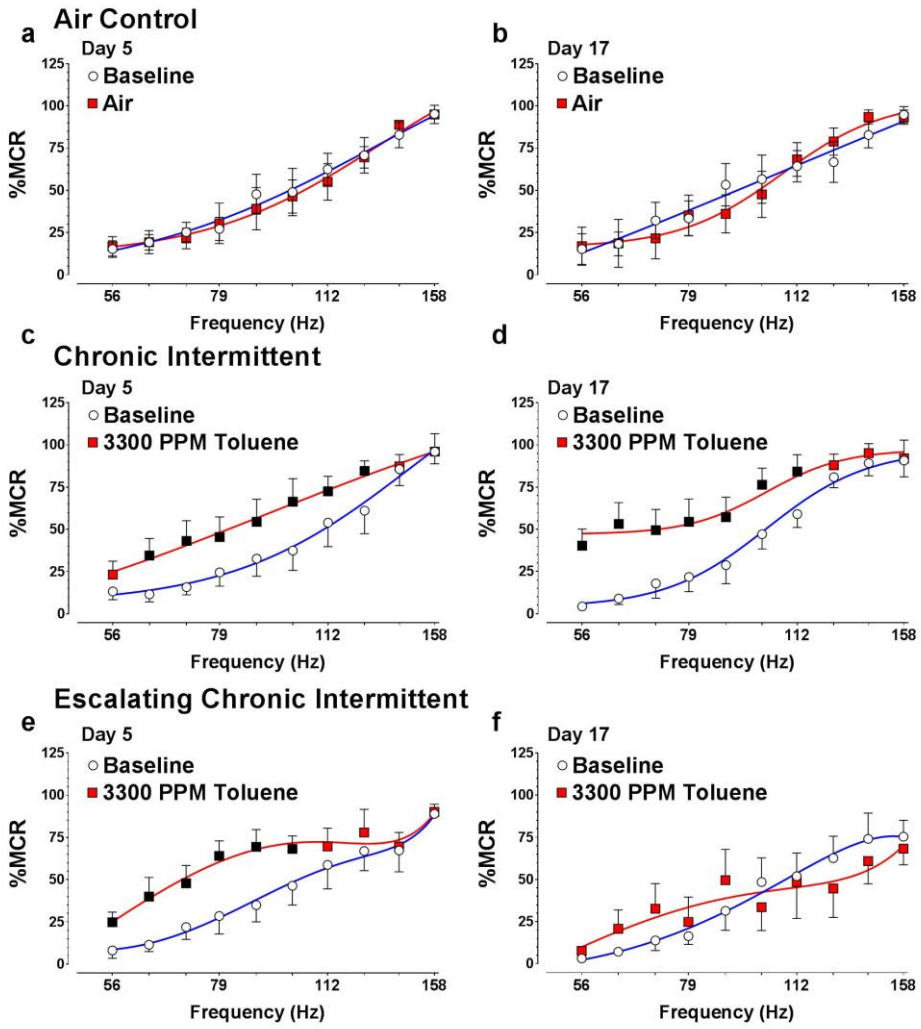

Figure 22: ICSS Rate-frequency data for test day 5 and test day 17 for air control, CIT and ECIT groups. Statistical significance between adjacent frequencies denoted by filled black symbol. a) Air control group baseline rate-frequency curves for baseline compared to air exposure on day 5. b) Air control group baseline rate-frequency curves for baseline compared to air exposure on day 17. c) CIT group baseline rate-frequency curves for baseline compared to 3300 ppm toluene exposure on day 5. d) CIT group baseline rate-

frequency curves for baseline compared to 3300 ppm toluene exposure on day 17. e) ECIT group baseline rate-frequency curves for baseline compared to 3300 ppm toluene exposure on day 5. f) ECIT group baseline rate-frequency curves for baseline compared to 3300 ppm toluene exposure on day 17.

Figure 23

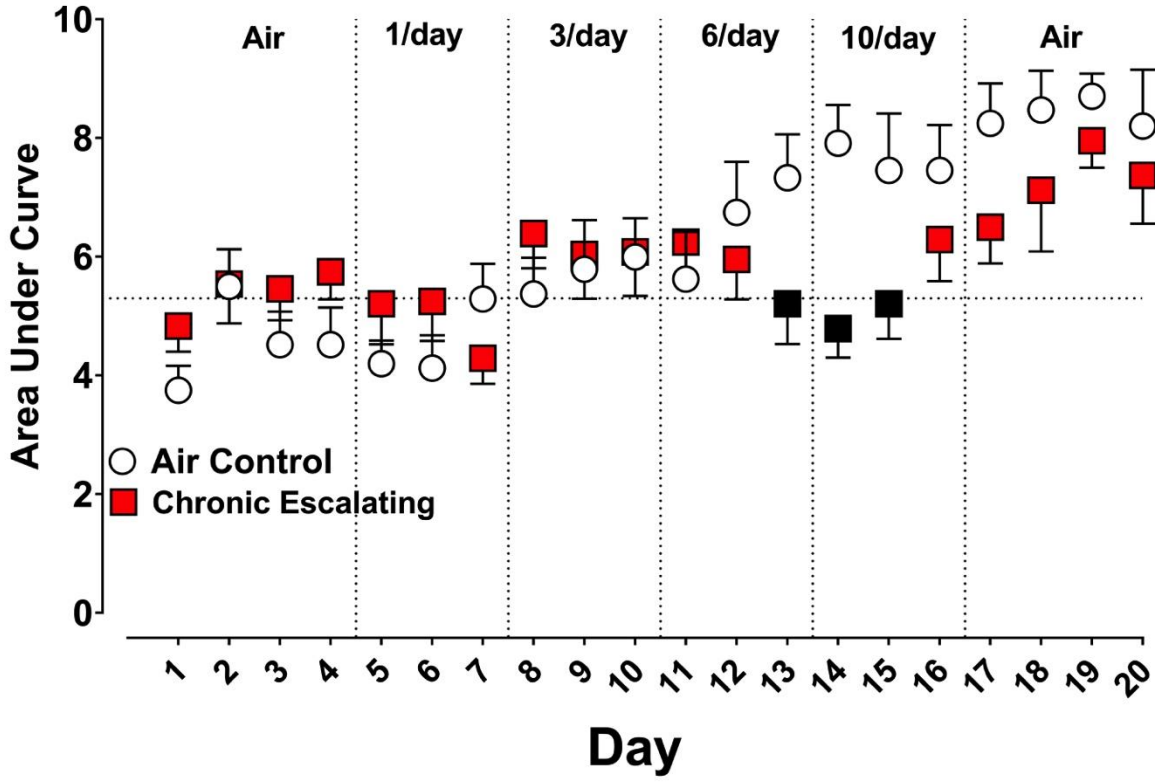

Figure 23: Comparison of the nesting data using the area under the curve for each day to compare air control and repeated chronic escalating toluene (ECIT) exposure in the nesting procedure. Statistical significance is denoted by filled black squares.

Figure 24

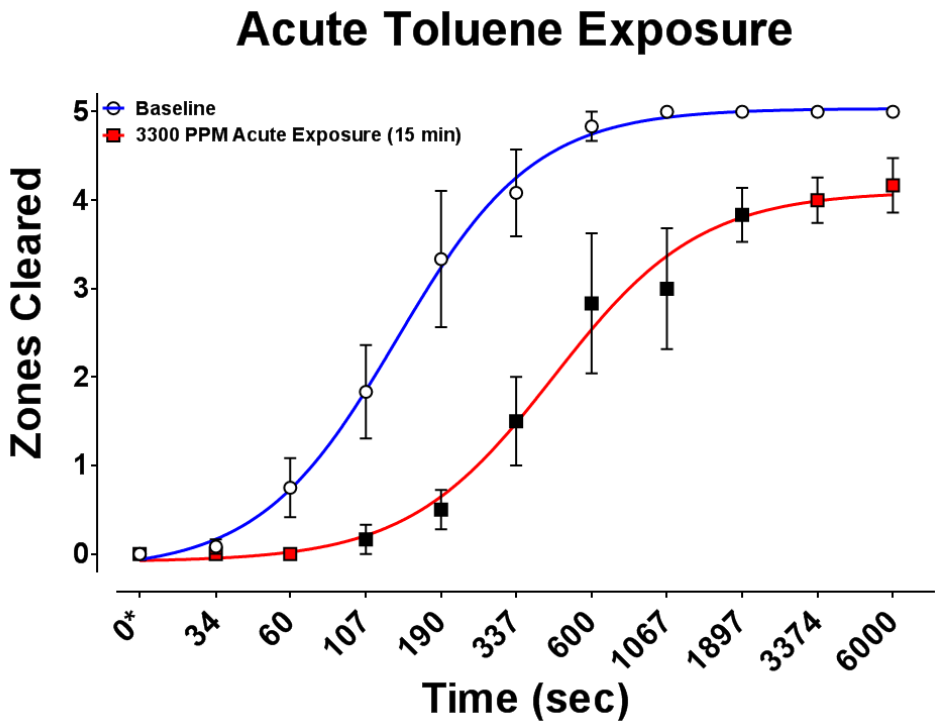

Figure 24: Number of zones cleared in the nesting assay during baseline and acute exposure from naïve mice. Statistical significance denoted by filled black symbols.

Discussion Aim 3

Previous literature has demonstrated that across a broad range of drug classes and ICSS test conditions, that following repeated administration of drugs of abuse there is an emergence of anhedonic-like effects. These effects can be quantified in ICSS using multiple response parameters such as curve shifts or increases in threshold that account for changes in responding for direct electrical stimulation of the mesolimbic reward pathway (Holtz *et al*, 2015; Markou and Koob, 1991; Paterson *et al*, 2000; Schulteis *et al*, 1995; Stoker and Markou, 2011). Based upon the results in figure 21, the ECIT toluene group produced a significant negative slope downwards relative to repeated exposures to CIT. The negative slope indicates that as a function of escalating repeated toluene exposures the reinforcing efficacy of brain self-stimulation decreases. This result would be consistent with the hypothesis that escalating the number of toluene exposures produces a cumulative decrease in overall responding for brain stimulation while in a drug free state. Indicating that repeated toluene exposure decreases the functionality of the mesolimbic dopamine system.

Interestingly, the decrease in the reinforcing efficacy of mesolimbic brain self-stimulation in ICSS in part agrees with a recent striatal dopamine dynamics study in which it was demonstrated that a significant decrease in levels of electrically evoked dopamine release in the nucleus accumbens core and shell occurred 24 hours after repeated 2000 or 4000 ppm toluene exposure for seven days (Apawu *et al*, 2015). However, it is difficult to draw conclusions comparing the results as the toluene concentrations, exposure times,

exposure schedules and withdrawal times were all different relative to the ones in this study.

Across the air and CIT groups, the results from the within-session comparisons in table 4 combined with baseline data over time (Figure 20a-b) showed that the baselines for these two groups remained relatively stable while ECIT produced a negative trend in baseline stimulations over time (Figure 20c and Figure 21). The first major comparison was within-groups. The cumulative effect of toluene exposure in the ECIT group decreased the degree of facilitation resulting from acute toluene (Figure 20c) as well as the number of frequencies that were significantly facilitated (Table 4). When acute toluene was tested in the CIT group, the cumulative effect of previous toluene exposures every three days did not appear to affect toluene's acute ability to facilitate ICSS (Figure 20b and Table 4).

The ECIT group's result is interesting in that the rate at which the groups baseline declined (negative slope Figure 20c) over repeated exposures was substantially less compared to the rate at which the ECIT's acute toluene exposure declined to baseline levels. Contrary to most observed effects in ICSS involving escalating dosages of a drug, most studies have reported tolerance to their rate-decreasing effects. An example of this phenomenon is with morphine, in which escalating dosages unmask otherwise initially weak drug-induced ICSS facilitation (Altarifi and Negus, 2011). Interestingly the tolerance-like effect observed with acute toluene in the study has not been reported in the literature for other drugs of abuse. However, sensitization to toluene in other behaviors such as locomotion has been observed (Apawu *et al*, 2015; Bowen *et al*, 2010), though the chronic effects of repeated toluene exposure have not been explored in great detail in

ICSS. Therefore, it is entirely possible that use of different exposure concentrations, exposure durations, or utilizing an intermediate schedule between the observed diminishing toluene facilitation in the ECIT group and the relatively unchanging toluene facilitation observed in the CIT may produce more diverse behavioral results in ICSS. For example, the significant increase in magnitude of facilitation seen by increasing the concentration of toluene and shortening the duration of exposure in the acute toluene ICSS studies described in Aim 1 and 2 may have similar parallels within chronic exposure ICSS paradigms described in Aim 3.

My third hypothesis was that following cessation from an escalating chronic intermittent toluene exposure schedule, responding for brain stimulation would be further suppressed. Presumably, following the last toluene exposure on day 20, any physiological compensatory changes induced to oppose the effects of repeated toluene exposure would not have returned to pre-exposure levels. Therefore, it was plausible that in the absence of toluene exposure the daily baselines measured in ICSS could further decrease, which would be indicative of a withdrawal syndrome. Twenty-four hours following the last exposure to escalating toluene exposures on day 21, the ECIT exposure group's baseline remained at levels consistent with the previous four days. Subsequently there was no significant shift in ECIT's baseline through day 24 indicating the absence of any spontaneous protracted withdrawal effects as compared to the acute withdrawal effects measured by the daily baselines on days 18, 19 and 20. Therefore at least under these set of conditions, the hypothesis that cessation of toluene from the escalating chronic intermittent exposure schedule would further suppress ICSS responding is rejected.

My fourth hypothesis was that repeated chronic intermittent exposure to toluene would produce gradual deficits in goal-directed behaviors other than ICSS as measured by the nesting procedure. The results showed that the ECIT group in the nesting procedure did significantly fall below the air control levels of completing the nesting procedure on three days. Figure 23 shows that after multiple trials for the air control group the baseline gradually shifted upwards, indicating a learned component in the procedure that enhances performance. The presence of a learned component complicates interpretation as repeated toluene exposure has been shown to produce adverse effects on learning and memory tasks (Dick *et al*, 2014) and this is why the nesting procedure was performed in a drug free state to minimize this direct effect as we demonstrated that acute exposure disrupts nesting behavior (Figure 24). Additionally, repeated escalating toluene exposure has been shown to alter glutamatergic and GABAergic in the hippocampus (Bale *et al*, 2005) which may impact or slow the rate of enhancing performance of nesting behavior. Therefore, it is entirely likely that multiple processes may be co-occurring for the escalating chronic intermittent group with manifestation of withdrawal effects at the same time as they continue to increase in performance as a result of repeated trials. By day 12 (figure 23), for the chronic intermittent group the separation between the two groups begins. Finally, by day 13-15 the groups have significantly separated as measured by the two-way repeated measures ANOVA post-hoc test. However, despite repeated chronic intermittent toluene from day 14 onwards the linear trend for the chronic intermittent exposure group is a constant climb through day 17 (first day no toluene exposure) until the data are not visually different from the air control group. Thus interpretation of nesting with all these possible

opponent processes co-occurring makes clear and simple interpretation difficult. In future studies however it would be highly advantageous to determine performance characteristics such that a ceiling effect could be reached to allow for a steady baseline for control and treatment groups prior to applying manipulations. Alternatively, if relatively stable performance enhancement rates can be established utilizing this rate of change as a metric to assess the influence of drugs or on it.

In summary, the chronic ICSS experiment demonstrated a number of key points. One, that regardless of treatment condition, baseline ICSS stimulations in a drug-free state did not significantly change over the course of treatment in the air and CIT groups. In the ECIT group, the regression trended downwards across the escalating exposure days, which may be indicative of a mild withdrawal-associated physical dependence. These experiments demonstrated the utility of this procedure to be further explored with alternative schedules of inhalant exposure or other inhalants all together.

Another remarkable feature that was captured on this initial first experimental run was the tolerance effect of toluene to facilitate ICSS by repeated escalating numbers of exposures. Given the wide availability of inhalants and the relatively rapid tolerance shown to its reward-like effects through escalating chronic intermittent use, these results may be directly relevant to helping understand substance abuse patterns in clinical populations, as inhalants are often reported to be one of the first types of substances abused among young individuals (Howard *et al*, 2011).

As for the utility and future uses for the nesting procedure, there are interesting aspects to explore in how it could be used and modified to model clinical relevant features

of inhalant abuse. One example may be one of the relevant diagnostic criteria within the DSM 5 for inhalant substance use disorders, the functional impairment associated with normal everyday tasks (American Psychiatric Association, 2013). One of the important questions that could be further explored is what parameters could be used to improve the reliability of stable generation of detailed behavioral curves. One possibility would be doubling or tripling the dependent measure, zones cleared, by increasing the number of nestlets. However, given the labor difficulty and time consuming nature of analyzing image data from the nesting procedure, adoption of image recognition tools to automate the process may be necessary.

Taken together, this exploratory aim searched for new ways in which procedures and technologies could be utilized to investigate the devastating effects of chronic inhalant abuse. These results extend the utility of intracranial self-stimulation procedures to investigate cumulative effects of repeated chronic inhalant exposure to measure acute withdrawal effects to investigate inhalants. Given the somewhat limited avenues available to study the full spectrum of inhalants abuse (Aim 1, Bespalov *et al*, 2003; Tracy *et al*, 2014, 2015; Yavich *et al*, 1994), ICSS may prove to be a very useful tool to explore chronic exposure effects.

References

Aickin M, Gensler H (1996). Adjusting for multiple testing when reporting research results: the Bonferroni vs Holm methods. *Am J Public Health* **86**: 726–8.

Akoijam BS, Jamir MN, Phesao E, Senjam GS (2013). Inhalant use among schoolchildren in northeast India: a preliminary study. *Subst Abuse* **7**: 185–90.

Alderson HL, Latimer MP, Blaha CD, Phillips AG, Winn P (2004). An examination of d-amphetamine self-administration in pedunculopontine tegmental nucleus-lesioned rats. *Neuroscience* **125**: 349–58.

Altarifi AA, Negus SS (2011). Some determinants of morphine effects on intracranial self-stimulation in rats: dose, pretreatment time, repeated treatment, and rate dependence. *Behav Pharmacol* **22**: 663–673.

American Psychiatric Association (American Psychiatric Publishing: Washington, DC, 2013). *Diagnostic and statistical manual of mental health disorders: DSM-5 (5th ed.).* .

Apawu AK, Mathews TA, Bowen SE (2015). Striatal dopamine dynamics in mice following acute and repeated toluene exposure. *Psychopharmacology (Berl)* **232**: 173–84.

Araki M, McGeer PL, McGeer EG (1984). Retrograde HRP tracing combined with a pharmacohistochemical method for GABA transaminase for the identification of presumptive GABAergic projections to the habenula. *Brain Res* **304**: 271–7.

Bale AS, Jackson MD, Krantz QT, Benignus VA, Bushnell PJ, Shafer TJ, *et al* (2007). Evaluating the NMDA-glutamate receptor as a site of action for toluene, in vivo.

Toxicol Sci An Off J Soc Toxicol **98**: 159–166.

Bale AS, Smothers CT, Woodward JJ (2002). Inhibition of neuronal nicotinic acetylcholine receptors by the abused solvent, toluene. *Br J Pharmacol* **137**: 375–383.

Bale AS, Tu Y, Carpenter-Hyland EP, Chandler LJ, Woodward JJ (2005). Alterations in glutamatergic and gabaergic ion channel activity in hippocampal neurons following exposure to the abused inhalant toluene. *Neuroscience* **130**: 197–206.

Balster RL, Cruz SL, Howard MO, Dell CA, Cottler LB (2009). Classification of abused inhalants. *Addiction* **104**: 878–882.

Barrot M, Sesack SR, Georges F, Pistis M, Hong S, Jhou TC (2012). Braking dopamine systems: a new GABA master structure for mesolimbic and nigrostriatal functions. *J Neurosci* **32**: 14094–101.

Bauer CT, Banks ML, Blough BE, Negus SS (2013). Use of intracranial self-stimulation to evaluate abuse-related and abuse-limiting effects of monoamine releasers in rats. *Br J Pharmacol* **168**: 850–862.

Beardsley PM, Hayes BA, Balster RL (1990). The self-administration of MK-801 can depend upon drug-reinforcement history, and its discriminative stimulus properties are phencyclidine-like in rhesus monkeys. *J Pharmacol Exp Ther* **252**: 953–9.

Beckley JT, Woodward JJ (2013). Volatile solvents as drugs of abuse: focus on the cortico-mesolimbic circuitry. *Neuropsychopharmacology* **38**: 2555–67.

Beckman NJ, Zacny JP, Walker DJ (2006). Within-subject comparison of the subjective and psychomotor effects of a gaseous anesthetic and two volatile anesthetics in healthy volunteers. *Drug Alcohol Depend* **81**: 89–95.

Beckstead MJ, Weiner JL, Eger EI 2nd, Gong DH, Mihic SJ (2000). Glycine and gamma-aminobutyric acid(A) receptor function is enhanced by inhaled drugs of abuse. *Mol Pharmacol* **57**: 1199–1205.

Belelli D, Pistis M, Peters JA, Lambert JJ (1999). General anaesthetic action at transmitter-gated inhibitory amino acid receptors. *Trends Pharmacol Sci* **20**: 496–502.

Benjamini Y, Krieger AM, Yekutieli D (2006). Adaptive linear step-up procedures that control the false discovery rate. *Biometrika* **93**: 491–507.

Bespalov A, Sukhotina I, Medvedev I, Malyshkin A, Belozertseva I, Balster R, *et al* (2003). Facilitation of electrical brain self-stimulation behavior by abused solvents. *Pharmacol Biochem Behav* **75**: 199–208.

Bielajew C, Shizgal P (1980). Dissociation of the substrates for medial forebrain bundle self-stimulation and stimulation-escape using a two-electrode stimulation technique. *Physiol Behav* **25**: 707–11.

Bielajew C, Shizgal P (1986). Evidence implicating descending fibers in self-stimulation of the medial forebrain bundle. *J Neurosci* **6**: 919–29.

Bishop BE, Laverty R (1989). Dose-dependent reduction by Ro 15-4513 in mice of the effects of ethanol and some other general depressant drugs. *Eur J Pharmacol* **162**: 265–71.

Blokhina EA, Dravolina OA, Bespalov AY, Balster RL, Zvartau EE (2004). Intravenous self-administration of abused solvents and anesthetics in mice. *Eur J Pharmacol* **485**: 211–218.

Bocklisch C, Pascoli V, Wong JCY, House DRC, Yvon C, Roo M de, *et al* (2013).

Cocaine disinhibits dopamine neurons by potentiation of GABA transmission in the ventral tegmental area. *Science* **341**: 1521–5.

Borghese CM, Werner DF, Topf N, Baron N V, Henderson LA, Boehm SL 2nd, *et al* (2006). An isoflurane- and alcohol-insensitive mutant GABA(A) receptor alpha(1) subunit with near-normal apparent affinity for GABA: characterization in heterologous systems and production of knockin mice. *J Pharmacol Exp Ther* **319**: 208–218.

Bossert JM, Franklin KBJ (2003). Reinforcing versus anticonvulsant drugs: effects on intracranial self-stimulation rate-frequency M50 indices. *Behav Brain Res* **144**: 243–247.

Bouchatta O, Ouhaz Z, Ba-Mhamed S, Kerekes N, Bennis M (2016). Acute and chronic glue sniffing effects and consequences of withdrawal on aggressive behavior. *Life Sci* doi:10.1016/j.lfs.2016.03.013.

Bowen SE (2006). Increases in amphetamine-like discriminative stimulus effects of the abused inhalant toluene in mice. *Psychopharmacology (Berl)* **186**: 517–524.

Bowen SE (2011). Two serious and challenging medical complications associated with volatile substance misuse: sudden sniffing death and fetal solvent syndrome. *Subst Use Misuse* **46 Suppl 1**: 68–72.

Bowen SE, Balster RL (1998). A direct comparison of inhalant effects on locomotor activity and schedule-controlled behavior in mice. *Exp Clin Psychopharmacol* **6**: 235–247.

Bowen SE, Charlesworth JD, Tokarz ME, Wright MJJ, Wiley JL (2007). Decreased

sensitivity in adolescent vs. adult rats to the locomotor activating effects of toluene. *Neurotoxicol Teratol* **29**: 599–606.

Bowen SE, Hannigan JH (2006). Developmental toxicity of prenatal exposure to toluene. *AAPS J* **8**: E419–424.

Bowen SE, Kimar S, Irtenkauf S (2010). Comparison of toluene-induced locomotor activity in four mouse strains. *Pharmacol Biochem Behav* **95**: 249–257.

Bowen SE, McDonald P (2009). Abuse pattern of toluene exposure alters mouse behavior in a waiting-for-reward operant task. *Neurotoxicol Teratol* **31**: 18–25.

Bowen SE, Wiley JL, Balster RL (1996). The effects of abused inhalants on mouse behavior in an elevated plus-maze. *Eur J Pharmacol* **312**: 131–136.

Bowen SE, Wiley JL, Jones HE, Balster RL (1999). Phencyclidine- and diazepam-like discriminative stimulus effects of inhalants in mice. *Exp Clin Psychopharmacol* **7**: 28–37.

Brauer LH, Ambre J, Wit H De (1996). Acute tolerance to subjective but not cardiovascular effects of d-amphetamine in normal, healthy men. *J Clin Psychopharmacol* **16**: 72–6.

Brosnan RJ, Pham TL (2014). Hydrocarbon molar water solubility predicts NMDA vs. GABAA receptor modulation. *BMC Pharmacol Toxicol* **15**: 62.

Brouette T, Anton R (2001). Clinical review of inhalants. *Am J Addict* **10**: 79–94.

Caplan JP, Pope AE, Boric CA, Benford DA Air conditioner refrigerant inhalation: a habit with chilling consequences. *Psychosomatics* **53**: 273–6.

Carlezon WA, Chartoff EH (2007). Intracranial self-stimulation (ICSS) in rodents to study

the neurobiology of motivation. *Nat Protoc* **2**: 2987–2995.

Chan M-H, Chung S-S, Stoker AK, Markou A, Chen H-H (2012). Sarcosine attenuates toluene-induced motor incoordination, memory impairment, and hypothermia but not brain stimulation reward enhancement in mice. *Toxicol Appl Pharmacol* **265**: 158–165.

Chan M-H, Tsai Y-L, Lee M-Y, Stoker AK, Markou A, Chen H-H (2015). The group II metabotropic glutamate receptor agonist LY379268 reduces toluene-induced enhancement of brain-stimulation reward and behavioral disturbances. *Psychopharmacology (Berl)* doi:10.1007/s00213-015-3973-3.

Cheeseman JF, Winnebeck EC, Millar CD, Kirkland LS, Sleigh J, Goodwin M, *et al* (2012). General anesthesia alters time perception by phase shifting the circadian clock. *Proc Natl Acad Sci U S A* **109**: 7061–6.

Cheong R, Wilson RK, Cortese ICM, Newman-Toker DE (2006). Mothball withdrawal encephalopathy: case report and review of paradichlorobenzene neurotoxicity. *Subst Abus Off Publ Assoc Med Educ Res Subst Abus* **27**: 63–67.

Collado V, Nicolas E, Faulks D, Hennequin M (2007). A review of the safety of 50% nitrous oxide/oxygen in conscious sedation. *Expert Opin Drug Saf* **6**: 559–571.

Corbett D (1989). Possible abuse potential of the NMDA antagonist MK-801. *Behav Brain Res* **34**: 239–246.

Cruz SL, Balster RL, Woodward JJ (2000). Effects of volatile solvents on recombinant N-methyl-D-aspartate receptors expressed in Xenopus oocytes. *Br J Pharmacol* **131**: 1303–1308.

Cruz SL, Domínguez M (2011). Misusing volatile substances for their hallucinatory effects: a qualitative pilot study with Mexican teenagers and a pharmacological discussion of their hallucinations. *Subst Use Misuse* **46 Suppl 1**: 84–94.

Cruz SL, Mirshahi T, Thomas B, Balster RL, Woodward JJ (1998). Effects of the abused solvent toluene on recombinant N-methyl-D-aspartate and non-N-methyl-D-aspartate receptors expressed in Xenopus oocytes. *J Pharmacol Exp Ther* **286**: 334–340.

Cruz SL, Rivera-García MT, Woodward JJ (2014). Review of Toluene Actions: Clinical Evidence, Animal Studies, and Molecular Targets. *J Drug Alcohol Res* **3**: 1–8.

D'Souza D (2015). Ability of Partial Inverse Agonist, Iomazenil, to Block Ethanol Effects in Humans. NCT01590277. *Clinicaltrials.gov* at <http://clinicaltrials.gov/show/NCT01590277>.

Depoortere R, Perrault G, Sanger DJ, Depoortere (1999). Intracranial self-stimulation under a progressive-ratio schedule in rats: effects of strength of stimulation, d-amphetamine, 7-OH-DPAT and haloperidol. *Psychopharmacology (Berl)* **142**: 221–229.

Dews PB (1977). Rate-dependency hypothesis. *Science* **198**: 1182–1183.

Diana M (2011). The dopamine hypothesis of drug addiction and its potential therapeutic value. *Front Psychiatry* **2**: 64.

Dias Cicarelli D, Rojas-Álvarez NE, Fuller P, Lacava Pagnocca M, Frerichs E, Martins Benseñor FE (2016). Effect of flumazenil on recovery from general anesthesia with isoflurane: A randomized controlled trial. *Colomb J Anesthesiol* **44**: 8–12.

Dick ALW, Axelsson M, Lawrence AJ, Duncan JR (2014). Specific impairments in

instrumental learning following chronic intermittent toluene inhalation in adolescent rats. *Psychopharmacology (Berl)* **231**: 1531–42.

Dixon CI, Morris H V, Breen G, Desrivieres S, Jugurnauth S, Steiner RC, *et al* (2010). Cocaine effects on mouse incentive-learning and human addiction are linked to alpha2 subunit-containing GABAA receptors. *Proc Natl Acad Sci U S A* **107**: 2289–94.

Duncan JR, Gibbs SJ, Lawrence AJ (2014). Chronic intermittent toluene inhalation in adolescent rats alters behavioural responses to amphetamine and MK801. *Eur Neuropsychopharmacol* **24**: 480–6.

Elkoussi A, Bakheet S (2011). Volatile substance misuse among street children in Upper Egypt. *Subst Use Misuse* **46 Suppl 1**: 35–39.

Emmanouil DE, Johnson CH, Quock RM (1994). Nitrous oxide anxiolytic effect in mice in the elevated plus maze: mediation by benzodiazepine receptors. *Psychopharmacology (Berl)* **115**: 167–172.

Engin E, Bakhurin KI, Smith KS, Hines RM, Reynolds LM, Tang W, *et al* (2014). Neural Basis of Benzodiazepine Reward: Requirement for α2 Containing GABAA Receptors in the Nucleus Accumbens. *Neuropsychopharmacology* **39**: 1805–15.

Fan L, Sonoda S, Watanabe M, Tsujikawa H, Okada T, Nishimura K, *et al* (2007). [Effects of nitrous oxide and isoflurane on the L-type calcium current of rabbit ventricular myocytes and their modulation by beta-adrenoceptor stimulation]. *Masui* **56**: 386–94.

File SE, Dingemanse J, Friedman HL, Greenblatt DJ (1986). Chronic treatment with Ro 15-1788 distinguishes between its benzodiazepine antagonist, agonist and inverse

agonist properties. *Psychopharmacology (Berl)* **89**: 113–7.

Fish EW, Riday TT, McGuigan MM, Faccidomo S, Hodge CW, Malanga CJ (2010). Alcohol, cocaine, and brain stimulation-reward in C57Bl6/J and DBA2/J mice. *Alcohol Clin Exp Res* **34**: 81–89.

Fish EW, Robinson JE, Krouse MC, Hodge CW, Reed C, Phillips TJ, *et al* (2012). Intracranial self-stimulation in FAST and SLOW mice: effects of alcohol and cocaine. *Psychopharmacology (Berl)* **220**: 719–730.

Flippo TS, Holder WDJ (1993). Neurologic degeneration associated with nitrous oxide anesthesia in patients with vitamin B12 deficiency. *Arch Surg (Chicago, Ill 1960)* **128**: 1391–1395.

Fry LE, Fry RA (2015). Anesthetic substance abuse: international perspectives and parallels. *Can J Anaesth = J Can d'anesthésie* **62**: 1346–7.

Fujimaki H, Win-Shwe T-T, Yoshida Y, Kunugita N, Arashidani K (2011). Dysregulation of immune responses in an allergic mouse model following low-level toluene exposure. *Toxicology* **286**: 28–35.

G J Schaefer, Michael RP (1988). An analysis of the effects of amphetamine on brain self-stimulation behavior. *Behav Brain Res* **29**: 93–101.

Gaiardi M, Bartoletti M, Bacchi A, Gubellini C, Babbini M (1985). Morphine and clonidine oral self-administration: a study in morphine dependent or abstinent rats. *Prog Neuropsychopharmacol Biol Psychiatry* **9**: 143–51.

Gardner EL, Vorel SR (1998). Cannabinoid transmission and reward-related events. *Neurobiol Dis* **5**: 502–33.

Garland EL, Howard MO (2010). Inhalation of computer duster spray among adolescents: an emerging public health threat? *Am J Drug Alcohol Abuse* **36**: 320–4.

Garland EL, Howard MO (2012). Volatile substance misuse : clinical considerations, neuropsychopharmacology and potential role of pharmacotherapy in management. *CNS Drugs* **26**: 927–35.

Garland EL, Howard MO, Perron BE (2009). Nitrous oxide inhalation among adolescents: prevalence, correlates, and co-occurrence with volatile solvent inhalation. *J Psychoactive Drugs* **41**: 337–47.

Gerasimov MR, Collier L, Ferrieri A, Alexoff D, Lee D, Gifford AN, *et al* (2003). Toluene inhalation produces a conditioned place preference in rats. *Eur J Pharmacol* **477**: 45–52.

Glickman ME, Rao SR, Schultz MR (2014). False discovery rate control is a recommended alternative to Bonferroni-type adjustments in health studies. *J Clin Epidemiol* **67**: 850–7.

Griffiths RR, Johnson MW (2005). Relative abuse liability of hypnotic drugs: a conceptual framework and algorithm for differentiating among compounds. *J Clin Psychiatry* **66 Suppl 9**: 31–41.

Gupta SR, Palmer CA, Curé JK, Balos LL, Lincoff NS, Kline LB (2011). Toluene optic neurotoxicity: magnetic resonance imaging and pathologic features. *Hum Pathol* **42**: 295–298.

Hannigan JH, Bowen SE (2010). Reproductive toxicology and teratology of abused toluene. *Syst Biol Reprod Med* **56**: 184–200.

Hapfelmeier G, Zieglgänsberger W, Haseneder R, Schneck H, Kochs E (2000). Nitrous oxide and xenon increase the efficacy of GABA at recombinant mammalian GABA(A) receptors. *Anesth Analg* **91**: 1542–1549.

Hatsukami DK, Fischman MW (1996). Crack cocaine and cocaine hydrochloride. Are the differences myth or reality? *JAMA* **276**: 1580–8.

Heinke W, Schwarzbauer C (2001). Subanesthetic isoflurane affects task-induced brain activation in a highly specific manner: a functional magnetic resonance imaging study. *Anesthesiology* **94**: 973–81.

Hillhouse TM, Porter JH, Negus SS (2014). Dissociable effects of the noncompetitive NMDA receptor antagonists ketamine and MK-801 on intracranial self-stimulation in rats. *Psychopharmacology (Berl)* **231**: 2705–16.

Hoet P, Lison D (2008). Ototoxicity of toluene and styrene: state of current knowledge. *Crit Rev Toxicol* **38**: 127–170.

Holtz NA, Radke AK, Zlebnik NE, Harris AC, Carroll ME (2015). Intracranial self-stimulation reward thresholds during morphine withdrawal in rats bred for high (HiS) and low (LoS) saccharin intake. *Brain Res* **1602**: 119–26.

Hondebrink L, Meulenbelt J, Kleef RGDM van, Berg M van den, Westerink RHS (2011). Modulation of human GABAA receptor function: a novel mode of action of drugs of abuse. *Neurotoxicology* **32**: 823–7.

Hout MC Van, Bingham T (2013). "Surfing the Silk Road": A study of users' experiences. *Int J Drug Policy* **24**: 524–529.

Howard MO, Balster RL, Cottler LB, Wu L-T, Vaughn MG (2008). Inhalant use among

incarcerated adolescents in the United States: prevalence, characteristics, and correlates of use. *Drug Alcohol Depend* **93**: 197–209.

Howard MO, Bowen SE, Garland EL, Perron BE, Vaughn MG (2011). Inhalant use and inhalant use disorders in the United States. *Addict Sci Clin Pract* **6**: 18–31.

Hwang JCF, Himel HN, Edlich RF (1996). Frostbite of the face after recreational misuse of nitrous oxide. *Burns* **22**: 152–153.

Invernizzi R, Pozzi L, Samanin R (1991). Release of dopamine is reduced by diazepam more in the nucleus accumbens than in the caudate nucleus of conscious rats. *Neuropharmacology* **30**: 575–8.

Jacob B, Heller C, Daldrup T, Bürrig KF, Barz J, Bonte W (1989). Fatal accidental enflurane intoxication. *J Forensic Sci* **34**: 1408–12.

Jason C. Hsu (Chapman & Hall: London, 1996). *Multiple Comparisons: Theory and methods*. .

Jevtović-Todorović V, Todorović SM, Mennerick S, Powell S, Dikranian K, Benshoff N, *et al* (1998). Nitrous oxide (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. *Nat Med* **4**: 460–3.

Jhou TC, Fields HL, Baxter MG, Saper CB, Holland PC (2009). The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron* **61**: 786–800.

Johnston, L. D., O'Malley, P. M., Miech, R. A., Bachman, J. G., & Schulenberg JE (Ann Arbor: Institute for Social Research, The University of Michigan.: 2016). *Monitoring the Future national survey results on drug use, 1975-2015: Overview, key findings on*

adolescent drug use. **1975-2015**: .

Jones HE, Balster RL (1998). Inhalant abuse in pregnancy. *Obstet Gynecol Clin North Am* **25**: 153–167.

June HL, Hughes RW, Spurlock HL, Lewis MJ (1994). Ethanol self-administration in freely feeding and drinking rats: effects of Ro15-4513 alone, and in combination with Ro15-1788 (flumazenil). *Psychopharmacology (Berl)* **115**: 332–9.

Jurd R, Arras M, Lambert S, Drexler B, Siegwart R, Crestani F, *et al* (2003). General anesthetic actions in vivo strongly attenuated by a point mutation in the GABA(A) receptor beta3 subunit. *FASEB J Off Publ Fed Am Soc Exp Biol* **17**: 250–252.

Kelley SP, Hodge CW (2003). The 5-HT3 antagonist Y-25130 blocks cocaine-induced lowering of ICSS reward thresholds in the rat. *Pharmacol Biochem Behav* **74**: 297–302.

Kempadoo KA, Tourino C, Cho SL, Magnani F, Leinninger G-M, Stuber GD, *et al* (2013). Hypothalamic neurotensin projections promote reward by enhancing glutamate transmission in the VTA. *J Neurosci* **33**: 7618–26.

Kida T, Noguchi J, Zhang M-R, Suhara T, Suzuki K (2003). Metabolite analysis of [11C]Ro15-4513 in mice, rats, monkeys and humans. *Nucl Med Biol* **30**: 779–784.

King GS, Smialek JE, Troutman WG (1985). Sudden death in adolescents resulting from the inhalation of typewriter correction fluid. *JAMA* **253**: 1604–6.

Koehler MM, Henninger CA (2014). Orofacial and digital frostbite caused by inhalant abuse. *Cutis* **93**: 256–60.

Kong J-T, Schmiesing C (2005). Concealed mothball abuse prior to anesthesia: mothballs,

inhalants, and their management. *Acta Anaesthesiol Scand* **49**: 113–6.

Kuhlman JJJ, Magluilo JJ, Levine B, Smith ML (1993). Two deaths involving isoflurane abuse. *J Forensic Sci* **38**: 968–971.

Lacy BW, Ditzler TF (2007). Inhalant abuse in the military: an unrecognized threat. *Mil Med* **172**: 388–392.

Larson EB, Akkentli F, Edwards S, Graham DL, Simmons DL, Alibhai IN, *et al* (2010). Striatal regulation of ΔFosB, FosB, and cFos during cocaine self-administration and withdrawal. *J Neurochem* **115**: 112–122.

Lecker I, Yin Y, Wang DS, Orser BA (2013). Potentiation of GABAA receptor activity by volatile anaesthetics is reduced by α5GABAA receptor-preferring inverse agonists. *Br J Anaesth* **110 Suppl** : i73–81.

Lee DE, Gerasimov MR, Schiffer WK, Gifford AN (2006). Concentration-dependent conditioned place preference to inhaled toluene vapors in rats. *Drug Alcohol Depend* **85**: 87–90.

Leitl MD, Onvani S, Bowers MS, Cheng K, Rice KC, Carlezon WA, *et al* (2013). Pain-Related Depression of the Mesolimbic Dopamine System in Rats: Expression, Blockade by Analgesics, and Role of Endogenous κ-opioids. *Neuropsychopharmacology* **39**: 614–624.

Levine B, Cox D, Jufer-Phipps RA, Li L, Jacobs A, Fowler D (2007). A fatality from sevoflurane abuse. *J Anal Toxicol* **31**: 534–6.

Li G-D, Chiara DC, Cohen JB, Olsen RW (2010). Numerous classes of general anesthetics inhibit etomidate binding to gamma-aminobutyric acid type A (GABAA) receptors. *J*

Biol Chem **285**: 8615–20.

Liang P, Zhou C, Li K-Y, Guo L-J, Liu B, Liu J (2014). Effect of flumazenil on sevoflurane requirements for minimum alveolar anesthetic concentration-awake and recovery status. *Int J Clin Exp Med* **7**: 673–9.

Lin D, Koob GF, Markou A (1999). Differential effects of withdrawal from chronic amphetamine or fluoxetine administration on brain stimulation reward in the rat--interactions between the two drugs. *Psychopharmacology (Berl)* **145**: 283–294.

Lin R-J, Chen H-F, Chang Y-C, Su J-J (2011). Subacute combined degeneration caused by nitrous oxide intoxication: case reports. *Acta Neurol Taiwan* **20**: 129–137.

Lo P-S, Chen H-H (2005). Immunohistochemical localization of toluene-induced c-Fos protein expression in the rat brain. *Toxicol Lett* **157**: 151–160.

Lopreato GF, Phelan R, Borghese CM, Beckstead MJ, Mihic SJ (2003). Inhaled drugs of abuse enhance serotonin-3 receptor function. *Drug Alcohol Depend* **70**: 11–5.

Lukas SE, Mendelson JH, Benedikt RA (1986a). Instrumental analysis of ethanol-induced intoxication in human males. *Psychopharmacology (Berl)* **89**: 8–13.

Lukas SE, Mendelson JH, Benedikt RA, Jones B (1986b). EEG alpha activity increases during transient episodes of ethanol-induced euphoria. *Pharmacol Biochem Behav* **25**: 889–95.

MacIver MB (2009). Abused inhalants enhance GABA-mediated synaptic inhibition. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol* **34**: 2296–2304.

Manbeck KE, Shelley D, Schmidt CE, Harris AC (2014). Effects of oxytocin on nicotine withdrawal in rats. *Pharmacol Biochem Behav* **116**: 84–9.

Markou A, Koob GF (1991). Postcocaine anhedonia. An animal model of cocaine withdrawal. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol* **4**: 17–26.

Markou A, Koob GF (1992). Bromocriptine reverses the elevation in intracranial self-stimulation thresholds observed in a rat model of cocaine withdrawal. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol* **7**: 213–224.

McBride WJ, Murphy JM, Lumeng L, Li TK (1988). Effects of Ro 15-4513, fluoxetine and desipramine on the intake of ethanol, water and food by the alcohol-preferring (P) and -nonpreferring (NP) lines of rats. *Pharmacol Biochem Behav* **30**: 1045–50.

Meadows R, Verghese A (1996). Medical complications of glue sniffing. *South Med J* **89**: 455–62.

Melón LC, Boehm SL (2011). GABAA receptors in the posterior, but not anterior, ventral tegmental area mediate Ro15-4513-induced attenuation of binge-like ethanol consumption in C57BL/6J female mice. *Behav Brain Res* **220**: 230–7.

Mennerick S, Jevtovic-Todorovic V, Todorovic SM, Shen W, Olney JW, Zorumski CF (1998). Effect of nitrous oxide on excitatory and inhibitory synaptic transmission in hippocampal cultures. *J Neurosci* **18**: 9716–26.

Miczek KA, Weerts EM (1987). Seizures in drug-treated animals. *Science* **235**: 1127a.

Mihic SJ, Ye Q, Wick MJ, Koltchine V V, Krasowski MD, Finn SE, *et al* (1997). Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* **389**: 385–389.

Miller DW, Yourick DL, Tessel RE (1989). Antagonism of methoxyflurane-induced

anesthesia in rats by benzodiazepine inverse agonists. *Eur J Pharmacol* **173**: 1–10.

Miller LL, Leitl MD, Banks ML, Blough BE, Negus SS (2015). Effects of the triple monoamine uptake inhibitor amitifadine on pain-related depression of behavior and mesolimbic dopamine release in rats. *Pain* **156**: 175–184.

Milner PM (1991). Brain-stimulation reward: a review. *Can J Psychol* **45**: 1–36.

Miyagawa M, Honma T, Sato M, Hasegawa H (1984). Conditioned taste aversion induced by toluene administration in rats. *Neurobehav Toxicol Teratol* **6**: 33–37.

Moody EJ, Skolnick P (1988). The imidazobenzodiazepine Ro 15-4513 antagonizes methoxyflurane anesthesia. *Life Sci* **43**: 1269–76.

Moser VC, Balster RL (1985). Effects of toluene, halothane and ethanol vapor on fixed-ratio performance in mice. *Pharmacol Biochem Behav* **22**: 797–802.

Moser VC, Balster RL (1986). The effects of inhaled toluene, halothane, 1,1,1-trichloroethane, and ethanol on fixed-interval responding in mice. *Neurobehav Toxicol Teratol* **8**: 525–531.

Motulsky H, Christopoulos A (Oxford University Press: Oxford, 2004). *Fitting models to biological data using linear and nonlinear regression: a practical guide to curve fitting*. .

Muelken P, Schmidt CE, Shelley D, Tally L, Harris AC (2015). A Two-Day Continuous Nicotine Infusion Is Sufficient to Demonstrate Nicotine Withdrawal in Rats as Measured Using Intracranial Self-Stimulation. *PLoS One* **10**: e0144553.

National Research Council (National Academies Press: Washington, D.C, 2011). *Guide for the care and use of laboratory animals*. .

Negus SS, Miller LL (2014). Intracranial Self-Stimulation to Evaluate Abuse Potential of Drugs. *Pharmacol Rev* **66**: 869–917.

Negus SS, Morrissey EM, Rosenberg M, Cheng K, Rice KC (2010). Effects of kappa opioids in an assay of pain-depressed intracranial self-stimulation in rats. *Psychopharmacology (Berl)* **210**: 149–159.

Negus SS, Neddenriep B, Altarifi AA, Carroll FI, Leitl MD, Miller LL (2015). Effects of ketoprofen, morphine, and kappa opioids on pain-related depression of nesting in mice. *Pain* **156**: 1153–60.

Nieh EH, Kim S-Y, Namburi P, Tye KM (2013). Optogenetic dissection of neural circuits underlying emotional valence and motivated behaviors. *Brain Res* **1511**: 73–92.

O'Neill KS, Todtenkopf MS (2010). Using a rate-frequency curve method to assess the rewarding properties of morphine in the intracranial self-stimulation paradigm in rats. *J Neurosci Methods* **189**: 75–79.

Olds J, Milner P (1954). Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *J Comp Physiol Psychol* **47**: 419–427.

Páez-Martínez N, Ambrosio E, García-Lecumberri C, Rocha L, Montoya GL, Cruz SL (2008). Toluene and TCE decrease binding to mu-opioid receptors, but not to benzodiazepine and NMDA receptors in mouse brain. *Ann N Y Acad Sci* **1139**: 390–401.

Paterson NE, Myers C, Markou A (2000). Effects of repeated withdrawal from continuous amphetamine administration on brain reward function in rats. *Psychopharmacology (Berl)* **152**: 440–446.

Pavlic M, Haidekker A, Grubwieser P, Rabl W (2002). Fatal accident caused by isoflurane abuse. *Int J Legal Med* **116**: 357–360.

Paxinos G, Franklin KBJ (Academic Press: San Diego, 2001). *The Mouse Brain in Stereotaxic Coordinates*. .

Pema PJ, Horak HA, Wyatt RH (1998). Myelopathy caused by nitrous oxide toxicity. *AJNR Am J Neuroradiol* **19**: 894–896.

Perit KE, Gmaz JM, Caleb Browne JD, Matthews BA, Dunn MBF, Yang L, *et al* (2012). Distribution of c-Fos immunoreactivity in the rat brain following abuse-like toluene vapor inhalation. *Neurotoxicol Teratol* **34**: 37–46.

Pokk P, Zharkovsky A (1997). The effects of flumazenil, Ro 15-4513 and beta-CCM on the behaviour of control and stressed mice in the plus-maze test. *J Physiol Pharmacol* **48**: 253–61.

Potier MC, Prado de Carvalho L, Dodd RH, Brown CL, Rossier J (1988). In vivo binding of (3H)Ro15-1788 in mice: comparison with the in vivo binding of (3H)flunitrazepam. *Life Sci* **43**: 1287–96.

Praharaj SK, Verma P, Arora M (2008). Inhalant abuse (typewriter correction fluid) in street children. *J Addict Med* **2**: 175–7.

Ramsay DS, Watson CH, Leroux BG, Prall CW, Kaiyala KJ (2003). Conditioned place aversion and self-administration of nitrous oxide in rats. *Pharmacol Biochem Behav* **74**: 623–633.

Ranft A, Kurz J, Becker K, Dodt H-U, Zieglgänsberger W, Rammes G, *et al* (2007). Nitrous oxide (N2O) pre- and postsynaptically attenuates NMDA receptor-mediated

neurotransmission in the amygdala. *Neuropharmacology* **52**: 716–723.

Rees DC, Knisely JS, Breen TJ, Balster RL (1987). Toluene, halothane, 1,1,1-trichloroethane and oxazepam produce ethanol-like discriminative stimulus effects in mice. *J Pharmacol Exp Ther* **243**: 931–7.

Reynolds LM, Engin E, Tantillo G, Lau HM, Muschamp JW, Carlezon WAJ, *et al* (2012). Differential roles of GABA(A) receptor subtypes in benzodiazepine-induced enhancement of brain-stimulation reward. *Neuropsychopharmacology* **37**: 2531–2540.

Reznikov R, Binko M, Nobrega JN, Hamani C (2016). Deep Brain Stimulation in Animal Models of Fear, Anxiety and Post-Traumatic Stress Disorder. *Neuropsychopharmacology* doi:10.1038/npp.2016.34.

Richardson KJ, Shelton KL (2014). Discriminative stimulus effects of nitrous oxide in mice: comparison with volatile hydrocarbons and vapor anesthetics. *Behav Pharmacol* **25**: 2–11.

Richardson KJ, Shelton KL (2015). N-methyl-D-aspartate receptor channel blocker-like discriminative stimulus effects of nitrous oxide gas. *J Pharmacol Exp Ther* **352**: 156–65.

Riegel AC, Zapata A, Shippenberg TS, French ED (2007). The abused inhalant toluene increases dopamine release in the nucleus accumbens by directly stimulating ventral tegmental area neurons. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol* **32**: 1558–1569.

Ritchie GD, Kimmel EC, Bowen LE, Reboulet JE, Rossi J (2001). Acute neurobehavioral

effects in rats from exposure to HFC 134a or CFC 12. *Neurotoxicology* **22**: 233–48.

Robinson JE, Agoglia AE, Fish EW, Krouse MC, Malanga CJ (2012). Mephedrone (4-methylmethcathinone) and intracranial self-stimulation in C57BL/6J mice: comparison to cocaine. *Behav Brain Res* **234**: 76–81.

Rosales CM, Young T, Laster MJ, Eger EI 2nd, Garg U (2007). Sevoflurane concentrations in blood, brain, and lung after sevoflurane-induced death. *J Forensic Sci* **52**: 1408–1410.

Rowlett JK, Lelas S (2007). Comparison of zolpidem and midazolam self-administration under progressive-ratio schedules: consumer demand and labor supply analyses. *Exp Clin Psychopharmacol* **15**: 328–337.

Rudolph U, Knoflach F (2011). Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. *Nat Rev Drug Discov* **10**: 685–697.

Rudolph U, Möhler H (2004). Analysis of GABAA receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annu Rev Pharmacol Toxicol* **44**: 475–98.

Russo SJ, Nestler EJ (2013). The brain reward circuitry in mood disorders. *Nat Rev Neurosci* **14**: 609–25.

Sagara H, Kitamura Y, Sendo T, Araki H, Gomita Y (2008). Effect of diazepam on the runway method using priming stimulation of intracranial self stimulation behavior. *J Pharmacol Sci* **107**: 355–360.

Scala G Di, Oberling P, Rocha B, Sandner G (1992). Conditioned place preference induced by Ro 16-6028, a benzodiazepine receptor partial agonist. *Pharmacol Biochem Behav*

41: 859–62.

Schaefer GJ, Michael RP (1987). Ethanol and current thresholds for brain self-stimulation in the lateral hypothalamus of the rat. *Alcohol* **4**: 209–213.

Schaefer GJ, Michael RP (1989). Interactions between RO 15-4513 and ethanol on brain self-stimulation and locomotor activity in rats. *Pharmacol Biochem Behav* **34**: 785–90.

Schulteis G, Markou A, Cole M, Koob GF (1995). Decreased brain reward produced by ethanol withdrawal. *Proc Natl Acad Sci U S A* **92**: 5880–4.

Shabel SJ, Proulx CD, Trias A, Murphy RT, Malinow R (2012). Input to the lateral habenula from the basal ganglia is excitatory, aversive, and suppressed by serotonin. *Neuron* **74**: 475–81.

Sharma S, Hryhorczuk C, Fulton S (2012). Progressive-ratio responding for palatable high-fat and high-sugar food in mice. *J Vis Exp JoVE* e3754doi:10.3791/3754.

Shelton KL (2007). Inhaled toluene vapor as a discriminative stimulus. *Behav Pharmacol* **18**: 219–229.

Shelton KL (2009). Discriminative stimulus effects of inhaled 1,1,1-trichloroethane in mice: comparison to other hydrocarbon vapors and volatile anesthetics. *Psychopharmacology (Berl)* **203**: 431–440.

Shelton KL, Balster RL (2004). Effects of abused inhalants and GABA-positive modulators in dizocilpine discriminating inbred mice. *Pharmacol Biochem Behav* **79**: 219–28.

Shelton KL, Grant KA (2001). Effects of naltrexone and Ro 15-4513 on a multiple

schedule of ethanol and Tang self-administration. *Alcohol Clin Exp Res* **25**: 1576–85.

Shelton KL, Nicholson KL (2010). GABA(A) positive modulator and NMDA antagonist-like discriminative stimulus effects of isoflurane vapor in mice. *Psychopharmacology (Berl)* **212**: 559–69.

Shelton KL, Nicholson KL (2012). GABAA-positive modulator selective discriminative stimulus effects of 1,1,1-trichloroethane vapor. *Drug Alcohol Depend* **121**: 103–109.

Shelton KL, Nicholson KL (2013). Benzodiazepine-like discriminative stimulus effects of toluene vapor. *Eur J Pharmacol* **720**: 131–137.

Singh J, Desiraju T, Raju TR (1997). Cholinergic and GABAergic modulation of self-stimulation of lateral hypothalamus and ventral tegmentum: effects of carbachol, atropine, bicuculline, and picrotoxin. *Physiol Behav* **61**: 411–418.

Slattery DA, Markou A, Cryan JF (2007). Evaluation of reward processes in an animal model of depression. *Psychopharmacology (Berl)* **190**: 555–568.

Smith AJ, Alder L, Silk J, Adkins C, Fletcher AE, Scales T, *et al* (2001). Effect of alpha subunit on allosteric modulation of ion channel function in stably expressed human recombinant gamma-aminobutyric acid(A) receptors determined using (36)Cl ion flux. *Mol Pharmacol* **59**: 1108–18.

Sonner JM, Werner DF, Elsen FP, Xing Y, Liao M, Harris RA, *et al* (2007). Effect of Isoflurane and Other Potent Inhaled Anesthetics on Minimum Alveolar Concentration, Learning, and the Righting Reflex in Mice Engineered to Express α1γ-Aminobutyric Acid Type A Receptors Unresponsive to Isoflurane. *J Am Soc Anesthesiol* **106**: 107–113.

Spencer JD, Raasch FO, Trefny FA (1976). Halothane abuse in hospital personnel. *JAMA* **235**: 1034–5.

Spielewoy C, Markou A (2003). Withdrawal from chronic phencyclidine treatment induces long-lasting depression in brain reward function. *Neuropsychopharmacology* **28**: 1106–16.

Stanton A. Glantz (McGraw-Hill Education / Medical: 2006). *Primer of Biostatistics*. .

Steffensen SC, Lee RS, Stobbs SH, Henriksen SJ (2001). Responses of ventral tegmental area GABA neurons to brain stimulation reward. *Brain Res* **906**: 190–197.

Stinchcomb A, Bowers BJ, Wehner JM (1989). The effects of ethanol and Ro 15-4513 on elevated plus-maze and rotarod performance in long-sleep and short-sleep mice. *Alcohol* **6**: 369–376.

Stoker AK, Markou A (2011). Withdrawal from chronic cocaine administration induces deficits in brain reward function in C57BL/6J mice. *Behav Brain Res* **223**: 176–181.

Straub CJ, Carlezon WAJ, Rudolph U (2010). Diazepam and cocaine potentiate brain stimulation reward in C57BL/6J mice. *Behav Brain Res* **206**: 17–20.

Suzuki T, Ueta K, Sugimoto M, Uchida I, Mashimo T (2003). Nitrous oxide and xenon inhibit the human (alpha 7)5 nicotinic acetylcholine receptor expressed in Xenopus oocyte. *Anesth Analg* **96**: 443–8, table of contents.

Svartling N, Ranta S, Vuola J, Takkunen O (1996). Life-threatening airway obstruction from nitrous oxide induced frostbite of the oral cavity. *Anaesth Intensive Care* **24**: 717–20.

Tan KR, Brown M, Labouèbe G, Yvon C, Creton C, Fritschy J-M, *et al* (2010). Neural

bases for addictive properties of benzodiazepines. *Nature* **463**: 769–774.

Tan KR, Yvon C, Turiault M, Mirzabekov JJ, Doehner J, Labouèbe G, *et al* (2012). GABA neurons of the VTA drive conditioned place aversion. *Neuron* **73**: 1173–83.

Tatum WO, Bui DD, Grant EG, Murtagh R (2010). Pseudo-guillain-barre syndrome due to "whippet"-induced myeloneuropathy. *J Neuroimaging Off J Am Soc Neuroimaging* **20**: 400–401.

Taylor SR, Badurek S, Dileone RJ, Nashmi R, Minichiello L, Picciotto MR (2014). GABAergic and Glutamatergic Efferents of the Mouse Ventral Tegmental Area. *J Comp Neurol* doi:10.1002/cne.23603.

Todtenkopf MS, O'Neill KS, Kriksciukaite K, Turncliff RZ, Dean RL, Ostrovsky-Day I, *et al* (2009). Route of administration affects the ability of naltrexone to reduce amphetamine-potentiated brain stimulation reward in rats. *Addict Biol* **14**: 408–418.

Tomasiewicz HC, Todtenkopf MS, Chartoff EH, Cohen BM, Carlezon WAJ (2008). The kappa-opioid agonist U69,593 blocks cocaine-induced enhancement of brain stimulation reward. *Biol Psychiatry* **64**: 982–988.

Tracy ME, Banks ML, Shelton KL (2015). Negative allosteric modulation of GABAA receptors inhibits facilitation of brain stimulation reward by drugs of abuse in C57BL6/J mice. *Psychopharmacology (Berl)* doi:10.1007/s00213-015-4155-z.

Tracy ME, Slavova-Hernandez GG, Shelton KL (2014). Assessment of reinforcement enhancing effects of toluene vapor and nitrous oxide in intracranial self-stimulation. *Psychopharmacology (Berl)* **231**: 1339–1350.

United Nations Environment Programme Environmental Effects Assessment Panel (2016).

Environmental effects of ozone depletion and its interactions with climate change: progress report, 2015. *Photochem Photobiol Sci* **15**: 141–74.

Venault P, Chapouthier G (2007). From the behavioral pharmacology of beta-carbolines to seizures, anxiety, and memory. *ScientificWorldJournal* **7**: 204–23.

Votey SR, Bosse GM, Bayer MJ, Hoffman JR (1991). Flumazenil: a new benzodiazepine antagonist. *Ann Emerg Med* **20**: 181–8.

Vry J De, Slangen JL (1985). The Ro 15-1788 cue: evidence for benzodiazepine agonist and inverse agonist properties. *Eur J Pharmacol* **119**: 193–7.

Wagner GA, Oliveira LG de, Barroso LP, Nishimura R, Ishihara LM, Stempliuk V de A, *et al* (2012). Drug use in college students: a 13-year trend. *Rev saúde pública* **46**: 497–504.

Walker DJ, Zacny JP (2001). Within- and between-subject variability in the reinforcing and subjective effects of nitrous oxide in healthy volunteers. *Drug Alcohol Depend* **64**: 85–96.

Weintraub E, Gandhi D, Robinson C (2000). Medical complications due to mothball abuse. *South Med J* **93**: 427–9.

Werner DF, Swihart A, Rau V, Jia F, Borghese CM, McCracken ML, *et al* (2011). Inhaled anesthetic responses of recombinant receptors and knockin mice harboring α2(S270H/L277A) GABA(A) receptor subunits that are resistant to isoflurane. *J Pharmacol Exp Ther* **336**: 134–44.

Wiley JL, Bale AS, Balster RL (2003). Evaluation of toluene dependence and cross-sensitization to diazepam. *Life Sci* **72**: 3023–3033.

Williams JM, Stafford D, Steketee JD (2005). Effects of repeated inhalation of toluene on ionotropic GABAA and glutamate receptor subunit levels in rat brain. *Neurochem Int* **46**: 1–10.

Winek CL, Wahba WW, Huston R, Rozin L (1997). Fatal inhalation of 1,1,1-trichloroethane. *Forensic Sci Int* **87**: 161–5.

Winston A, Kanzy A, Bachuwa G (2015). Air Duster abuse causing rapid airway compromise. *BMJ Case Rep* **2015**: .

Wise RA, Bauco P, Carlezon WAJ, Trojniar W (1992). Self-stimulation and drug reward mechanisms. *Ann N Y Acad Sci* **654**: 192–198.

Wood RW, Grubman J, Weiss B (1977). Nitrous oxide self-administration by the squirrel monkey. *J Pharmacol Exp Ther* **202**: 491–9.

Woodward JJ, Nowak M, Davies DL (2004). Effects of the abused solvent toluene on recombinant P2X receptors expressed in HEK293 cells. *Brain Res Mol Brain Res* **125**: 86–95.

Wu L-T, Pilowsky DJ, Schlenger WE (2004). Inhalant abuse and dependence among adolescents in the United States. *J Am Acad Child Adolesc Psychiatry* **43**: 1206–14.

Wu M-S, Hsu Y-D, Lin J-C, Chen S-C, Lee J-T (2007). Spinal myoclonus in subacute combined degeneration caused by nitrous oxide intoxication. *Acta Neurol Taiwan* **16**: 102–105.

Xia Y, Driscoll JR, Wilbrecht L, Margolis EB, Fields HL, Hjelmstad GO (2011). Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. *J Neurosci* **31**: 7811–6.

Yamakura T, Harris RA (2000). Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels. Comparison with isoflurane and ethanol. *Anesthesiology* **93**: 1095–1101.

Yavich L, Patkina N, Zvartau E (1994). Experimental estimation of addictive potential of a mixture of organic solvents. *Eur Neuropsychopharmacol J Eur Coll Neuropsychopharmacol* **4**: 111–118.

Yavich L, Tiihonen J (2000). Patterns of dopamine overflow in mouse nucleus accumbens during intracranial self-stimulation. *Neurosci Lett* **293**: 41–44.

Yavich L, Zvartau E (1994). A comparison of the effects of individual organic solvents and their mixture on brain stimulation reward. *Pharmacol Biochem Behav* **48**: 661–664.

Yücel M, Takagi M, Walterfang M, Lubman DI (2008). Toluene misuse and long-term harms: a systematic review of the neuropsychological and neuroimaging literature. *Neurosci Biobehav Rev* **32**: 910–926.

Zacny JP, Yajnik S, Lichtor JL, Klafta JM, Young CJ, Thapar P, *et al* (1996). The acute and residual effects of subanesthetic concentrations of isoflurane/nitrous oxide combinations on cognitive and psychomotor performance in healthy volunteers. *Anesth Analg* **82**: 153–7.

Zessen R van, Phillips JL, Budygin EA, Stuber GD (2012). Activation of VTA GABA neurons disrupts reward consumption. *Neuron* **73**: 1184–1194.

Zhou C, Liu J, Chen X-D (2012). General anesthesia mediated by effects on ion channels. *World J Crit care Med* **1**: 80–93.

VITA

Matthew E. Tracy was born in Stillwater, Oklahoma on March 5th, 1984 a United States citizen. He was an activist that supports open source and access adoption in science regardless of nationality or geographical background. He enthusiastically embraces the principles outlined in the "Guerilla Open Access Manifesto" by the late Aaron Swartz.

EDUCATION

Virginia Commonwealth University SOM – Richmond, VA (2010-present)

- PhD Candidate, Dept. of Pharmacology & Toxicology
- Anticipated graduation – 05/2016

University of Nebraska, Lincoln, NE (2005-2009)

- Bachelor of Science in Biochemistry – 12/2009
- Minors: Chemistry, Mathematics, Philosophy, Psychology

University of Nebraska, Kearney, NE (2002-2004)

PUBLICATIONS

Tracy, M. E., Banks, M. L., & Shelton, K. L. (2015). Negative allosteric modulation of GABAA receptors inhibits facilitation of brain stimulation reward by drugs of abuse in

C57BL6/J mice. *Psychopharmacology*. doi:10.1007/s00213-015-4155-z

Bagdas D, AlSharari SD, Freitas K, Tracy M, Damaj MI (2015). The role of alpha5 nicotinic acetylcholine receptors in mouse models of chronic inflammatory and neuropathic pain. *Biochem Pharmacol* doi:10.1016/j.bcp.2015.04.013

Tracy ME, Slavova-Hernandez GG, Shelton KL. Assessment of reinforcement enhancing effects of toluene vapor and nitrous oxide in intracranial self-stimulation. *Psychopharmacology*. 2014 Apr;231(7):1339-50. doi: 10.1007/s00213-013-3327-y

Charntikov S, Tracy ME, Zhao C, Li M, Bevins RA. Conditioned Response Evoked by Nicotine Conditioned Stimulus Preferentially Induces c-Fos Expression in Medial Regions of Caudate-Putamen. *Neuropsychopharmacology*. 2012 Mar;37(4):876-84. doi: 10.1038/npp.2011.263

GRANTS/AWARD HISTORY

Predoctoral Ruth L. Kirschstein National Research Service Award (NRSA)

- National Institute of Drug Abuse, National Institutes of Health, "Acute and Chronic Effects of Inhalants in ICSS" 1F31DA034469
- 04/10/2013 – 04/09/2016

SciPy 2015 Travel Award

- Travel Expenses for the SciPy Python Conference 05/2015 – Austin, Texas.

Undergraduate Creative Activities & Research Experiences (UCARE) Grant

- University of Nebraska-Lincoln, Lincoln, NE "Subjective Effects of the Opioid Drug Tramadol"
- 09/2009 – 12/2010

CONFERENCE PRESENTATIONS

Tracy ME, Shelton KL. Effect of $GABA_A$ partial inverse agonists on methamphetamine-facilitated ICSS reward. Poster session presented at Experimental Biology; 2015 Apr 28-May 1; Boston, MA.

Tracy ME, Shelton KL. Pharmacological Modulation of the Reinforcement Enhancing Effects of Toluene Vapor in Intracranial Self-stimulation. Poster session presented at 44th Annual Conference of Society for Neuroscience; 2014 Nov 15-19; Washington, DC.

Tracy ME, Shelton KL. Acute N_2O Exposure Affects Brain Self-stimulation in C57BL6/J Mice. Poster session presented at 15th biennial meeting of the European Behavioral Pharmacology Society; 2013 Sept 6-9; La Rochelle, France.

Tracy ME, Shelton KL. Toluene and Diazepam: Potentiation of the Reinforcing Effects of Brain Self-stimulation in C57BL/6J Mice. Poster session presented at 42nd Annual Conference of Society for Neuroscience; 2012 Oct 17-21; New Orleans, LA.

Tracy ME, Shelton KL. Assessment of the abused inhalant toluene on intracranial self-stimulation in a dynamic exposure model of inhalant delivery. Poster session presented at Experimental Biology; 2012 Apr 22; San Diego, CA.

Tracy ME, Younkin J, Shelton KL. Intracranial Self Stimulation: Examining the Rewarding Properties of Abused Inhalants. Poster session presented at 28th Annual Watts Day Research Symposium; 2011 Oct 19; Richmond, VA.

Polewan RJ, Tracy ME, Hoffman L, Bevins RA. Conditioned stimulus effects of nicotine and varenicline substitution in female rats. Poster session presented at: Nicotine: Neural Mechanisms of Addiction. 40th Annual Conference of Society for Neuroscience; 2010 Oct 15-19; San Diego, CA.

Charntikov S., Tracy ME, Bevins, RA. A c-Fos immunoreactivity study of the nicotine state as a stimulus in an appetitive conditioning task. Poster session presented at: Nicotine: Molecular Mechanisms of Addiction. 40th Annual Conference of Society for Neuroscience; 2010 Oct 15-19; San Diego, CA.

Barrett ST, Tracy ME, & Bevins RA. Nicotine enhances resistance to extinction of operant responding in rats. Annual Conference of Midwestern Psychological Association; 2010 Apr 30- May 2; Chicago, IL.

Tracy ME, Barret ST, & Bevins RA. The Behavioral Effects of Tramadol in an Unbiased Place Conditioning Procedure. Poster session presented at: Behavioral Pharmacology II. 39th Annual Conference of Society for Neuroscience; 2009 Oct 17-21; Chicago, IL.

Reichel CM, Murray JE, Barr JD, Sanderson SC, Tracy ME, & Bevins RA. Transfer of Extinction Learning for the Conditioned Stimulus of Nicotine by Nicotinic Receptor Agonists. Poster session presented at: 71st Annual Conference of The College on Problems of Drug Dependence; 2009 June 20-25; Reno/Sparks, NV.