Published in final edited form as:

Psychopharmacology (Berl). 2016 February ; 233(4): 715–725. doi:10.1007/s00213-015-4155-z.

Negative allosteric modulation of GABA_A receptors inhibits facilitation of brain stimulation reward by drugs of abuse in C57BL6/J mice

Matthew E. Tracy, BS¹, Matthew L. Banks, PhD, PharmD², and Keith L. Shelton, PhD³

¹Department of Pharmacology and Toxicology, Virginia Commonwealth University

²Dept. of Pharmacology and Toxicology, Virginia Commonwealth University

³Department of Pharmacology and Toxicology, Virginia Commonwealth University

Abstract

Rationale—There is an emerging body of evidence that implicates a crucial role of γ -aminobutyric acid subtype A (GABA_A) receptors in modulating the rewarding effects of a number of abused drugs. Modulation of GABA_A receptors may therefore represent a novel drug-class independent mechanism for the development of abuse treatment pharmacotherapeutics.

Objectives—We tested the hypothesis that the GABA_A receptor benzodiazepine-site (BDZ) negative modulator Ro15-4513 would reduce the reward-related effects of three pharmacologically dissimilar drugs; toluene vapor, d-methamphetamine and diazepam using intracranial self-stimulation (ICSS) in mice. We also examined whether Ro15-4513 attenuated dopamine release produced by d-methamphetamine in an *in vivo* microdialysis procedure.

Results—Ro15-4513 abolished ICSS reward facilitation produced by all three abused drugs at Ro15-4513 doses which had no effect on ICSS when administered alone. In contrast, the BDZ antagonist flumazenil only attenuated the ICSS-facilitating effects of diazepam. Administration of the same dose of Ro15-4513 which abolished drug-facilitated ICSS produced a 58% decrease in d-methamphetamine stimulated dopamine in the nucleus accumbens of mice relative to d-methamphetamine alone.

Conclusions—These results demonstrate that negative modulation of GABA_A receptors can produce profound reductions in reward-related effects on a diverse group of drugs that activate the mesolimbic reward pathway through different mechanisms. These data suggest that pharmacological modulation of GABA_A receptors may represent a viable pathway for the development of drug abuse pharmacotherapies.

⁹Correspondence Author: Keith L. Shelton, PhD, 410 North 12th Street, Room 746, P.O. Box 980613, Richmond, Virginia 23298-0613, Phone: (804) 827-2104, FAX: (804) 828-2117, kshelto@vcu.edu.

Conflict of interest: Dr. Banks declares that the present study was not related to this professional relationship and should not be perceived as constituting a conflict of interest.

Introduction

The illicit use and abuse of drugs in spite of their detrimental effects remains an ongoing societal problem. It is estimated that drug abuse in the United States costs upwards of 700 billion dollars per year in lost productivity, increased healthcare costs, and criminal activity (National Drug Intelligence Center 2011). However, despite the huge economic and health-related toll that drugs can take on individuals and society, most classes of abused drugs have no FDA approved pharmacotherapies. Stimulants such as methamphetamine and abused inhalants such as toluene are among the substances which lack effective treatment medications.

A common thread underlying drugs of abuse are the effects they produce on the mesolimbic dopamine (DA) system, which is critical in reward processes (Koob 1992; Ikemoto and Bonci 2014). A key component of this pathway are dopaminergic neurons which project from the ventral tegmental area (VTA) through the medial forebrain bundle to the nucleus accumbens (NAc) (Oades and Halliday 1987). While DA is the primary neurotransmitter within the mesolimbic system, other neurotransmitter receptors play a role in regulating DA release (Creed et al. 2014). 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 (van Zessen et al. 2012; Tan 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) (Di Scala et al. 1992; Bossert and Franklin 2003; Rowlett and Lelas 2007). 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 (Moody and Skolnick 1988; Bishop and Laverty 1989; 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, BDZ-site negative modulators may also have utility in reducing abuse-related behavioral effects of other drugs that do not act directly at the BDZ site. In order to test this hypothesis we examined the effect of Ro15-4513 to attenuate the ICSS facilitation produced by the DA releaser d-methamphetamine, the abused inhalant toluene, and the GABA_A BDZ-site positive modulator diazepam. We contrasted the effects of Ro15-4513 on drug-facilitated ICSS to those of the BDZ-site antagonist flumazenil to determine the degree to which negative modulation of GABA_A receptors as opposed to neutral antagonism was required to produce the effects noted. In addition to our behavioral tests we employed a microdialysis procedure to determine if Ro15-4513 would attenuate DA release in the NAc evoked by d-methamphetamine, a defining neurochemical characteristic of many drugs of abuse.

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 addressed 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).

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 for ICSS as described previously (Tracy et al. 2014). Following stable responding at 30 responses/min, mice progressed 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 (Potier et al. 1988; Kida et al. 2003).

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) (Negus and Miller 2014). 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 with 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 (Jason C. Hsu 1996; Aickin and Gensler 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 μ L/min. Prior to beginning sample collection, probes equilibrated for at least two hours or until stable DA 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 μ L 0.01M HCl. DA 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 $\mu\text{L}/\text{min}$. After establishing a stable baseline, saline was administered to examine the effects of injection on DA 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. DA 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 DA 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 $\times$ 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 (Fig. 1a). The ANOVA found a significant treatment $\times$ 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 (Fig. 1b) producing a significant treatment $\times$ 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 (Fig. 1c). There was neither a significant main effect of flumazenil treatment [$F(3, 18) = 1.810, p = 0.1815$] nor treatment $\times$ frequency interaction [$F(27, 162) = 1.252, p = 0.1968$]. Ro15-4513 when administered alone significantly reduced responding for ICSS (Fig. 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 (Fig. 2a). The ANOVA indicated a significant treatment $\times$ 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 increase %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 2b). The ANOVA indicated a significant main effect of treatment [$F(2, 12) = 7.949, p = 0.0063$] but no treatment $\times$ 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 (Fig. 2c). The ANOVA found a significant treatment $\times$ 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 (Fig. 2d). The ANOVA indicated a significant treatment $\times$ 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 (Fig. 2d). 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 (Fig. 2e). The ANOVA found a significant main effect of treatment [$F(3, 18) = 8.296, p = 0.0011$] but no treatment $\times$ 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 (Fig. 2e). 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 (Fig. 2e).

Pretreatment with 0.3 mg/kg Ro15-4513 also completely blocked facilitation of ICSS produced by 3 mg/kg diazepam (Fig. 2f). The ANOVA found a significant treatment $\times$ 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 (Fig. 2c) 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 1. 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 DA levels ($\pm$ 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 DA levels peaking at 903% of baseline 21 min after d-methamphetamine injection (Fig. 3). Administration of 1 mg/kg of Ro15-4513 1-min prior to d-methamphetamine attenuated maximal extracellular DA levels in the NAc which peaked

at 434% of baseline. A mixed 2-way ANOVA demonstrated a treatment $\times$ time interaction [$F(52, 338) = 4.978, p < 0.0001$]. Post hoc analysis indicated that DA 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. DA 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 4.

Discussion

As expected based on prior studies, methamphetamine, toluene and diazepam all significantly facilitated ICSS responding (Robinson et al. 2012; Chan et al. 2012; Bauer et al. 2013; Tracy et al. 2014; Chan et al. 2015). 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 we did not explore this interaction systematically our 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 (Lukas et al. 1986a; Lukas et al. 1986b; Brauer et al. 1996).

It was our 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 drugs that do not themselves bind at this site. It was therefore important to differentiate the effects of Ro15-4513 from those produced by a BDZ-site antagonist. 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 but flumazenil. However, a dose of flumazenil sufficient to block a high efficacy benzodiazepine-site agonist had no effect on ICSS facilitation produced by either methamphetamine or 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). In contrast, Ro15-4513 completely blocked d-methamphetamine, toluene and diazepam-facilitated

ICSS. While toluene has positive GABA_A modulatory effects (Beckstead et al. 2000; Williams et al. 2005; Shelton and Nicholson 2013; Beckley and Woodward 2013), d-methamphetamine does not (Hondebrink et al. 2011), supporting our hypothesis that negative allosteric GABA_A modulation via the BDZ site can strongly affect the reward-related effects of drugs of abuse, 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 produced 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, we assessed the effects of Ro15-4513 and flumazenil alone on ICSS responding. In the subsequent interaction study we 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. We therefore are 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 produced a measurable effect although at these doses any effect might be attributable to either inverse agonist properties (De Vry and Slangen 1985; File et al. 1986) or even to its weak positive modulatory effects at α_{1-2} -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 (Tan et al. 2010; Rudolph and Knoflach 2011; Engin et al. 2014).

To extend our behavioral results to a direct measure of mesolimbic dopaminergic activity, we examined whether Ro15-4513 would attenuate d-methamphetamine stimulated DA release in the NAc. We focused only on d-methamphetamine as it produces much more robust DA release than either toluene or diazepam, and therefore provided sufficient experimental dynamics to detect changes in DA levels (Invernizzi et al. 1991; Riegel et al. 2007; Apawu et al. 2015). To allow direct comparison with our ICSS data, we utilized the same dose combination of 1 mg/kg Ro15-4513 with 3 mg/kg d-methamphetamine that was examined in the ICSS procedure. Administration of 1 mg/kg Ro15-4513 with 3 mg/kg d-methamphetamine produced a ~58% reduction in peak DA 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. Time course differences between testing in ICSS and the observed peak dopaminergic activity were minimally separated when accounting for lag time between microdialysis probe tip and 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). These data confirm that negative modulation of GABA_A receptors will attenuate drug-stimulated DA 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 DA 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 DA 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.

Our 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 we 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 our 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 (MSNs) (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). Other examples of more distant projections 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 activation of RMTg neurons inhibit the locomotor and reward-like functions found in midbrain dopaminergic cells (Jhou et al. 2009). 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 show that GABAergic negative allosteric modulation attenuates drug facilitated ICSS in mice across three abused drugs with differing underlying mechanisms of action. Given our *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 $\alpha_{1-3,5}$ -containing GABA_A receptors (BDZ-sensitive) which are ubiquitously expressed through the mammalian brain, 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, our 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).

Acknowledgments

Dr. Shelton and Matt Tracy declare that the NIH has funded their research under R01DA-020553 and F31DA-034469, respectively. They receive no compensation nor do they have any conflicting financial interests in regards to the work described in this manuscript. Dr. Banks declares that NIH has funded his research. During the past 3 years, he has received compensation as a collaborator with Perdue Pharmaceuticals for projects related to opioid pharmacology and drug development.

References

- Aickin M, Gensler H. Adjusting for multiple testing when reporting research results: the Bonferroni vs Holm methods. *Am J Public Health*. 1996; 86:726–8. [PubMed: 8629727]
- Apawu AK, Mathews TA, Bowen SE. Striatal dopamine dynamics in mice following acute and repeated toluene exposure. *Psychopharmacology (Berl)*. 2015; 232:173–84.10.1007/s00213-014-3651-x [PubMed: 24994552]
- Bauer CT, Banks ML, Blough BE, Negus SS. Use of intracranial self-stimulation to evaluate abuse-related and abuse-limiting effects of monoamine releasers in rats. *Br J Pharmacol*. 2013; 168:850–862.10.1111/j.1476-5381.2012.02214.x [PubMed: 22978626]
- Beckley JT, Woodward JJ. Volatile solvents as drugs of abuse: focus on the cortico-mesolimbic circuitry. *Neuropsychopharmacology*. 2013; 38:2555–67.10.1038/npp.2013.206 [PubMed: 23954847]
- Beckstead MJ, Weiner JL, Eger EI 2nd, et al. Glycine and gamma-aminobutyric acid(A) receptor function is enhanced by inhaled drugs of abuse. *Mol Pharmacol*. 2000; 57:1199–1205. [PubMed: 10825391]
- Bishop BE, Laverty R. Dose-dependent reduction by Ro 15-4513 in mice of the effects of ethanol and some other general depressant drugs. *Eur J Pharmacol*. 1989; 162:265–71. [PubMed: 2498108]
- Bossert JM, Franklin KBJ. Reinforcing versus anticonvulsant drugs: effects on intracranial self-stimulation rate-frequency M50 indices. *Behav Brain Res*. 2003; 144:243–247. [PubMed: 12946613]
- Brauer LH, Ambre J, De Wit H. Acute tolerance to subjective but not cardiovascular effects of d-amphetamine in normal, healthy men. *J Clin Psychopharmacol*. 1996; 16:72–6. [PubMed: 8834422]
- Carlezon WA, Chartoff EH. Intracranial self-stimulation (ICSS) in rodents to study the neurobiology of motivation. *Nat Protoc*. 2007; 2:2987–2995.10.1038/nprot.2007.441 [PubMed: 18007634]
- Chan M-H, Chung S-S, Stoker AK, et al. Sarcosine attenuates toluene-induced motor incoordination, memory impairment, and hypothermia but not brain stimulation reward enhancement in mice. *Toxicol Appl Pharmacol*. 2012; 265:158–165.10.1016/j.taap.2012.10.004 [PubMed: 23067721]

- Chan M-H, Tsai Y-L, Lee M-Y, et al. The group II metabotropic glutamate receptor agonist LY379268 reduces toluene-induced enhancement of brain-stimulation reward and behavioral disturbances. *Psychopharmacology (Berl)*. 2015;1007/s00213-015-3973-3 [PubMed: 24478655]
- Creed MC, Ntamati NR, Tan KR. VTA GABA neurons modulate specific learning behaviors through the control of dopamine and cholinergic systems. *Front Behav Neurosci*. 2014; 8:8.10.3389/fnbeh.2014.00008 [PubMed: 24478655]
- D'Souza D. Ability of Partial Inverse Agonist, Iomazenil, to Block Ethanol Effects in Humans. *NCT01590277*. 2015
- De Vry J, Slangen JL. The Ro 15-1788 cue: evidence for benzodiazepine agonist and inverse agonist properties. *Eur J Pharmacol*. 1985; 119:193–7. [PubMed: 3937736]
- Di Scala G, Oberling P, Rocha B, Sandner G. Conditioned place preference induced by Ro 16-6028, a benzodiazepine receptor partial agonist. *Pharmacol Biochem Behav*. 1992; 41:859–62. [PubMed: 1317585]
- Dixon CI, Morris HV, Breen G, et al. Cocaine effects on mouse incentive-learning and human addiction are linked to alpha2 subunit-containing GABAA receptors. *Proc Natl Acad Sci U S A*. 2010; 107:2289–94.10.1073/pnas.0910117107 [PubMed: 20133874]
- Engin E, Bakhurin KI, Smith KS, et al. Neural Basis of Benzodiazepine Reward: Requirement for $\alpha 2$ Containing GABAA Receptors in the Nucleus Accumbens. *Neuropsychopharmacology*. 2014; 39:1805–15.10.1038/npp.2014.41 [PubMed: 24553732]
- File SE, Dingemans J, Friedman HL, Greenblatt DJ. Chronic treatment with Ro 15-1788 distinguishes between its benzodiazepine antagonist, agonist and inverse agonist properties. *Psychopharmacology (Berl)*. 1986; 89:113–7. [PubMed: 3090585]
- Fish EW, Riday TT, McGuigan MM, et al. Alcohol, cocaine, and brain stimulation-reward in C57Bl/6J and DBA/2J mice. *Alcohol Clin Exp Res*. 2010; 34:81–89.10.1111/j.1530-0277.2009.01069.x [PubMed: 19860803]
- Hondebrink L, Meulenbelt J, van Kleef RGDM, et al. Modulation of human GABAA receptor function: a novel mode of action of drugs of abuse. *Neurotoxicology*. 2011; 32:823–7.10.1016/j.neuro.2011.05.016 [PubMed: 21729720]
- Ikemoto S, Bonci A. Neurocircuitry of drug reward. *Neuropharmacology*. 2014; 76 Pt B:329–41.10.1016/j.neuropharm.2013.04.031 [PubMed: 23664810]
- Invernizzi R, Pozzi L, Samanin R. Release of dopamine is reduced by diazepam more in the nucleus accumbens than in the caudate nucleus of conscious rats. *Neuropharmacology*. 1991; 30:575–8. [PubMed: 1922681]
- Hsu, Jason C. Multiple Comparisons: Theory and methods. First. Chapman & Hall; London: 1996.
- Jhou TC, Fields HL, Baxter MG, et al. The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron*. 2009; 61:786–800.10.1016/j.neuron.2009.02.001 [PubMed: 19285474]
- Kida T, Noguchi J, Zhang M-R, et al. Metabolite analysis of [^{11}C]Ro15-4513 in mice, rats, monkeys and humans. *Nucl Med Biol*. 2003; 30:779–784. [PubMed: 14499337]
- Koob GF. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol Sci*. 1992; 13:177–184.10.1016/0165-6147(92)90060-J [PubMed: 1604710]
- Leitl MD, Onvani S, Bowers MS, et al. Pain-Related Depression of the Mesolimbic Dopamine System in Rats: Expression, Blockade by Analgesics, and Role of Endogenous κ -opioids. *Neuropsychopharmacology*. 2013; 39:614–624.10.1038/npp.2013.236 [PubMed: 24008352]
- Lukas SE, Mendelson JH, Benedikt RA. Instrumental analysis of ethanol-induced intoxication in human males. *Psychopharmacology (Berl)*. 1986a; 89:8–13. [PubMed: 3090597]
- Lukas SE, Mendelson JH, Benedikt RA, Jones B. EEG alpha activity increases during transient episodes of ethanol-induced euphoria. *Pharmacol Biochem Behav*. 1986b; 25:889–95. [PubMed: 3786347]
- McBride WJ, Murphy JM, Lumeng L, Li TK. 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*. 1988; 30:1045–50. [PubMed: 3265788]

- Melón LC, Boehm SL. 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*. 2011; 220:230–7.10.1016/j.bbr.2011.02.014 [PubMed: 21320533]
- Miczek KA, Weerts EM. Seizures in drug-treated animals. *Science*. 1987; 235:1127a.10.1126/science.235.4793.1127a [PubMed: 3823870]
- Miller DW, Yourick DL, Tessel RE. Antagonism of methoxyflurane-induced anesthesia in rats by benzodiazepine inverse agonists. *Eur J Pharmacol*. 1989; 173:1–10. [PubMed: 2558027]
- Miller LL, Leidl MD, Banks ML, et al. Effects of the triple monoamine uptake inhibitor amitifadine on pain-related depression of behavior and mesolimbic dopamine release in rats. *Pain*. 2015; 156:175–184.10.1016/j.pain.0000000000000018 [PubMed: 25599313]
- Moody EJ, Skolnick P. The imidazobenzodiazepine Ro 15-4513 antagonizes methoxyflurane anesthesia. *Life Sci*. 1988; 43:1269–76. [PubMed: 2845216]
- National Drug Intelligence Center. The Economic Impact of Illicit Drug Use on American Society. Washington D.C.: United States Department of Justice; 2011.
- National Research Council. Guide for the care and use of laboratory animals. 8th. National Academies Press; Washington, D.C: 2011.
- Negus SS, Miller LL. Intracranial Self-Stimulation to Evaluate Abuse Potential of Drugs. *Pharmacol Rev*. 2014; 66:869–917.10.1124/pr.112.007419 [PubMed: 24973197]
- Nieh EH, Kim S-Y, Namburi P, Tye KM. Optogenetic dissection of neural circuits underlying emotional valence and motivated behaviors. *Brain Res*. 2013; 1511:73–92.10.1016/j.brainres.2012.11.001 [PubMed: 23142759]
- Oades RD, Halliday GM. Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. *Brain Res*. 1987; 434:117–65. [PubMed: 3107759]
- Páez-Martínez N, Ambrosio E, García-Lecumberri C, et al. Toluene and TCE decrease binding to mu-opioid receptors, but not to benzodiazepine and NMDA receptors in mouse brain. *Ann N Y Acad Sci*. 2008; 1139:390–401.10.1196/annals.1432.031 [PubMed: 18991886]
- Paxinos, G.; Franklin, KBJ. The Mouse Brain in Stereotaxic Coordinates. 2nd. Academic Press; San Diego: 2001.
- Pokk P, Zharkovsky A. 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*. 1997; 48:253– 61. [PubMed: 9223029]
- Potier MC, Prado de Carvalho L, Dodd RH, et al. In vivo binding of (3H)Ro15-1788 in mice: comparison with the in vivo binding of (3H)flunitrazepam. *Life Sci*. 1988; 43:1287–96. [PubMed: 2845217]
- Riegel AC, Zapata A, Shippenberg TS, French ED. The abused inhalant toluene increases dopamine release in the nucleus accumbens by directly stimulating ventral tegmental area neurons. *Neuropsychopharmacol Off Publ Am Coll Neuropsychopharmacol*. 2007; 32:1558–1569.10.1038/sj.npp.1301273
- Robinson JE, Agoglia AE, Fish EW, et al. Mephedrone (4-methylmethcathinone) and intracranial self-stimulation in C57BL/6J mice: comparison to cocaine. *Behav Brain Res*. 2012; 234:76–81.10.1016/j.bbr.2012.06.012 [PubMed: 22728726]
- Rowlett JK, Lelas S. Comparison of zolpidem and midazolam self-administration under progressive-ratio schedules: consumer demand and labor supply analyses. *Exp Clin Psychopharmacol*. 2007; 15:328–337.10.1037/1064-1297.15.4.328 [PubMed: 17696679]
- Rudolph U, Knoflach F. Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. *Nat Rev Drug Discov*. 2011; 10:685–697.10.1038/nrd3502 [PubMed: 21799515]
- Russo SJ, Nestler EJ. The brain reward circuitry in mood disorders. *Nat Rev Neurosci*. 2013; 14:609–25.10.1038/nrn3381 [PubMed: 23942470]
- Schaefer GJ, Michael RP. Interactions between RO 15-4513 and ethanol on brain self-stimulation and locomotor activity in rats. *Pharmacol Biochem Behav*. 1989; 34:785–90. [PubMed: 2623031]
- Shelton KL, Grant KA. Effects of naltrexone and Ro 15-4513 on a multiple schedule of ethanol and Tang self-administration. *Alcohol Clin Exp Res*. 2001; 25:1576–85. [PubMed: 11707632]

- Shelton KL, Nicholson KL. Benzodiazepine-like discriminative stimulus effects of toluene vapor. *Eur J Pharmacol.* 2013; 720:131–137. [PubMed: 24436974]
- Smith AJ, Alder L, Silk J, et al. 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.* 2001; 59:1108–18. [PubMed: 11306694]
- Glantz, Stanton A. *Primer of Biostatistics.* 6th. McGraw-Hill Education / Medical; 2006.
- Steffensen SC, Lee RS, Stobbs SH, Henriksen SJ. Responses of ventral tegmental area GABA neurons to brain stimulation reward. *Brain Res.* 2001; 906:190–197. [PubMed: 11430879]
- Stinchcomb A, Bowers BJ, Wehner JM. The effects of ethanol and Ro 15-4513 on elevated plus-maze and rotarod performance in long-sleep and short-sleep mice. *Alcohol.* 1989; 6:369–376. [PubMed: 2510766]
- Straub CJ, Carlezon WAJ, Rudolph U. Diazepam and cocaine potentiate brain stimulation reward in C57BL/6J mice. *Behav Brain Res.* 2010; 206:17–20.10.1016/j.bbr.2009.08.025 [PubMed: 19716385]
- Tan KR, Brown M, Labouèbe G, et al. Neural bases for addictive properties of benzodiazepines. *Nature.* 2010; 463:769–774.10.1038/nature08758 [PubMed: 20148031]
- Tan KR, Yvon C, Turiault M, et al. GABA neurons of the VTA drive conditioned place aversion. *Neuron.* 2012; 73:1173–83.10.1016/j.neuron.2012.02.015 [PubMed: 22445344]
- Taylor SR, Badurek S, Dileone RJ, et al. GABAergic and Glutamatergic Efferents of the Mouse Ventral Tegmental Area. *J Comp Neurol.* 2014;10.1002/cne.23603
- Tracy ME, Slavova-Hernandez GG, Shelton KL. Assessment of reinforcement enhancing effects of toluene vapor and nitrous oxide in intracranial self-stimulation. *Psychopharmacology (Berl).* 2014; 231:1339–1350.10.1007/s00213-013-3327-y [PubMed: 24186077]
- van Zessen R, Phillips JL, Budygin EA, Stuber GD. Activation of VTA GABA neurons disrupts reward consumption. *Neuron.* 2012; 73:1184–1194.10.1016/j.neuron.2012.02.016 [PubMed: 22445345]
- Venault P, Chapouthier G. From the behavioral pharmacology of beta-carbolines to seizures, anxiety, and memory. *ScientificWorldJournal.* 2007; 7:204–23.10.1100/tsw.2007.48 [PubMed: 17334612]
- Votey SR, Bosse GM, Bayer MJ, Hoffman JR. Flumazenil: a new benzodiazepine antagonist. *Ann Emerg Med.* 1991; 20:181–8. [PubMed: 1996802]
- Williams JM, Stafford D, Steketee JD. Effects of repeated inhalation of toluene on ionotropic GABAA and glutamate receptor subunit levels in rat brain. *Neurochem Int.* 2005; 46:1–10.10.1016/j.neuint.2004.07.006 [PubMed: 15567510]
- Xia Y, Driscoll JR, Wilbrecht L, et al. Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. *J Neurosci.* 2011; 31:7811–6.10.1523/JNEUROSCI.1504-11.2011 [PubMed: 21613494]

Figure 1.

Mean ($\pm$ 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 2.

Mean ($\pm$ 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, # denote significant difference for labeled point vs pretreatment with 3 mg/kg flumazenil or 1 mg/kg Ro15-4513.

Figure 3.

Mean baseline ($\pm$ 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 ($\pm$ 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 4.

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).

Table 1
Maximum Response Rates

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 multiple comparisons test.

* Indicates significant difference.