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Effects of the abused inhalant toluene on mPFC-dependent cognitive behaviors and associated neural activity

By

Kevin Michael Braunscheidel, B.A.

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences in the College of Graduate Studies
Department of Neuroscience

July 2020

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DEDICATION

to Kelsey
to John & A
to Kiwi
and to Karpé

ACKNOWLEDGEMENTS

I would like to first thank my parents, John and Adrianne Braunscheidel, for their unwavering support of my goals throughout my life. I hope that I have made you proud.

Thank you to my peers, especially Alex Smith, Maggie Mae Mell, Jess Breedlove, Logan Dowdle, J.R. Haun, and others. Commiseration is the best medicine. Beer helps, too.

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ABSTRACT

KEVIN M. BRAUNSCHEIDEL. Effects of the abused inhalant toluene on mPFC-dependent cognitive behaviors and associated neural activity . Under the direction of JOHN J. WOODWARD.

Volatile organic solvents like toluene induce euphoria and intoxication when inhaled at high concentrations. Inhalant misuse is linked to behavioral, cognitive, and anatomical deficits in humans leading to a reduced productivity and quality of life. Yet, preclinical studies on the effect of inhalants on executive control in animal models are limited. We address this gap in knowledge using rodent models in two ways: first, by examining the long-lasting effects of repeated toluene inhalation during adolescence on several measures of executive function in adulthood and second, by studying the effects of acute toluene inhalation on risk/reward decision making and related neurocircuitry. Repeated inhalation of toluene during adolescence blunted acquisition of operant and Pavlovian learning in adulthood without affecting probabilistic discounting, progressive ratio breakpoint, latent inhibition or reversal learning. Acute toluene vapor inhalation, however, caused a dose-dependent, sex-independent deficit in behavioral flexibility during probabilistic discounting, a pattern that implicates dysfunctional medial prefrontal cortex (mPFC) activity. To address this hypothesis, we virally expressed the genetically encoded calcium sensor GCaMP6f in glutamatergic mPFC neurons and monitored calcium transients during during task performance using in vivo fiber photometry. Peaks in GCaMP6f activity shifted from pre-risky to pre-safe choice during contingency updating, an effect that was eliminated by acute toluene exposure. mPFC activity in toluene-treated animals also did not distinguish between risky/large wins and safe/small wins. Interestingly, previous studies from our lab demonstrated a toluene-induced long-term depression of AMPA-mediated synaptic activity in deep-layer mPFC neurons. This effect was dependent on endocannabinoids (EC) synthesis and presynaptic cannabinoid receptor (CB1R) function. Here, we found that pharmacological inhibition of CB1Rs in the mPFC or systemically did not mitigate toluene's effect on probabilistic discounting. Behavioral flexibility in this task also depends on functional mPFC-basolateral amygdala (BLA) neurocircuitry. Electrophysiological interrogation of BLA neurons innervated by the mPFC using ex vivo slice electrophysiology and optogenetics revealed a CB1R-dependent decrease in excitatory synaptic transmission following toluene application. These data elucidate learning and behavioral flexibility deficits caused by toluene, including insights on potential mPFC-BLA- and CB1R-dependent mechanisms.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Introduction

The term “inhalants” refers to any highly volatile, non-combusted substance that is inhaled to experience a euphoric high (Balster et al., 2009; Siegel et al., 2009; Gigengack, 2014; Johnston et al., 2018). Human abusers commonly achieve this high by either “sniffing” fumes directly from a canister or concentrating the substance in a bag and “huffing” the vapor (3000 – 15,000 ppm), several times over the course of 15 minutes to several hours (Bowen et al., 2006). Inhalants are chemically and pharmacologically diverse and include anesthetics, alkyl nitrates, nitrous oxide, and volatile organic solvents (Marsolek et al., 2010; Beckley and Woodward, 2013; Johnston et al., 2018). Whereas other illicit substances are often difficult or expensive to obtain, inhalants are an ingredient in many common household products (e.g. spray paints, cleaners, and adhesives). As a result of their accessibility, it is not surprising that adolescents constitute a significant percentage of the overall inhalant-abusing population. In 2018, 662,000 American adolescents reported abusing inhalants (Substance Abuse and Mental Health Services Administration, 2019). This number is likely an underestimation as it does not account for delinquent and homeless individuals, populations that are particularly susceptible to inhalant abuse (see *Inhalant abuse: epidemiology*). Despite the prevalence of inhalant abuse only a handful of NIH funded grants are devoted to their study (NIH Reporter).

Inhalant Abuse: Epidemiology

The National Institute on Drug Abuse estimates that roughly 23.4 million American adults have used inhalants at least once in their lives (Tice, 2016) while 846,000 adults are active users (Substance Abuse and Mental Health Services Administration, 2019). However, given their ease of accessibility, low cost, and low rates of testing, inhalant abuse is particularly high among adolescent populations. Inhalant abuse in the United States peaked in the 1970's and has been largely in decline ever since (Marsolek et al., 2010; Halliburton and Bray, 2016). Despite this, it has been suggested that usage rates may be on the rise in middle school populations (Johnston et al., 2018) and currently, the lifetime prevalence in American primary school populations remains 6-8% (Kann et al., 2016; Johnston et al., 2018), second to marijuana in terms of illicit substance abuse.

Inhalant abuse is a global phenomenon. Other well-developed nations including Canada, Japan and several Western European countries report comparable or lower lifetime usage rates compared to the United States (Kikuchi and Wada, 2003; Adlaf et al., 2004; ESPAD Group, 2016). A particular burden, however, is placed on impoverished or isolated communities (Perron and Howard, 2009). For example, groups of young Native Americans have been found "bagging" gasoline in rural Alaska (Eggertson, 2014). In India, 35% of homeless children reported huffing toluene-containing whitener (Praveen et al., 2012) and a staggering 91% of children living on the streets of Upper Egypt have been reported to abuse inhalants (Elkoussi and Bakheet, 2011). Native populations in the United

states (Kaufman, 1973; Beauvais et al., 2002), Australia (Cairney et al., 2002) and eastern Slovakia (Važan et al., 2011) also report some of the highest rates of inhalant abuse. These findings extend to the most impoverished countries of Europe, as high lifetime prevalence has been reported in Croatia (25%), Slovenia (14%), Greece (13%), Estonia (13%), Georgia (12%) and Austria (10%) (ESPAD Group, 2016). A similar trend is observed across the United States where some of the poorest states (Alabama, Arkansas, Mississippi, West Virginia) report the highest lifetime prevalence (all > 9.9%) of inhalant abuse (Kann et al., 2016). This socio-economic correlation is also supported by Kann et al.'s finding of higher lifetime inhalant abuse rates in large urban school districts relative to the entire state. For instance, there is a much higher inhalant use prevalence in teens residing in Baltimore (11.6%) and the District of Columbia (11.5%) compared to the state of Maryland's overall prevalence (8.5%).

The driving force of these high rates of inhalant abuse are undoubtedly multi-faceted, but poor or absent parenting in these regions may be one common factor (Fleschler et al., 2002; Siegel et al., 2009; Elkoussi and Bakheet, 2011). In fact, a 2011 longitudinal study of 8,182 American teenagers (89,219 person-period observations) found that poor parenting is associated with initiation of inhalant use, with parental drug use being a risk factor and parental monitoring being protective (Nonnemaker et al., 2011). It is, therefore unsurprising that socially marginalized youths (including those in juvenile detention or socially impoverished conditions) have high rates of inhalant abuse across the Americas (Tapia-Conyer et al., 1995; Howard and Jenson, 1999; Dell and Hopkins, 2011)

Not all low socio-economic status areas report high rates of inhalant abuse. In fact, in some of South America's poorest countries, teenagers report lower average lifetime prevalence of inhalant abuse (2.7-5.5%) than the United States (6.7%) (Hynes-Dowell et al., 2011; Johnston et al., 2018). Conversely, the lifetime prevalence of inhalant abuse is 16.55% in Brazil despite their relatively high GDP. This could be explained by the sociocultural history of individuals with higher socioeconomic status abusing lança and loló (ether containing perfumes), especially during *Carnival* (Hynes-Dowell et al., 2011; Sanchez et al., 2013). Similarly, 27.9% of Kingston University students in London admitted to past-year "hippy crack" (nitrous oxide) abuse and many of these students intended to continue use of the drug (Ehirim et al., 2018). If this finding extends across other English populations it may help explain the relatively high lifetime prevalence of inhalant use (9%) across all UK adolescents (ESPAD Group, 2016).

Sex differences in usage rates have been reported for other drugs of abuse (ESPAD Group, 2016; Johnston et al., 2018). With regards to inhalants, these differences have also been reported but are largely inconsistent across specific populations and time (Batis, 2017). For example, in American middle schools, girls have a higher lifetime prevalence than boys, but this observation is reversed by the end of high school (Johnston et al., 2018). In Europe, lifetime prevalence in male adolescents has historically been higher than females, yet recent trends have clouded these differences (ESPAD Group, 2016). Therefore, future investigations should consider sex-related differences in inhalant abuse on a population-by-population basis.

Toluene Pharmacology

Organic solvents are perhaps the most accessible inhalant as illustrated by U.S. Poison Control data which describes cases involving over 3000 different solvent containing products (Marsolek et al., 2010). Despite the general dearth of basic neuroscience research on inhalants, there is a relative abundance of data on toluene (methylbenzene). This was driven by initial studies examining the toxicity of high concentrations of toluene-containing products like adhesives, paints and paint thinners (Malm and Lying-Tunell, 1980; King et al., 1981; Streicher et al., 1981; Fornazzari et al., 1983; Hormes et al., 1986). Other research was concerned with exposure by industry workers to chronic, sub-abuse vapor concentrations (Apostoli et al., 1982; Foo et al., 1991; Morata et al., 1993; Neubert et al., 2001a, 2001b). The original hypothesis on the mechanism for toluene was that due to its small, lipophilic structure, it altered neuronal activity as a result of cellular membrane disruption (Oh and Kim, 1976; Lebel and Schatz, 1990). This idea has since been challenged by numerous studies describing the interaction between toluene and a variety of neuronal receptor systems.

Bioavailability

Following inhalation of toluene fumes, the majority of vapor is exhaled unchanged. The rest enters the bloodstream through the alveoli and distributes through the body (Garcia, 1994). Blood concentration in rats reaches 60% of the peak concentration about 10 minutes following inhalation and declines to 30% about 40 minutes following inhalation (Benignus et al., 1981). Only about 3% of

inhaled toluene reaches the brain (Benignus et al., 1981) and it is mostly eliminated from the central nervous system 30 min following inhalation (Gerasimov et al., 2002).

Glutamate Receptors

Glutamate is the major excitatory neurotransmitter in the brain and the glutamatergic system is critically involved in developing and maintaining an addiction to abused substances (Kalivas, 2009). A seminal series of electrophysiology experiments using expression of recombinant receptors showed that toluene dose-dependently inhibited the *N*-methyl-D-aspartate subtype of ionotropic glutamate receptor (GluNs) at concentrations that did not affect membrane integrity (Cruz et al., 1998). These effects were rapid, reversible, and subunit selective, with GluN1/2B receptors being more sensitive than GluN1/2A or GluN1/2C. These authors also showed that toluene does not act as a competitive inhibitor of glutamate, but may reduce channel function by interfering with the domains involved in channel gating. Although a specific toluene-sensitive domain has not been identified, toluene inhibition is not altered by transmembrane domain mutations that reduce the inhibitory potency of alcohol (Smothers and Woodward, 2016). Toluene inhibition of native neuronal NMDARs has since been shown in rodent medial prefrontal cortex (mPFC), nucleus accumbens, and hippocampus (Bale et al., 2005; Beckley and Woodward, 2011; Beckley et al., 2016). Upregulation of glutamatergic signaling has been reported following repeated drug treatment. For instance, the NMDA component of glutamate-mediated synaptic

events was enhanced following four days of bath-applied toluene to hippocampal cell cultures (Bale et al., 2005) Repeated inhalation also increased expression of GluN subunits in the mPFC and nucleus accumbens (Williams et al., 2005). Increases in hippocampal GluN protein levels persist following a protracted drug abstinence (Furlong et al., 2016). In contrast to these findings, other studies have shown that repeated episodes of toluene inhalation reduces ifenprodil binding to mesocorticolimbic GluN2B-containing receptors without affecting overall protein expression (Dick et al., 2015), and impairs NMDAR-mediated synaptic plasticity in the hippocampus (Bale et al., 2005) and mPFC (Cruz et al., 2019). Taken together, these results suggest that toluene acutely inhibits NMDA signaling, but that homeostatic mechanisms following multiple drug treatments may be in place to counteract this inhibition.

In contrast to NMDA receptors, the other major ionotropic glutamate receptors including 2-amino-3-(3-hydroxy5-methyl-isoxazol-4-yl) propanoic acid (GluA) or kainate receptors are not inhibited by toluene (Cruz et al., 1998; Bale et al., 2005). Likewise, bath-applied toluene does not have immediate effects on AMPAR function in rodent brain slices (Bale et al., 2005; Beckley and Woodward, 2011; Beckley et al., 2016). Rather, when toluene is applied to rodent prefrontal cortex or accumbens, GluA-mediated excitatory signaling slowly begins to decrease, an effect that persists long after toluene clearance. Toluene's effect on GluA signaling was blocked by chelating internal calcium stores, inhibiting internal calcium release by ryanodine receptors, and blocking cannabinoid receptor type 1, which, together, support an endocannabinoid-mediated mechanism (Beckley

and Woodward, 2011; Beckley et al., 2016). Following repeated toluene treatment, changes in GluA protein or mRNA are largely absent. Williams and colleagues (2005) described one exception where increased mPFC GluA2/3 was detected following multiple toluene treatments, but they note that this particular subunit is often co-expressed with GluNs (which are also increased). Alternatively, increases in calcium-impermeable GluA2/3 could be a neuroprotective homeostatic response to prevent excitotoxicity (Talos et al., 2006)

Few studies exist on the interaction between toluene and metabotropic glutamate receptors (mGluR). In one of three such studies, Del Re and colleagues determined that toluene does not alter mGluR(Gi)-dependent GIRK signaling (Del Re et al., 2006). In a second study, a single toluene exposure caused differential changes in gene expression of Gq-protein coupled mGluR1 and mGluR7 receptors (Hester et al., 2011). While the behavioral consequences of this effect are currently unknown, another Gq-protein coupled mGluR (mGluR5) has been shown to be involved in toluene-induced motor incoordination and learning impairments (Chan et al., 2012). Taken together, the existing data suggest that toluene may alter signaling by Gq-, but not Gi-protein coupled mGluRs.

GABA Receptors

In addition to its inhibition of excitatory glutamatergic signaling, toluene enhances the activity of recombinant inhibitory gamma-aminobutyric acid ($GABA_A$) receptors (Beckstead et al., 2000). This inhibition also occurs in brain slices as toluene enhances GABA-mediated synaptic inhibition in the CA1 of the

hippocampus (MacIver, 2009). Similar to toluene's effect on glutamate receptors, repeated exposure has been shown to alter GABA receptor expression throughout the brain. For instance, following toluene treatment/use, $GABA_A$ α1 subunit protein expression is decreased in the substantia nigra and ventral tegmental area (Williams et al., 2005) and $GABA_A$-mediated currents are diminished (Bale et al., 2005). Curiously, however, following a protracted period of drug abstinence, $GABA_A$ receptor protein is upregulated in the medial prefrontal cortex, ventromedial striatum, and dorsolateral striatum (Williams et al., 2005; Furlong et al., 2016). The compensatory response of the central nervous system to repeated toluene exposures are, therefore, complex and should not be generalized.

Other Receptors

Toluene interacts with several other receptor systems, although the literature is sparse in comparison with that for glutamate and GABA; for review, see (Beckley and Woodward, 2013). Many of toluene's effects are consistent with its role as a CNS depressant. For example, toluene is a positive allosteric modulator of ionotropic glycine receptors (Beckstead et al., 2000, 2001) and stimulates 5HT2C receptors (Rivera-garcía et al., 2015), while it inhibits nicotinic acetylcholine receptors (Bale et al., 2002), L-type neuronal calcium channels (Tillar et al., 2002; Shafer et al., 2005), gap junction connexins (Del Re and Woodward, 2005), and P2X2- or P2X4- ATP receptors (Woodward et al., 2004). Paradoxically, toluene may lead to increases in overall neuronal activity as it enhances 5-HT3 receptor function (Lopreato et al., 2003) and 5HT2A signaling (Rivera-garcía et al.,

2015), inhibits BK and GIRK potassium channels (Del Re et al., 2006), potentiates P2X3 ATP receptor function (Woodward et al., 2004), and promotes release of intracellular calcium stores (MacIver, 2009; Beckley and Woodward, 2011). However, research on the effect of toluene on these systems is still nascent and more studies are required to fully understand how these actions contribute to toluene's effect on brain function.

Inhalant Use Disorder: Replicating Symptomology in Preclinical Models

Clinical Presentation

Intoxication occurs after inhaling concentrated vapors (3000 – 15,000 ppm) of inhalants several times over the course of 15 minutes to several hours (Bowen et al., 2006). Individuals report positive effects including euphoria, disinhibition, and excitement that lasts 15 – 60 minutes following use of the drug (Flanagan and Ives, 1994; Anderson and Loomis, 2003; Gigengack, 2014). While the addictive potential of inhalants was first posited in the mid-1900s (Clinger and Johnson, 1951; Glaser and Massengale, 1962), a specific disorder related to inhalant use was underdiagnosed through the early 2000s, with one meta-analysis classifying only 8% of inhalant users as individuals with a disorder (Wu and Ringwalt, 2006). With the adoption of the DSM-5, inhalant abuse and dependence is now defined using similar diagnostic criteria as traditional drugs of abuse including symptoms such as drug tolerance, drug cravings, and physical withdrawal during drug abstinence (American Psychiatric Association, 2013). Comparison of two similarly sourced populations shows that approximately 46% of individuals who use

inhalants now meet criteria for an inhalant abuse disorder (Ridenour et al., 2015), up from 35% in 2006 (Ridenour et al., 2007).

Preclinical modeling of drug reward-related behaviors

In order to better address the cellular, molecular, and systems neuroscience basis of addiction, researchers have developed numerous preclinical models (Sanchis-Segura and Spanagel, 2006) with operant-based drug self-administration being the gold standard. In this paradigm, animals learn to self-administer a drug (orally or intravenously via a chronically implanted catheter) for multiple hours per day over the course of several weeks. This is usually followed by drug-cue extinction, reinstatement or relapse trials to detect changes in drug seeking or craving. The development of a rodent model of inhalant self-administration has lagged behind other drugs of abuse presumably due to the difficulty in controlling inhalant concentrations and overcoming initial aversive effects associated with solvent odor. In the first of exactly two published attempts to do so, four squirrel monkeys outfitted with a custom inhalation helmets were trained to press a lever to receive toluene vapor infusions (Weiss et al., 1979). This approach required a significant investment of time and economic resources and has not been repeated. The second study involved a single, 30 min intravenous administration of a toluene-containing solution in mice (Blokhina et al., 2004). This approach is problematic for studying inhalant addiction for several reasons: 1) it does not mimic the inhaled route of administration in humans, 2) as a solvent, toluene can induce significant vein damage (Kulkarni et al., 2015), and 3) it prevents the study of

critical aspects of addiction that require multiple administration sessions; as such, this study has not been repeated.

With the difficulties noted above in the use of inhalant self-administration protocols, researchers have relied on other techniques to describe the effect of toluene on addiction-related behaviors. For instance, drugs of abuse cause characteristic changes in locomotor activity both acutely, and over time (sensitization). Toluene inhalation has been shown to cause biphasic effects on goal-directed behavior that is typical of central nervous system (CNS) depressants: low concentrations of toluene increase (and high concentrations decrease) fixed-rate responding for a food reward in mice, rats and pigeons (Weiss et al., 1979; Glowa, 1981; Glowa et al., 1983; Wood et al., 1983). Toluene generates an inverted-U dose-response curve on locomotion, similar to that produced by other CNS depressants. (Kjellstrand et al., 1985; Wood and Colotla, 1990; Riegel and French, 1999a; Lo et al., 2009). Brief, repeated schedules of high-concentration toluene vapor exposures designed to model human consumption patterns also causes locomotor sensitization (Himnan, 1984; Batis et al., 2010; Páez-martínez et al., 2020). Behavioral sensitization has also been reported in several strains of mice, with the ethanol-avoiding DBA/2J strain being particularly susceptible (Bowen et al., 2010). Locomotor sensitization cross-sensitizes between toluene and other drugs of abuse including diazepam and cocaine (Wiley et al., 2003) and in a drug discrimination task, toluene substitutes for CNS depressants including ethanol and pentobarbital (Rees et al., 1987a, 1987b). Paradoxically, toluene also cross-sensitizes with cocaine and will substitute for amphetamine-driven operant

behavior (Beyer et al., 2001; Bowen, 2006). While toluene has CNS depressant effects, these results suggest that toluene is not strictly a depressant, supporting clinical observations and its mixed pharmacological profile discussed above(Flanagan and Ives, 1994; Anderson and Loomis, 2003; Gigengack, 2014).

Although studies of drug-induced changes in locomotor activity are important, they do not fully encapsulate motivated behaviors relating to drug memories. To address this, scientists often use a conditioned place preference test to measure the association of a rewarding experience with the context in which it was experienced. Like all other drugs of abuse, toluene generates this hedonic association in rodents (Funada et al., 2002; Wayman and Woodward, 2018). In addition to CPP, intracranial self-stimulation (ICSS) can be used to assess the effect of drugs on reward-processing. To conduct this task, rodents are implanted with an electrode in the median forebrain bundle and then trained to operantly respond for different frequencies of activation. Stimulation of this tract is highly reinforcing, as it activates limbic structures involved in reward (Stevens Negus and Miller, 2014). The response rate in the presence of a drug compared to baseline is a good measure of how primed the reward neurocircuitry is compared to baseline. Animals treated with any of the following substances have lower ICSS response rates (i.e. the animal needs less stimulation to feel reward): amphetamine, methamphetamine, MDMA, cocaine, nicotine, diazepam, and caffeine (Bauer et al., 2013, 2014; Stevens Negus and Miller, 2014) . This effect represents drug-primed sensitization of reward signaling in the brain (Stevens Negus and Miller, 2014). Surprisingly, initial reports showed that glue vapors

containing 25% toluene increased ICSS threshold (Yavich et al., 1994). Studies using pure toluene vapor, however, have demonstrated the expected decreases in ICSS response rate, an effect that requires mGluR2/3 and $GABA_A$ activity. (Chan et al., 2012, 2015; Tracy et al., 2014, 2016; Wu et al., 2018). Further research is needed to identify the specific glutamate and GABA neurocircuitry mediating this toluene induced sensitization.

Striatal dopamine release dictates reward-related behaviors and is pathologically disrupted in individuals with drug and alcohol dependence and in rodents undergoing preclinical models of addiction (Volkow et al., 2019). Toluene evokes the release of dopamine in the ventral striatum (Stengård et al., 1994; Gerasimov et al., 2002; Riegel et al., 2007) via direct stimulation of ventral tegmental neurons, the main dopaminergic input to the nucleus accumbens (Riegel and French, 1999b; Nimitvilai et al., 2016). Extracellular dopamine is responsible for toluene's effects on locomotion (Riegel et al., 2003; Apawu et al., 2015). Acute toluene also increases dopaminergic neuronal activation measured by increases in c-Fos expression in the striatum and ventral tegmental area (Lo et al., 2009; Perit et al., 2012).

Toluene effects on the striatum may have neurotransmitter system specificity. For instance, the enhancing effects of toluene on the ventral tegmental area are 100x more potent than alcohol and accomplished via independent signaling involving muscarinic and GABA-(A and B) receptors (Nimitvilai et al., 2016). Toluene also induces an endocannabinoid mediated inhibition of glutamatergic transmission in D2- but not D1-receptor-containing nucleus

accumbens core neurons (Beckley et al., 2016) and D1 receptor expression is not altered by repeated exposure to toluene vapor (Páez-martínez et al., 2020). Further, blocking D2 receptors in the accumbens prevents toluene-induced locomotor activity (Riegel and French, 1999a). These results suggest a major role for D2-containing striatal neurons in mediating changes in reward processing by toluene.

Studies on the neurobiology of reward processing in human inhalant users did not exist until recently when Jain and colleagues measured BOLD fMRI responses in response to drug cues to compare craving among inhalant users and control subjects (Jain et al., 2020). Among other regions, inhalant cues increased activity in the dorsal striatum and prefrontal cortex, dopamine-rich regions that are involved in habit formation. These results are an important finding in the pursuit of treatments for inhalant abuse in humans. For instance, it was recently reported that substitution therapy using lavender oils and perfume reduced cravings in inhalant abusers although these comparisons were only made against baseline cravings and thus do not control for a placebo effect (Kalayasiri et al., 2018).

Emotional Regulation

Abstinence from all major drugs of abuse results in a negative emotional state (American Psychiatric Association, 2013) that can increase an individual's risk of relapse (Koob and Moal, 2005; Wise and Koob, 2013). The term "amotivational syndrome" has even been suggested as a characteristic feature in inhalant users (Wada et al., 2005). Inhalant users have a high lifetime prevalence

of mood (48%) and anxiety (36%) disorders that developed following onset of inhalant abuse (Wu and Howard, 2007). It follows that people with inhalant use disorders are more likely to be receiving professional treatment for emotional issues (Wu and Ringwalt, 2006). Negative affect has been consistently replicated in animal models on drug relapse (Wise and Koob, 2013; Volkow et al., 2019). Although in rodents toluene is acutely anxiolytic (Lo et al., 2000), abstinence from binge-like toluene inhalation regimen increases vertical rearing (Duncan et al., 2012), a marker of fear and anxiety (Lever et al., 2006). However chronic intraperitoneal injections of toluene in mice does not affect anxiety or depression-like behaviors measured by an elevated plus maze or emergence tests (Lin et al., 2010).

Emotional distress leading to drug relapse is associated with enhanced sensitivity to brain stress systems including the amygdala, habenula, and hypothalamus (Koob et al., 2014; Roberto et al., 2017). Although well studied for other drugs of abuse, there is a paucity of data on the effects of toluene on these systems. A single study by Perit and colleagues (2012) found that an acute 10 or 30 min exposure to 5000 ppm toluene increased c-Fos immunoreactivity, a proxy for cellular activity, in the rat amygdala (basolateral and central), hypothalamus (anterior, paraventricular, and medial preoptic), but not the habenula 1.5 hours after drug exposure. More studies of this type are needed to identify the cellular and molecular determinants involved in the negative emotional state that can accompany inhalant abuse. Together with the other findings discussed in this

section, data from these types of studies are critical for developing effective strategies for treating disorders related to inhalant abuse.

The Prefrontal Cortex: Executive Function & Top-Down Control of Behavior

The prefrontal cortex (PFC), a specialized sub-division of the frontal lobe, is a hub for executive function and exerts control over a variety of sub-cortical areas (Kesner and Churchwell, 2011; Widge et al., 2019). Interrogation of the PFC and its non-human mammalian homologues has led to at least four overlapping theorems on its functionality: *"domain specificity", "level of processing", "rule-learning based on complexity",* and *"top-down control"* (Kesner and Churchwell, 2011). Domain specificity posits that the main role of the PFC is to support working memory defined as a specialized process by which internalized stimuli are held "online" to guide behavior in the absence of external stimuli (Goldman-Rakic, 1996). Owen, Petrides, and colleagues suggest subdividing regions of the PFC on the basis of different levels or complexity of processing (Petrides, 1996; Owen, 1997) . A third model also suggests subdividing the PFC into at least five regions, but these regions each dictate discrete rules or strategies rather than working memory (Wise et al., 1996; Wise, 2008). Finally, according to the top-down executive control model, the PFC engages in executive functions by maintaining patterns of activity that represent goals (Miller and Cohen, 2001). This model also suggest subdivisions of the PFC based not only on anatomy, but also subcortical (especially limbic) projections. Under this model, the rodent PFC is broken down into the following subdivisions, each of which have human homologues (Kesner

and Churchwell, 2011): dorsomedial (anterior cingulate and precentral), ventromedial (prelimbic, infralimbic, medial orbitofrontal), lateral (anterior insula and lateral orbitofrontal), and ventral (ventral orbitofrontal and ventrolateral orbitofrontal). Throughout this dissertation the PFC will be discussed in the context of mediating top-down cognitive control with a focus on the ventromedial PFC since this region appears to be involved in maladaptive goal-based decision making repoprted in individuals with substance use disorders (Kalivas et al., 2009; Volkow et al., 2019)

Cognitive “top-down” control of executive function refers to the ability to regulate cognitive activity and behaviors needed to achieve a central goal, especially in the presence of intermediate steps or distractions (Botvinick and Braver, 2015; Shenhav et al., 2017). This incredibly complex process includes monitoring interactions with the world, evaluating the results of actions, and making adjustments in behaviors to better reach the intended goals. Thus, executive control includes functions such as working memory, temporal processing, planning, flexibility, and decision making. Pathological failures of cognitive control spans many psychiatric illnesses (Gruner and Pittenger, 2017; Ryman et al., 2018; Yang et al., 2018) including substance use disorders (Butler and Le Foll, 2019), where drug relapse is common despite known negative consequences and expressly wanting to quit.

Drugs of abuse impair PFC function consistent with the notion of weakened top-down control in individuals with substance use disorders. For instance, reduced PFC reactivity to negative reinforcers predicts marijuana and cocaine use

in humans (Blair et al., 2018) and activating the PFC can reduce drug craving in humans (Terraneo et al., 2016; Coles et al., 2018). Descending cortico-striatal pathways seem especially important (Winstanley et al., 2010; Tang et al., 2015) as imaging studies in humans have demonstrated that activity in the PFC is linked to striatal function, and inversely associated with sensitivity to hedonic effects of psychostimulants (Volkow et al., 1999, 2002; Blair et al., 2018). Further, reduced expression or function of striatal D2Rs is linked to decreased activity in the dorsolateral PFC in human subjects with substance use disorders (Volkow et al., 2013). Moreover, the function of the PFC in addicted individuals has been shown to predict clinical outcomes, with disrupted connectivity between PFC and striatal regions being a consistent finding among individuals addicted to various drug classes (Tomasi and Volkow, 2013).

Studies in animals corroborate the importance of the PFC and its descending striatal projects in regulating addiction-related behaviors (Kalivas, 2009; Peters et al., 2009; Rocha and Kalivas, 2010; Shen and Kalivas, 2013). For instance, mPFC activity is inhibited in cocaine seeking rodents, and optogenetic excitation of the mPFC during abstinence results in reduced drug seeking (Chen et al., 2013). In line with human studies, specific fronto-striatal pathways are involved in drug-seeking behaviors in rodents. For instance, nucleus accumbens core-projecting prelimbic neurons (PL-NAcc) promote, while nucleus accumbens shell-projecting infralimbic neurons (IL-NAcs) block psychostimulant seeking behavior (Peters et al., 2009; Rocha and Kalivas, 2010; Ball and Slane, 2012; Augur et al., 2016). These effects may be drug-specific, as reinstatement of heroin

seeking was enhanced by activation of IL-NAcs neurons (Bossert et al., 2011, 2012) and reinstatement of alcohol self-administration was facilitated by inactivation of the PL-NAcc (Willcocks and McNally, 2013). These data strongly support the involvement of PFC-NA in regulating substance abuse-related behaviors in rodents, although the specific sub-circuitry does not generalize among different drugs of abuse.

Recent work from our lab and others have described the effect of toluene on electrophysiological properties in the mPFC. Bath-applied toluene does not alter current-evoked spiking in rat mPFC slices (Beckley and Woodward, 2011) but does alter the function of numerous ligand- and voltage-gated channels in this region (see: *Toluene Pharmacology).* This includes a persistent, endocannabinoid-mediated depression of AMPA signaling in deep-layer PL pyramidal neurons (Beckley and Woodward, 2011). Mimicking human inhalant exposure patterns in rats with repeated toluene inhalation treatments in vivo, however, increases the excitability of deep-layer PL neurons, an effect that is mediated by a decrease in afterhyperpolarization current (Armenta-Resendiz et al., 2018). This treatment also increased synaptic strength and reduced inhibitory transmission in the PL as measured by extracellular recordings (Cruz et al., 2019).

The effect of toluene vapor inhalation on mPFC-NA signaling is circuit specific. Following a single binge-like exposure to toluene, current-evoked firing of deep layer PL-NAcs neurons is reduced while that of deep layer PL-NAcc neurons is enhanced (Wayman and Woodward, 2017). Moreover, the expression of toluene CPP was blocked when IL-NAcs were chemogenetically silenced (Wayman and

Woodward, 2018). The mPFC also indirectly impacts striatal circuitry by regulating toluene-induced synaptic strengthening of accumbens projecting dopamine neurons in the VTA. Pharmacologically enhancing mPFC activity prior to toluene inhalation prevents increases in the AMPA/NMDA ratio of NAc-projecting VTA DA neurons, while pharmacologically inhibiting the mPFC prior to exposure to a previously ineffective (and less than abused) concentration of toluene vapor increases the AMPA/NMDA ratio of these neurons (Beckley et al., 2013) Interestingly, this study also showed that toluene inhalation has no effect on the AMPA/NMDA ratio of VTA DA neurons that project to the mPFC. These studies provide strong evidence that toluene alters the function of specific frontostriatal networks that likely underly maladaptive decision making in inhalant abusers.

Toluene-induced Cognitive Dysfunction

The sequelae of inhalant addiction extend beyond changes in reward value processing and cue reactivity. Early studies on recreational inhalant users mention impairments in memory, attention and judgements compared with control and even polydrug users (Korman et al., 1980; Hormes et al., 1986). Impaired processing speeds can even be detected after a single drug experience (Stollery, 1996). Other studies have demonstrated reductions in IQ, working memory, behavioral flexibility, attention, and response inhibition in inhalant abusers (Howard et al., 2008; Lubman et al., 2008; Yuncu et al., 2015). Some toluene-induced impairments in cognition appear to recover (e.g. paired-associations, response inhibition) while others (e.g. visual motor speed, learning and memory, and

executive control) persist despite a protracted period of drug abstinence in inhalant abusers (Dingwall et al., 2011; Takagi et al., 2011).

These lasting deficits are likely due in part to long-lasting damage to white matter, tissue that is particularly susceptible to toluene due to its high lipid content and toluene's high lipid:water coefficient. Toluene damages cortical white matter, as well as the corpus callosum, areas that are essential for executive function (Rosenberg et al., 1988, 2002; Kornfeld et al., 1994; Filley et al., 2004; Marulanda and Colegial, 2005). Prolonged inhalant abuse can even produce toluene leukoencephalopathy, a form of dementia (Hormes et al., 1986; Rosenberg et al., 1988; Filley et al., 1990, 2004; Filley, 2013). In fact, the degree of cerebral white matter injury correlates with the severity of cognitive impairment (Filley et al., 1990).

In line with human studies, chronic exposure of rodents to toluene causes a wide range of impairments in cognitive behaviors including, impaired novel object recognition, spatial learning, and inhibitory avoidance when tested with little or no drug abstinence (Baydas et al., 2005; Batis et al., 2010; Tin et al., 2012). Persistent behavioral effects following drug abstinence are mixed: deficits in learning and memory, have been reported, but more complicated tasks such as outcome devaluation and Pavlovian-to-instrumental transfer are not altered (Dick et al., 2014; Furlong et al., 2016; Braunscheidel et al., 2017). Motivational changes were not observed in two of these studies (Dick et al., 2014; Braunscheidel et al., 2017). However, Furlong et. al (2016a) did note an increase in progressive ratio breakpoint in animals with a history of toluene inhalation. Subtle deficits in

behavioral flexibility were also reported although these might generalize to overall learning deficits, and not flexible decision making impairments, per se. (Furlong et al., 2016; Braunscheidel et al., 2017).

Stark changes in delay discounting, where rats are tasked with choosing to wait progressively longer periods of time for a large food reward, are noted in animals with a distant history of toluene inhalation (Furlong et al., 2016). Other drugs of abuse cause a leftward shift in delay discounting as rodents are unwilling to wait long periods of time for a large reward (Simon et al., 2007; Mendez et al., 2010). This reflects the human condition (Petry, 2001; Kirby and Petry, 2004) and is usually interpreted as increased impulsivity in drug treated animal. Toluene, however, causes a rightward shift in delay discounting, as animals wait longer for a large reward (Furlong et al., 2016). This change was accompanied by increased inhibitory $GABA_A\alpha 1$ protein in the PL. Similar rightward shifts in delayed discounting have been observed following pharmacological lesioning of the mPFC (Churchwell et al., 2009; Gill et al., 2010) and recent reports show that subsets of PL neurons encode reward value during this task (Sackett et al., 2019). Interestingly, Bowen and colleagues previously reported that gestational toluene treatments (every other day from gestational day 8 to 20) altered performance in a waiting-for-reward task in the resulting brood. In this appropriately named task, rats simply need to wait progressively longer periods of time for reward delivery following a series of lever presses. Rats whose mothers were treated with toluene received significantly more rewards than their untreated counterparts (Bowen et al., 2009). Taken together, these results suggest that the effects of toluene on

mPFC-dependent complex decisions where time is a salient factor persist despite protracted drug abstinence.

With regards to modeling toluene-induced dementia, leukoencephalopathy was not observed in rats with a near lifetime of toluene exposure (Ranson and Del Bigio, 2018). However, treating rodents with chronic, intermittent toluene vapor binges does result in region-specific white matter abnormalities, with deficits in the anterior commissure noted following a month of treatments (Duncan 2012). Rearing (vertical plane exploration) deficits were also identified, but they preceded any changes in white matter abnormalities and persisted following white matter recovery suggesting separate underlying mechanisms. Rearing more likely reflects either fear and anxiety or non-specific increases in motivation and arousal and not higher ordered executive function (Lever et al., 2006). As such, and in agreement with the general lack of an effect on complicated decision making in other preclinical studies, the corpus callosum was not affected in this study while other white matter deficits showed recovery following 8 weeks of drug abstinence (Duncan 2012) .

Concluding Remarks

Much of our understanding on the pharmacology and neurobiology of volatile organic solvents abuse comes from studies of toluene, a methylated form of benzene found in a variety of paints, paint thinners, and glues. Still, inhalants are a severely understudied drug of abuse despite their worldwide use and addictive potential. Maladaptive decision making in substance use disorders are

driven by drug-induced changes in the prefrontal cortex, a key brain area involved in top-down control over complex decision making. The following chapters add to an emerging literature on the effects of toluene on cognitive function and prefrontal cortical physiology. Understanding how toluene vapor inhalation impacts the prefrontal cortex is critically important for developing targeted treatment strategies for inhalant abusers.

CHAPTER 2: PERSISTENT COGNITIVE AND MORPHOLOGICAL ALTERATIONS INDUCED BY REPEATED EXPOSURE OF ADOLESCENT RATS TO THE ABUSED INHALANT TOLUENE

INTRODUCTION

Since drug addiction may develop following chronic use, it is important to understand the effects of repeated, long-term toluene exposure on behavior and cognition. Results from preclinical studies reveal that chronic exposure to toluene can cause a wide range of cognitive and behavioral impairments including sensitization to drug-induced hyperlocomotion, impaired novel object recognition, spatial learning, and inhibitory avoidance (Batis et al., 2010; Baydas et al., 2005; Huerta-Rivas et al., 2012). In addition, clinical studies in human toluene abusers also report cognitive deficits such as decreases in IQ and impairments in executive functions such response inhibition, behavioral flexibility, working memory, and attention (Howard et al., 2008; Lubman et al., 2008; Yuncu et al., 2015). Although these studies of chronic toluene exposure are essential to understanding the drug's effects on cognition, most of them assessed behavioral performance shortly following the last toluene exposure. While important, it is also critical to examine whether there are changes in cognitive function following a more protracted period of abstinence.

Drug abstinence results in a negative emotional state, increased anxiety, and social withdrawal – all of which increase an individual's risk of relapse (Goodwin et al., 2002; Mcgregor et al., 2008; Wise and Koob, 2013).

Understanding the behavioral profile during drug abstinence is essential for effective treatment of substance use disorders. The few studies concerning toluene's effects following protracted abstinence are somewhat inconsistent and results vary based on the cognitive measure tested. For example, while deficits in object recognition, operant conditioning, delay discounting, progressive ratio responding, and contingency monitoring have been observed, protracted abstinence from chronic toluene exposure does not affect Pavlovian-to-instrumental transfer, outcome devaluation, anxiety or spatial memory (Lin et al., 2010; Dick et al., 2014; Furlong et al., 2016). Further, while inhalant-induced deficits in behavioral flexibility have been detected in humans after a short abstinence period (5-9 days), their effects in a protracted abstinence rodent model are subtle (Dick et al., 2014; Yuncu et al., 2015; Furlong et al., 2016).

One of the more commonly studied forms of behavioral flexibility involves training a subject to respond to a certain set of rules for a reward, and measuring the ability to adjust behavior when a new rule is introduced unexpectedly. Efficient completion of these tasks is critically-dependent on the integrity of the prefrontal cortex (Hamilton and Brigman, 2015). Moreover, disrupting communication between the medial prefrontal cortex (mPFC) and nucleus accumbens core (NAc) impairs shifting between strategies by increasing perseverative responding (Block et al., 2007). This circuitry is part of a larger network that controls the transition to habitual drug use, where prelimbic mPFC-NAc connectivity is essential for the initiation of drug-seeking behaviors (Everitt and Robbins, 2005; Stefanik et al., 2013). Both behavioral flexibility and drug addiction require structural

modifications in the mPFC and NAc to permit the formation and maintenance of new synapses. The postsynaptic dendritic spine is a key component of this neuroplasticity, with long-thin immature spines giving way to mushroom-headed spines over the course of excitatory synaptic growth (Holtmaat et al., 2006). While nearly every drug of abuse examined to date alters dendritic spine morphology in the mPFC and NAc (Mulholland, Chandler, & Kalivas, 2016; Spiga et al., 2014), it is not known whether similar changes occur following toluene exposure.

There is a particularly high incidence of inhalant abuse in adolescents due to the low cost and high availability of toluene-containing products (e.g. paint thinners, nail polish, permanent markers) (Johnston et al., 2015). In the present study, adolescent rats were chronically exposed to abuse levels of toluene vapor and then allowed to recover in their home cage for a protracted abstinence (CTA). When rats reached adulthood, we assessed two types of behavioral flexibility – strategy set-shifting and reversal learning – and examined the density and sub-types of dendritic spines in mPFC and NAc. The results from these studies show that toluene exposure during adolescence produces selective impairments in cognitive function during adulthood that are accompanied by alterations in dendritic spine morphology that are region- and spine-subtype specific.

MATERIALS AND METHODS

Animals

Sixty-seven male Sprague-Dawley Rats (post-natal day (P) 32 on arrival; Harlan Laboratories, Indianapolis, IN) were housed in pairs in polypropylene

cages on a reverse light cycle (lights off at 0900 h) in a climate controlled room with *ad libitum* access to food and water unless otherwise noted. Each rat was acclimated to handling for 5 min per day for at least 2 days prior to toluene exposure. All procedures were performed in compliance with the Medical University of South Carolina IACUC protocols.

Toluene Inhalation

On the day before the first toluene exposure, adolescent rats (P38) were habituated to the exposure chamber (30x30x30cm) for 15 min. On each of the following 5 days (P39-43), a binge-like regimen was used to mimic adolescent human toluene abuse. Sessions consisted of two, 15 min exposures to 10,500 ppm toluene generated using a sevoflurane vaporizer (Penlon Limited; flow rate 4L/min, 8% volume). Each exposure was separated by 2 h of recovery in the home cage. We have previously used gas chromatography to validate this protocol for generating abuse-level toluene concentrations (Beckley et al., 2013). Importantly, these exposures fall within human consumption patterns: 15 min to several hours at 5000 to 15000 ppm (Brouette and Anton, 2001; Bukowski, 2001). Interestingly, Gmaz et al. (2012) exposed Long-Evans rats to 5000 ppm toluene for 30 min and determined that the resulting brain toluene concentrations (500-1000 μmol/l) would be similar to those experienced by humans inhaling toluene-containing products. Similar exposure protocols have been used to study the effect of chronic exposure to abuse levels of toluene vapor (Moser and Balster, 1981; Bowen et al., 2009; Dick et al., 2014; Furlong et al., 2016). Control rats were exposed to chambers filled with air on the same schedule as above. Housing pairs were

placed in the same drug treatment group to avoid potential exposure of air-treated controls to toluene. Animal weights were recorded every day following toluene exposure, and once every 3-5 days thereafter.

Operant Conditioning

Lever Press Training

Rats (eighteen toluene-, seventeen air-treated) were first habituated to 20% sweetened condensed milk (SCM), the reward used throughout these studies. During reward exposure, each rat pair was given free access to 10 ml SCM for two days before exposure to the operant chambers. Subjects were monitored to ensure both rats sampled the SCM. Lever press training was subsequently conducted in operant chambers (Med Associates, St. Albans, VT) that began during adulthood (P60) and proceeded as described previously (Brady and Floresco, 2015). Briefly, rats were first trained to lever press on a fixed-ratio (FR) 1 schedule for 45 µl SCM dispensed from a central feeding well over the course of three phases (1-3). Phase 1 (30 min session) began with both levers extending, each of which were reinforced on an FR1 schedule. Rodents moved on to phase 2 if they made 50 responses for two consecutive days. Phase 2 was identical to phase 1, except that levers retracted 20 s when pressed and then were presented again. Rodents progressed to phase 3 if they made 50 responses for two consecutive days. Phase 3 lasted 30 to 45 min and consisted of 100 trials. During each trial, one of the two levers were extended for 10 s in a pseudorandom order. If the rat responded on the lever, it was retracted for 20 s and a reward was delivered to the feeding well. If the extended lever was not pressed during a trial,

it was retracted, and the house light was illuminated for a 30 s time out period which was recorded as an “omission”. Once a subject reached criteria (10 or fewer omissions per session for two consecutive days) a side preference test was performed as previously described (Brady & Floresco, 2015; Floresco, Block, & Tse, 2008). For each of 60 trials, both levers extended simultaneously and were reinforced on an FR1 schedule. A trial concluded when two presses occurred, which resulted in lever retraction for 20 s. The preferred side was defined as the side that a rat pressed first most often across trials.

Visual Cue Discrimination

Rats were trained to respond to only the lever under an illuminated light (visual cue) in order to receive reinforcement. Rats received daily session of 100 trials. Each trial started with a visual cue light turning on above one of two lever slots. Three s later the house light turned on and both levers were inserted into the chamber. The visual cue was presented in a pseudorandom order across trials to indicate which lever would elicit a reward when pressed. Responses on this “active” lever delivered reward on a FR1 schedule. Responding on either lever caused both levers to retract for 20 s. If neither lever was pressed within 10 s, both were retracted and the house light was illuminated for a 30 s time out period, recorded as an “omission”. Rats were trained to a criterion of two consecutive sessions with less than 10 omissions. They were then subjected to the strategy shift to response discrimination.

Strategy Set-Shift to a Response Discrimination

During this phase, rats were required to shift their strategy and use an egocentric spatial response strategy, wherein responding on one lever (i.e. left vs right lever) now delivered the reward irrespective of the position of the visual cue (Brady and Floresco, 2015). We chose to use this visual cue-response shift because our primary interest was in ascertaining how toluene exposure may affect PFC functioning, and previous studies have shown that performance on this type of shift is more sensitive to disruption following PFC inactivation (Floresco et al., 2008). Reinforced levers were counter-balanced against the rats preferred location as determined by the side preference task. The manner in which the set-shift was administered was varied across two experiments. Group "A" consisted of eight toluene- and nine air-treated rats that received a "within-session" shift, where the session started with 20 "reminder trials" of the visual cue rule. On the 21st trial, rats were required to use a response rule to obtain reward. A separate group ("B") consisted of eight toluene- and eight air-treated rats that completed this task "between-sessions", where the strategy shift occurred without any reminder trials (i.e. the last training trial and first training trial were separated by 24 h). Sessions ended once at least 30 trials were completed and a rat achieved criterion performance (8 consecutive correct responses). Primary dependent variables for this task were trials to criterion and errors to criterion. Incorrect responses on illuminated levers were further categorized as either perseverative errors (if >4 in a block of 16 trials) or regressive errors (if ≤4 in a block of 16). During the strategy shift, an incorrect response on an unlit lever was classified as

a never reinforced error. Lever press latency, and accuracy during reminder trials were also recoded.

Reversal Learning

Once rats achieved criterion performance (8 consecutive correct lever presses under 30 trials for two days in a row) on the strategy shift task, the response discrimination was reversed so that responses on the previously incorrect lever were now reinforced, as described previously (Brady and Floresco, 2015). Again, the visual cue lights were illuminated above one of the levers on each trial, but here, they served as distractors. Rats from group A were tested for reversal learning using a within-session reversal shift while rats from group B were tested using a between-session task design. Primary dependent variables for this task were trials to completion and errors to criterion. Sessions ended once 10 consecutive correct responses were made and at least 30 trials had occurred. Errors were further categorized as either congruent or noncongruent with visual cue. Lever press latency, and accuracy during reminder trials were also recorded.

Progressive Ratio Test

After achieving criterion performance on the reversal phase of the task, animals from both group A and group B performed a progressive ratio task using the following schedule of reinforcement: responses per reward (rounded) $= 5e^{\text{reward number}*0.2} - 5$ (Richardson and Roberts, 1996). Each reward delivery

preceded a 4 s timeout period. All testing occurred over a single 16 h period that terminated if no rewards were delivered within a 1 h period.

Classical Conditioning

A separate cohort of 32 rats were divided into 4 groups (n=8/group) to test the interaction between drug history (CTA vs air) and cue pre-exposure (pre exposed, “PE” vs non pre exposed, “NPE”) on classical conditioning using a procedure based on Nonkes et al. (2012). CTA and air-treated rats were acclimatized to SCM one day before training began (see 2.3.1). On P60, rats were placed in operant chambers (Med Associates, St. Albans VT) for two sessions of food well location training. During these 60 min sessions, 45 µl of SCM were delivered on a variable interval (VI, 3 min average inter-trial interval) for 15 trials to ensure frequent visits to the well during future testing. For the next 6 days, half of the rats underwent cue pre exposure sessions. Rats were food deprived for 2 h before each session. These 60 min sessions consisted of a 60 s compound cue (tone + stimulus lights) delivered on a VI (3 min average inter-trial interval) for 15 trials with no reward delivery. On these training days, NPE rats were placed in operant boxes without any cues for the same amount of time as their PE peers. This training resulted in four groups (air-PE, air-NPE, CTA-PE, CTA-NPE).

Rats next underwent eight days of classical conditioning sessions. Rats were food deprived for 2 h before each session. During these 40 min test sessions, a 60 s compound cue was delivered on a VI (3 min average inter-trial interval) for 10 trials. This cue was paired with the delivery of 45 µl SCM 30 s into

cue presentation. The primary dependent variable used to measure cue-reward association strength was the elevation ratio, X/(X+Y). “X” equals the number of food well entries during the 30 s period following cue onset, when reward was available. “Y” equals the number of entries during the 30 s preceding cue onset, when reward was not available. Latency to approach the food well following cue onset and SCM delivery were also recorded. Cue-reward pairing was extinguished over the next three sessions using the same program in the absence of the SCM reward.

Dendritic Spine Analysis

At PD 93-97 (7 days following the progressive ratio task, and approximately 7 weeks after the last toluene exposure) rats from the within-session behavioral flexibility test (group A) were processed for dendritic spine labeling and classification as previously reported (Uys et al., 2015). First, rats were anesthetized with urethane (3g/kg, i.p.) and perfused with 300ml saline-free 0.1M phosphate buffer (PB) followed by 300 ml 1.5% paraformaldehyde (PFA). Brains were blocked and post-fixed for 1 h in 1.5% PFA and then sliced into 150 µm coronal sections using a vibratome. Tungsten particles (1.3 µm diameter) were coated with DiI and then applied to coronal slices using a Helio Gene gun (Bio-Rad, Hercules, CA) fitted with a 3.0 µm polycarbonate filter (BD Biosciences, San Jose, CA). The lipophilic dye was allowed to spread through the samples overnight in PB at 4°C. The next day, slices were washed once in PB and then mounted onto slides with ProLong Gold (Life Technologies, Carlsbad, CA) and

coverslipped. Slices were imaged using a Zeiss LSM 510 confocal microscope and 63x oil immersion objective (Plan-Apochromat; NA = 1.4). Voxel size (49 x 49 x 100 nm) was set according to the Nyquist theorem. On average, three to five dendritic segments 50 µm in size from basal dendrites were collected and imaged. Each segment was second-order, 25 µm from any branch point, and began 50-100 µm from the soma. Images were deconvolved using AutoQuant (Media Cybernetics, Rockville, MD) and subsequently modeled using Imaris (Bitplane, Zurich, Switzerland) software. Based on previously reported specifications (Trantham-Davidson et al., 2016; Lin, Lo, Lyu, & Lai, 2017), spines were classified into subtypes using the following parameters: spine length < 0.75 µm, stubby; spine length between 0.75 µm and 3.0 µm, long-thin; spine length < 3.5 µm, head width minimum > 0.3 µm, and head width maximum > minimum neck width *1.5, mushroom; spine length ≥ 3.0 µm, filopodia. Two-headed spines were counted manually.

Statistics

Lever press and visual discrimination data were analyzed with two-tailed unpaired t-tests using Prism 7 (Graphpad Software San Diego, CA). One rat (CTA, group B) failed to progress through phase 1 of training, assigned a value of 10 days for this training phase (max number of days to criteria observed), and then removed from the remainder of the study. Three rats (two air, group A; one CTA, group B) failed to meet criteria in phase 3 after 8 days, but subsequently passed visual cue training criteria and were included in the remainder of

behavioral studies. Two outliers (one CTA, group A; one air, group A) as determined by Grubbs test were removed from the statistical analysis of figure 2.1A. Behavioral flexibility data were analyzed with 2-way ANOVA with task as the within subject factor and drug experience as the between subject factor (Prism 7). Error data from these experiments were analyzed with two-tailed unpaired t-tests. Classical conditioning and extinction data were analyzed using a three-way ANOVA using SPSS (SPSS, Armonk, NY) with cue and drug experience as between subject factors and test session (time) as the within subject, repeated factor. Since the purpose of these experiments was to explore the effects of CTA over time under a single set of cue experiences, we further analyzed any drug experience x time interactions revealed by the three-way ANOVA within each cue exposure condition using a two-way repeated measures ANOVA. Dendritic spine analyses were conducted using a mixed model (SAS Proc Mixed, SAS Institute Inc., Cary, NC) with a first order autoregressive covariance matrix across the sequential slices within rats.

RESULTS

Toluene Vapor Inhalation Attenuates Weight Gain

CTA rats weighed significantly less than air-treated controls by the fifth day of treatment. This difference persisted until the first day of lever press training (P60), but weights were not significantly different during behavioral flexibility testing (supplementary figure 2.1).

CTA Causes Persistent Operant Conditioning Deficits

In order to identify potential drug-induced deficits in operant conditioning, we noted the initial operant responding and number of days required to meet criteria during training. During initial lever press training, CTA rats pressed for reward significantly fewer times than air treated controls [$t(31)=2.095$, $p<0.05$] (Figure 2.1A). CTA rats took significantly longer to reach lever pressing criteria during phase 1 compared to air treated controls [$t(33)=3.05$, $p<0.01$] (Figure 2.1B). This deficit was not present during subsequent training: lever press training phase 2 [$t(32)=0$, $p>0.999$], lever press training phase 3 [$t(32)=0.72$, $p=0.923$], visual cue training [$t(32)=0.36$, $p=0.994$] (figure 2.1C-E). Both test groups (i.e. "A" or "B", see methods) progressed through training at comparable rates (supplementary table 1). Finally, the number of training days did not correlate with future strategy shifting

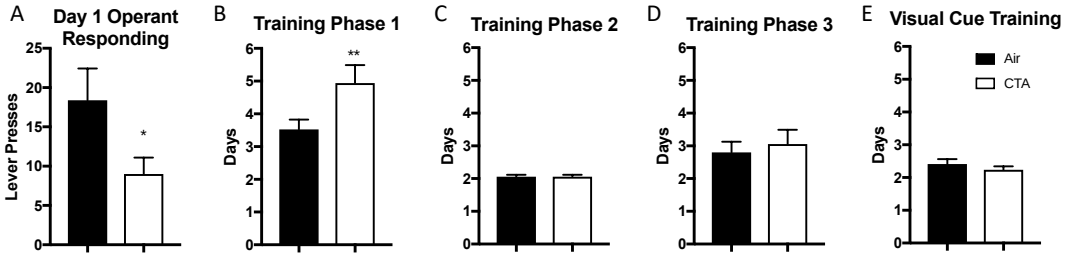


Figure 2.1. A history of toluene exposure during adolescence impairs acquisition of operant behavior in adulthood. A) CTA exposed rats had fewer lever presses for a reward (20% sweetened condensed milk) on day one of operant training. B) CTA rats reached lever press training phase 1 criteria in significantly more days compared to control. C-E) CTA and air exposed rats progressed through the remainder of operant training in equivalent number of days. Data shown are mean ± SEM; *$p<0.05$, **$p<0.01$; air n=17, CTA n=17.

(Pearson r=-0.033, p=0.86) or reversal learning (Pearson r=0.096, p=0.61) performance. Taken together, these data suggest that there is a toluene-induced deficit in operant conditioning, but not visual discrimination.

Within-Session Tests of Behavioral Flexibility:

Strategy Set-Shifting

Eighteen rats (nine air, nine toluene; group A) were trained to lever press for a SCM reward in response to a visual cue. Once rats met the criteria of 8 correct lever presses in a row in under 30 trials, they were tested for their ability to shift to a new discrimination strategy (i.e. switch to a location based rule) using a within-session test design (i.e. testing occurred immediately after 20 training reminder trials at the start of the test session). Analysis of these data revealed no detectable differences in performance during the first twenty reminder trials on test day as measured by overall accuracy and response latency (all *t's*< 1.09, *p's*>0.29, supplementary figure 2.2A,B). A 2-way ANOVA revealed a main effect of task ($F_{1,16}$=116.6, p<0.0001), but no task x drug interaction ($F_{1,16}$=0.0373, p=0.8494) or main effect of drug ($F_{1,16}$=0.3939, p=0.5397; figure 2.2A). Sidak's post hoc revealed no differences in the number of trials to criteria between CTA rats and air treated controls during the reminder trials or the strategy set shift task itself (all *t's* <0.59, *p's* >0.81; figure 2.2A). There were no group differences in errors to criteria, response latency or errors committed in CTA rats compared to air treated controls (all *p's* >0.05; figure 2.2B, supplementary figure 2.2C, 2.3).

Reversal Learning

Following completion of the set shift to the location-based rule, rats were tested on a reversal of this discrimination (i.e. learn that the previously inactive lever was now the only active lever) using a within-session test design (i.e. the reversal shift occurred immediately after 20 training reminder trials within the same session). There were no detectable differences between air and CTA rats in performance during the first twenty reminder trials on test day as measured by overall accuracy and response latency (all *t's*<1.16, *p's*>0.26; supplementary figure 2.2D,E). A 2-way ANOVA revealed a main effect of task ($F_{1,16}$=56.6, *p*<0.0001), but no task x drug interaction ($F_{1,16}$=0.0829, *p*=0.7772) or main effect of drug ($F_{1,16}$=0.0004, *p*=0.9835; figure 2.2C). Sidak's post hoc revealed no differences in the number of trials to criteria between CTA rats and air treated controls during the reminder trials or the reversal learning task itself (all *t's* <0.21, *p's* >0.97; figure 2.2C). There were no group differences in errors to criteria or error subtypes committed in CTA rats compared to air treated controls (*p* >0.05; figure 2.2D, supplementary figure 2.2F, 2.3).

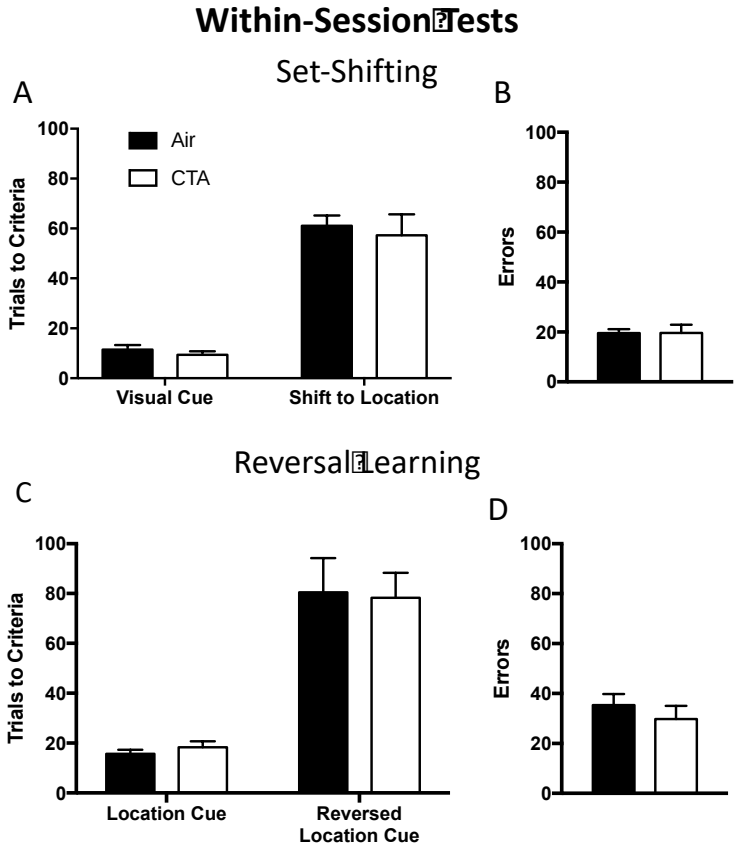


Figure 2.2. A history of adolescent toluene exposure did not affect within-session set-shifting or reversal learning in adulthood. (A & B) CTA and air exposed rats reached set-shift criteria in the same number of trials and committed the same number of errors during a within-session test design. (C & D) CTA and air exposed rats reached reversal learning criteria in the same number of trials and committed the same number of errors during a within-session test design. Data shown are mean ± SEM; air n=9, CTA n=9.

Between-Session Tests of Behavioral Flexibility:

Test of behavioral flexibility involve suppression of responding of an initial rule, exploring alternative rules, and the establishment/maintenance of an new rule (Block et al., 2007). Learning and extinction deficits have been observed both in acute and chronic toluene use in both humans (Yuncu et al., 2015) and rodents (Dick et al., 2014). Since a history of toluene exposure could be affecting the

strength of the existing rule memory (Floresco & Jentsch, 2011) we expanded the amount of time between training and testing ("between-session" tests) and in a separate cohort of animals measured their behavioral flexibility. In this paradigm, testing occurred 24h after the last visual set training session.

Strategy Set-Shifting

A separate group of sixteen rats (eight air, eight toluene; group B) was trained to lever press in response to a visual cue. Subjects were then tested on their ability to shift strategy between-session (i.e. with no reminder trials before the set shift and during the same session). A 2-way ANOVA revealed a task x drug interaction ($F_{1,14}$=7.842, p<0.05) as well as main effects of task ($F_{1,14}$=197.6, p<0.0001) and drug ($F_{1,14}$=5.955, p<0.05). This effect was driven by CTA rats reaching criterion performance more rapidly than control rats when the rule switched to being dependent on lever-location (Sidak's *post-hoc* p<0.01; figure 2.3A). There was no difference in performance during the last day of visual cue discrimination training (Sidak's *post-hoc* p=0.9372; figure 2.3A). The enhanced performance was also reflected in the trend towards decreased errors committed by CTA rats compared to controls [t(14)=2.00, p=0.066; figure 2.3B]. There were no significant differences in the types of errors committed or number of trials omitted during testing (all p's >0.08; supplementary figure 2.4).

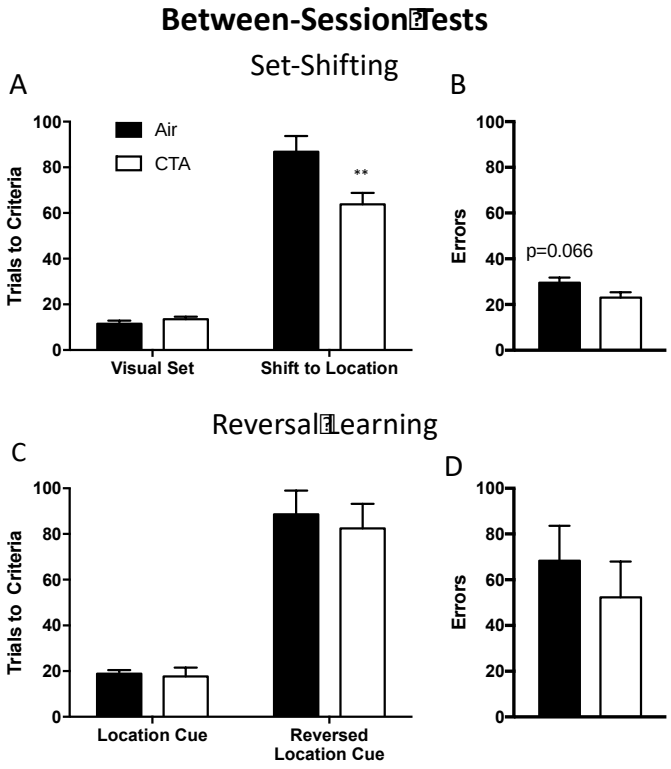


Figure 2.3. Toluene exposure during adolescence improved between-session set-shifting, but not reversal learning in adulthood. CTA rats reached set-shift criteria in fewer trials as compared to air-exposed controls (A) and showed a trend towards fewer errors (B). (C & D) CTA rats reached reversal learning criteria in the same number of trials and committed the same number of errors as air-exposed controls. Data shown are mean ± SEM; ***p*<0.01. Data shown are mean ± SEM; air n=8, CTA n=8.

Reversal Learning

Once trained on the location-based rule, rats were tasked to reverse their responding. A 2-way ANOVA revealed a main effect of task ($F_{1,14}$=84.55, *p*<0.001), but no task x drug interaction ($F_{1,14}$=0.1186, p=0.7361) or main effect of drug $F_{1,14}$=0.2026, *p*=0.6601; figure 2.3C). Sidak's post hoc revealed no differences in the number of trials to criteria between CTA rats and air treated controls during last training task or during the reversal learning task itself (all *t's* <0.57, *p's* >0.82; figure

3c). There were no significant differences in total errors during testing [$t(14)=0.73$, $p=0.4807$; figure 2.3D]

Progressive Ratio

In order to determine if the operant conditioning deficits observed were due to a decreased motivation for obtaining SCM reward, we measured responding using a progressive ratio reinforcement regimen in these same rats. All rats from the behavioral flexibility experiments were included in this study. There were no differences between CTA and air-treated controls in response breakpoint, active lever presses, or inactive lever presses (all t's<0.85, p's>0.05; figure 2.4). These results suggest that the deficits in operant conditioning are not easily attributable to deficits in motivation to obtain a food reward.

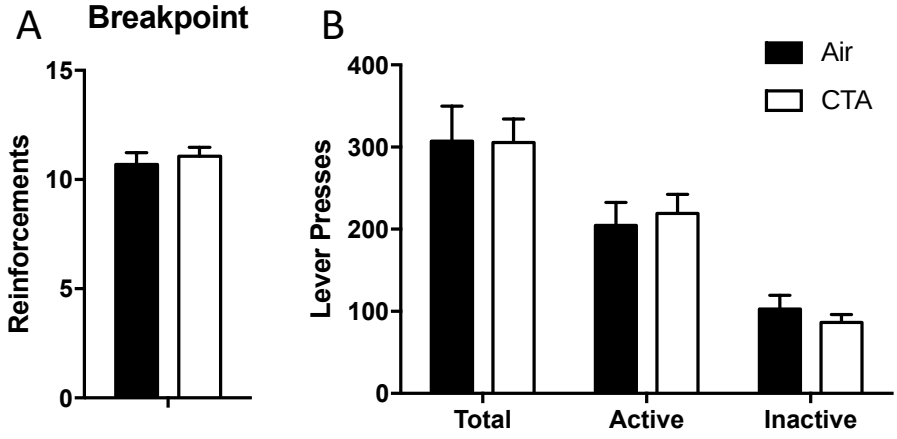


Figure 2.4. A history of toluene exposure during adolescence does not alter motivation for a food reward. A) CTA rats had a comparable breakpoint compared to controls using a progressive ratio schedule of reinforcement. B) The number of presses of active and inactive levers was not different between CTA and air-exposed rats. Data shown are mean ± SEM; air n=17, CA n=17.

Toluene Vapor Inhalation Causes Persistent Classical Conditioning Deficits

Acquisition and Latent Inhibition

The associative learning phenomena of latent inhibition – where previous exposure to a non-reinforced cue blunts future conditioning to that cue – has also been proposed to play a role in behavioral flexibility (Chess et al., 2012; Nonkes et al., 2012). Blunted latent inhibition may increase exploration of previously irrelevant rule sets during a strategy shift that could lead to an apparent facilitation of shifting. Given that CTA resulted in somewhat more rapid shifting to a novel rule when rats were tested using a between-session protocol, we tested whether latent inhibition was altered in adult rats with CTA. In so doing, we characterized classically conditioned approach behavior, and extinction of this behavior, in both cue naïve and cue pre-exposed rats.

Results from the between-session strategy shifting test suggest that toluene exposure may cause long term deficits in latent inhibition of a previously irrelevant cue to future cue-reward associations. By including both a cue pre-exposed (PE) and a non-cue pre-exposed (NPE) group of air and CTA rats, this classical conditioning task design allowed us to test the effect of CTA on both classical conditioning and latent inhibition. We tested 8 rats in each of the drug x cue exposure combinations (air-PE, air-NPE, CTA-PE, CTA-NPE).

Conditioning was primarily measured using an elevation ratio, (X/(X+Y)), where X equals the number of food well approaches during the first 30 s of cue (when reward was available) and Y equals the number of approaches during the 30 s prior to cue onset (when reward was unavailable). As predicted by latent

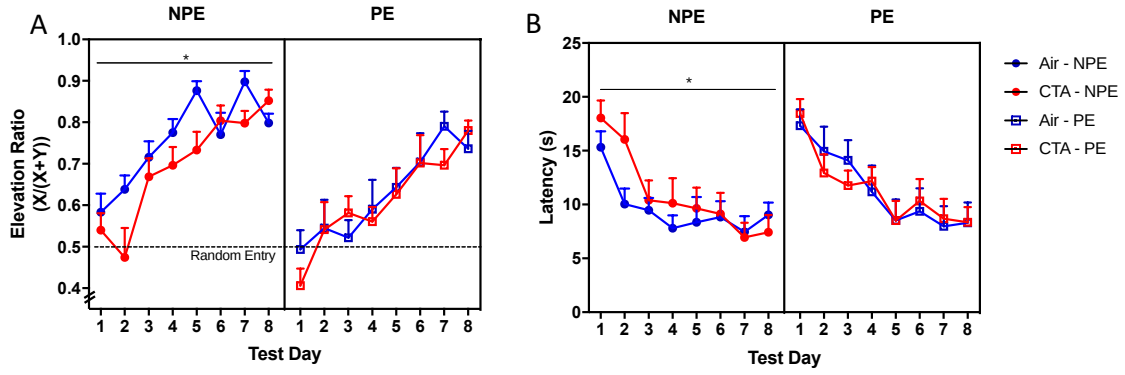


Figure 2.5. CTA mitigates the acquisition rate of classical conditioning without impairing latent inhibition. CTA and air treated animals were either naïve to the CS (NPE) or pre-exposed (PE) to the CS during the training phase. Conditioning was measured as the elevation ratio defined as X/(X+Y) where X is the number of food well entries during the first 30 s of cue and Y is the number of entries 30 s prior to cue onset. A) CTA blunted the conditioning acquisition curve over the course of eight test days in NPE but not PE rats. B) CTA also delayed food well approach in response to CS, indicative of blunted CS-US association. Data shown are mean + SEM; drug x test day interaction *p<0.05; all groups n=8.

inhibition cue pre-exposure blunted elevation ratios over the course of 8 test sessions (main effect of cue, $F_{1,7}$=15.70, p<0.001). While there was no main effect of drug ($F_{1,7}$=2.599, p=0.118), there was a drug x time interaction ($F_{1,7}$=2.04, p<0.05). Subsequent partitioning of this interaction revealed a significant difference between treatment groups in the NPE condition (main effect of drug; $F_{1,7}$=2.331, p<0.05) but not in PE condition (main effect of drug; $F_{1,7}$=0.98, p=0.450] (Figure 2.5A). This interaction was reflected by the decreased latency in initiating food well approach behavior in response to cue over time in NPE (drug x time $F_{1,7}$=2.626, p<0.05), but not PE (drug x time $F_{1,7}$=0.689, p=0.681; Figure 2.5B). CTA decreased elevation ratio within the first day of training as well (main effect of drug; $F_{1,2}$ = 4.597, p<0.05), but there was no evidence of a drug x time

interaction ($F_{1,7}$=0.124, *p*=0.727; supplementary figure 5). These data indicate that CTA retards classical conditioning of a cue to a reward, but does not affect how a pre-exposure to a cue impedes subsequent associative learning about that cue.

Extinction of Classically Conditioned Approach Behavior

There were no main effects of drug ($F_{1,7}$=0.599, *p*=0.455), cue ($F_{1,7}$=0.011, *p*=0.918) or drug x time interactions ($F_{1,7}$=0.507, *p*=0.605) on elevation ratios over the course of three extinction trials (Figure 2.6A). Interestingly, there was a drug x time interaction within the first extinction session ($F_{1,2}$=4.711, *p*<0.05; figure 2.6B) that reflected a significant difference between groups in the PE condition ($F_{1,2}$=6.117, p<0.05), but not the NPE animals ($F_{1,7}$=0.5637, *p*=0.47; figure 2.6A). Neither a history of cue exposure nor CTA alone affected elevation ratios during this first extinction session (main effect of cue $F_{1,2}$=0.591; main effect of drug, $F_{1,2}$=0.448, *p*=0.593). Further, the drug x time interaction was not observed during acquisition of approach behavior ($F_{1,7}$=0.124, *p*=0.727 supplementary figure 2.5). These data suggest that CTA enhances extinction of classically conditioned approach behavior only when the subject has previous experience of a non-reinforced cue within the first extinction session.

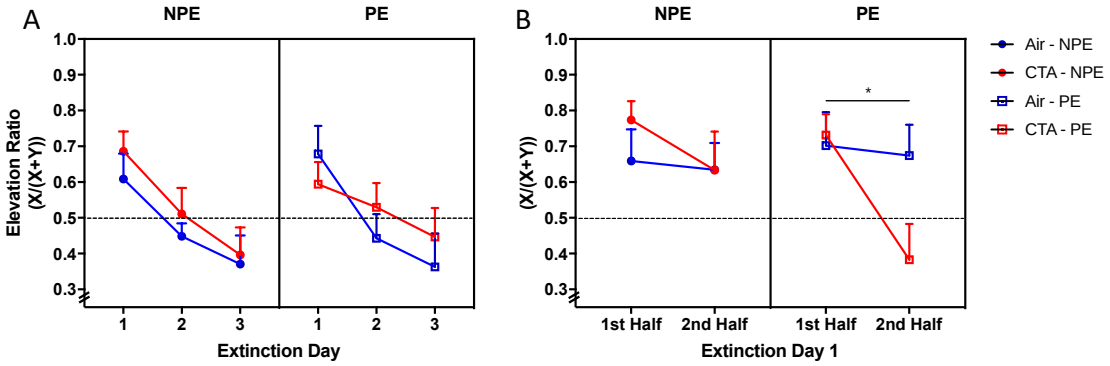


Figure 2.6. CTA enhances extinction of classically conditioned approach behavior only within the first extinction session. CTA and air treated animals were either cue-naïve (NPE) or pre-exposed (PE) to cue during the training phase. Conditioning was measured as the elevation ratio defined as X/(X+Y) where X is the number of food well approaches during the first 30 s of cue and Y is the number 30 s prior to cue onset. A) CTA did not affect extinction progression over the course of three days. B) However, CTA-PE rats extinguished responding quicker than their air-PE counterparts within the first day of extinction testing. Data shown are mean + SEM; random well entry (dotted line); drug x time interaction *p<0.05; all groups n=8.

Changes in Dendritic Spine Morphology Caused by Toluene Vapor Inhalation

To supplement findings from the behavioral studies, we measured dendritic spine density and morphology in neurons from two critical nodes in appetitive behavior, the mPFC and NAc (figure 2.7A-E). There were no detectable differences in dendritic spine density on basal dendrites of layer 5 prelimbic mPFC of CTA vs control rats (figure 2.7E). Conversely, toluene exposure during adolescence caused a lasting increase in dendritic spine density of NAc medium spiny neurons (main effect of drug $F_{1,4}$=6.10, p<0.05; figure 2.7F) *Post-hoc* analysis revealed that this effect was driven by an increased prevalence of long-

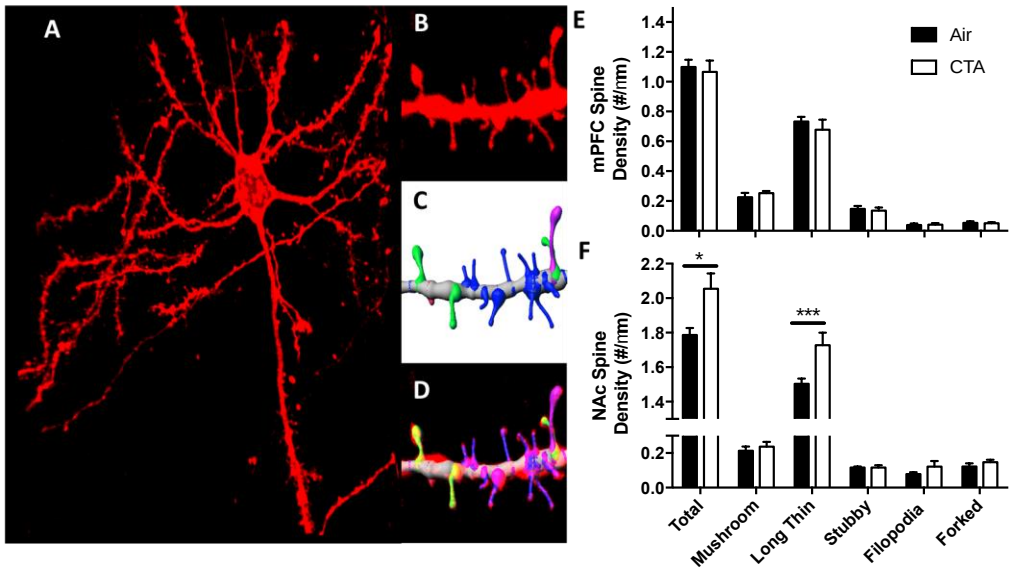


Figure 2.7. Chronic exposure to toluene during adolescence causes persistent increases in dendritic spine density in a spine subtype and region-specific manner. (A) Representative image of a DiI-labeled pyramidal neuron from the prelimbic medial prefrontal cortex. Insets show representative spine segment image (B) after deconvolution, (C) modeling, and (D) overlay. (E) Chronic adolescent toluene exposure does not alter basal dendritic spine density in deep-layer mPFC neurons in adults. (F) Chronic adolescent toluene exposure increases spine density in medium spiny neurons of the NAc, an effect driven by the long-thin spine subtype. Data shown are mean +SEM; main effect $F_{1,4}$=6.10 **p*<0.05; *post-hoc* t(60)=4.68, *** *p*<0.001; air n=8, CTA n=9.

thin spines [t(60)=4.68; *p*<0.001]. There were no detectable differences in the length or head diameter of any spine subtype in either region tested (data not shown). Interestingly, there was a strong positive correlation between strategy-shifting (but not reversal learning) performance and medial PFC (but not NAc) spine density, further implicating the reliance of this behavior on the mPFC (Spearman *r*=0.667, n=17, *p*<0.01).

DISCUSSION

The Effect of Toluene on Motivated Behavior

In this study, we investigated whether repeated exposures to abuse-level concentrations of toluene vapor (10,500 ppm) during adolescence induced persistent cognitive effects (Lubman et al., 2008; Gmaz et al., 2012; Dick et al., 2014). The results indicate that adolescent exposure to toluene retarded operant conditioning in adulthood consistent with observed instrumental learning deficits reported by Dick et al. (2014). The lever press training protocol used in this study had three phases. Phase 1 is a simple operant conditioning setup where levers remain extended throughout each session, and it was during this phase of training where deficits induced by toluene exposure were apparent. The next two phases contain an occasion setter, namely lever extension/retraction that signals reward availability. Here, no differences between groups were observed. The lack of effect during this phase may reflect the fact that the retraction of levers recruits cue-related attentional mechanisms that aid in task performance and could compensate for deficits in simple operant conditioning. The deficits shown in the present study are not likely attributable by a decreased motivation for SCM reward, as CTA rats reached a similar breakpoint under a progressive ratio level of responding compared to control, similar to that previously reported using a sucrose reward (Dick et al., 2014). Rather, these effects likely reflect impairments in the initial formation of action-outcome associations.

Exposure of adolescent rats to toluene vapor resulted in an initial reduction in weight gain. This finding mirrors reports in humans indicating that inhalant

abusers often present as emaciated and is consistent with results from other rodent studies (Ryu et al., 1998; Duncan et al., 2012; Dick et al., 2014). The reduced body weight of CTA rats at the beginning of operant training could contribute to the observed deficits, as underweight rats might reach satiety quicker than heavier counterparts. However, both CTA and Air rats quickly consumed 5 ml of SCM in the days preceding operant training as part of the reward exposure protocol. This volume far exceeds the mean volumes consumed in lever press phase 1 (~1 ml air, ~0.5 ml CTA). Weight differences disappeared soon after operant training began and thus likely did not confound strategy shifting or reversal learning.

Behavioral Flexibility and Memory Retrieval

We next studied the effect of CTA on two types of behavioral flexibility – strategy set-shifting and reversal learning – in adulthood using either a within-session or between-session test design. There were no differences in behavioral flexibility in CTA rats that completed tasks within-session, suggesting that chronic toluene exposure does not produce long term deficits in set-shifting or reversal learning. Interestingly CTA rats performed a strategy shift quicker compared to controls when tested using a between-session design.

On the surface, the apparent improvement in set-shifting displayed by CTA rats may be interpreted as an enhancement in flexibility. While this is a possibility, it is important to note that CTA only accelerated shifting using a between-session shift, suggesting that these effects may not reflect a uniform enhancement in the

mechanisms underlying shifting. Tasks which require behavioral flexibility typically present situations necessitating suppression of a previously relevant strategy alongside simultaneous acquisition and maintenance of a new strategy. With regards to the former, suppression of old strategies may be facilitated if there are underlying impairments in the consolidation, maintenance or retrieval of these strategies. In fact, pharmacologically destabilizing initial memories leads to enhanced reversal learning (Weiner et al., 1986) and extradimensional set-shifting (Crofts et al., 2001). Furthermore, increasing the time between training and testing can also degrade memories and lead to poorer retrieval (Floresco & Phillips, 2001). With respect to the present study, retrieving the previously held response strategy is theoretically more important to completing between-session compared to within-session shifts because there are no reminder trials to jumpstart responding to the initial attentional set (visual cue). Interestingly, chronic exposure to 2000PPM toluene produces hippocampal cell loss that does not recover following 90 days of abstinence (Zhvania et al.,2012), and CTA alters hippocampus-dependent behaviors such as operant conditioning, delay discounting and contingency monitoring (Dick et al., 2014, Furlong et al., 2016). In attempting to explain the apparent improvement in set-shifting observed in one of our experiments, it is possible that CTA disrupted access to memories regarding the previously acquired discrimination rule via perturbations in hippocampal functioning. In turn, during between-session testing, poorer retrieval of this rule within the appropriate task context would lead to more rapid learning of the novel rule. On the other hand, the lack of improvement in shifting in CTA rats given reminder trials of the within-

session shifts may be attributable to a reactivation of memories associated with the old rule that caused a comparable amount of interference of learning of the new rule in both groups. As such, when the within- and between- session data are considered together, it appears that CTA rats have persistent deficits in appetitive memory recall, but not necessarily enhanced behavioral flexibility.

One important caveat of these conclusions is the lack of differences when comparing within- vs between-session reversal learning. Two factors could explain this discrepancy. First, reversal learning was assessed after strategy set-shifting and side preference testing. This ensured that tasks proceeded in order of increasing difficulty, but also resulted in an incorrect lever choice that was 1) naturally preferred and 2) illuminated 50% of trials (a previously reinforced cue). Second, strategy set-shifting and reversal learning are dependent on two distinct prefrontal regions, the mPFC and orbitofrontal cortex, respectively (Floresco, Zhang, & Enomoto, 2009; Ghods-Sharifi, Haluk, & Floresco, 2008; Hamilton & Brigman, 2015). Nevertheless, the finding that CTA only affected set-shifting and not reversal learning suggests that impairments in memory retrieval induced by these manipulations may be more apparent when distinct discrimination strategies are required, rather than situations requiring the use of the same basic strategy (i.e. always press a lever in one location) and a mere shift between stimulus-reward associations.

The Effect of Latent Inhibition on Behavioral Flexibility

Upon changes in reinforcement contingencies that occur during strategy or reversal shifts, animals must acquire and then maintain a new strategy. Previous exposure to a non-reinforced cue has been repeatedly shown to block future conditioning to that cue, a phenomenon called latent inhibition. Although latent inhibition has traditionally been studied using Pavlovian conditioning paradigms, this idea can be conceptualized as "learned irrelevance" of cues in an operant conditioning scenario (although this definition is simplistic, for review: see (Meyer and Louilot, 2014)). Blunted latent inhibition has been observed in serotonin knockout (5HTT-/-) mice (Nonkes et al., 2012) and in spontaneously hypertensive rats, which are used as a in a rodent model of ADHD (Calzavara et al., 2009). Interestingly, both of these animal models also induce more rapid shifts in behavior upon changes in reinforcement contingencies that occurred between sessions (Chess et al.,2012; Nonkes et al., 2012). Furthermore, the enhanced shifting observed in spontaneously hypertensive rats was not observed following a within-session shift, in a manner similar to the present findings (Chess et al., 2012). The present studies reveal that CTA does not cause persistent latent inhibition deficits indicating that memory of previously non-reinforced cues likely do not play a role in the acquisition of new response strategies in the between-session tests of behavioral flexibility.

An additional novel finding from the latent inhibition study is that CTA enhances extinction progression within the first day of extinction compared to air-treated controls only when rats have previous experience with non-reinforced cues. This group was most similar to rats in the between-session behavioral

flexibility tasks in that both had experience with a non-reinforced cue, and were tested 24h after the last training session. The enhanced extinction specific to CTA-NPE rats might be the cause of the decreased perseveration (and thus, enhanced performance) by CTA rats in between-session set-shifting.

Prolonged Abstinence from Addictive Substances Alters Postsynaptic Neuron Morphology in the Nucleus Accumbens

Addictive substances affect frontal-striatal pathways and are thought to impair "top-down" regulation of compulsive drug-seeking behaviors. Chronic toluene exposure has been reported to decrease dendritic complexity in superficial cortical layers following 48 h of abstinence (Pascual and Bustamante, 2010, 2011; Pascual et al., 2011). The present findings add to these data by showing that protracted abstinence from toluene exposure (7 weeks) is associated with increases in long-thin spines in the NAc. The effect of drugs of abuse on spine morphology is variable, presumably due to different mechanisms of action, differences in drug treatment (chronic vs acute), and time of measurement (no abstinence vs hours or weeks; for review see: Mulholland, Chandler, & Kalivas, 2016; Spiga et al., 2014). Similar to toluene, chronic treatment with cocaine (Rasakham et al., 2014), alcohol (Uys et al., 2013; Peterson et al., 2015) and nicotine (Gipson et al., 2013) causes selective and persistent enhancements in the NAc dendritic spine morphology.

The increased presence of long thin spines in the NAc might reflect an increase in silent synapses and thus deactivation of the region (Grueter et al., 2013). This could explain the impaired acquisition of operant and classical

conditioning induced by CTA, both of which are blunted by NA lesions (Meredith et al., 2008). This explanation does not, however, address the specific enhancements in both extinction learning and delay dependent strategy set shifting caused by CTA since extinction is considered a novel memory rather than degradation of an initial memory (although unlearning can occur; for review see: Todd, Vurbic, & Bouton, 2014).

CONCLUSIONS

While strategy set-shifting was enhanced in CTA rats, this effect was only observed if there was long period of time before training and testing. This enhancement was not observed when a simple reversal of stimulus-reward associations was made. To assess the generality of these findings, future research should test whether CTA enhances behavioral flexibility exclusively when other dissimilar discrimination strategies are used (e.g. by using different textured levers or nose poke holes associated with different odors). Deficits in operant conditioning, classical conditioning and enhanced extinction following classical conditioning suggest that the behavioral flexibility “enhancements” in CTA rats may actually reflect specific impairments in appetitive memory recall. This would explain the lack of differences in behavioral flexibility when using a within-session test design that relies less on memory recall. Lasting changes in appetitive behavior may reflect specific anatomical alterations in neuroanatomy responsible for goal directed action. To this end, we observed an increase in immature dendritic spines in the NAc of CTA rats. This could prevent synaptic plasticity and thus behavioral

flexibility in certain situations. Future studies should explore the cellular/molecular changes (e.g. enhanced GABAergic interneuron activity, upregulated D2 receptor expression, etc.) driving this aberrant NAc morphology.

CHAPTER 3 THE ABUSED INHALANT TOLUENE IMPAIRS MEDIAL PREFRONTAL CORTEX ACTIVITY AND RISK/REWARD DECISION MAKING DURING A PROBABILISTIC DISCOUNTING TASK

INTRODUCTION

Preclinical models of toluene intoxication report that acute exposure to toluene impairs simple behaviors such as locomotion and object recognition (Batis et al., 2010; Huerta-Rivas et al., 2012; Montes et al., 2017). Deficits in more complex forms of cognition that are mediated by the medial prefrontal cortex (mPFC) have also been observed following chronic exposure to toluene vapor, despite varying lengths of abstinence (Baydas et al., 2005; Dick et al., 2014; Furlong et al., 2016; Braunscheidel et al., 2017). These effects may be mediated in part by neurophysiological perturbations within the frontal lobes, as previous work in our lab has shown that toluene causes long-term depression of AMPA currents in mPFC neurons (Beckley and Woodward, 2011).

Little is known regarding the effects of inhalants on evaluative processes involving decisions under conditions of uncertainty or risk. One way to assess these functions in rats is with a probabilistic discounting task. In this task, rats are trained to choose between two levers, one a small, certain “safe” reward that always delivers a smaller reward, while the other delivers a large, uncertain “risky” reward under varying schedules of reinforcement probability. The neural circuitry that mediates this form of decision making has been studied in some detail and includes the basolateral amygdala, nucleus accumbens shell, lateral habenula,

medial orbitofrontal cortex and notably, the mPFC (Ghods-Sharifi et al., 2009; St. Onge and Floresco, 2010; Stopper and Floresco, 2011, 2014; Stopper et al., 2014). Pharmacological inactivation of the mPFC impairs flexible decision making on this task, in that rats are slower to update choice biases as reward probabilities change (St. Onge and Floresco, 2010). This profile is distinct from those induced by inactivation of cortical or subcortical brain regions mentioned above. Given these considerations, the present study used a probabilistic discounting task to test the hypothesis that acute toluene exposure induces a hypoactive mPFC, leading to behavioral inflexibility during probabilistic discounting. To identify toluene-induced changes in mPFC activity during the task, we virally expressed the genetically encoded calcium sensor GCaMP6f in glutamatergic mPFC neurons and monitored calcium transients in real-time using in vivo fiber photometry while rats performed the task. Our results provide physiological evidence for the mPFC's theorized role in updating expected values of actions and implicates mPFC dysfunction in the decision-making deficits caused by toluene.

MATERIALS AND METHODS

Animals

Sprague-Dawley Rats (45 male, 34 female, post-natal day (P) 32 on arrival; Envigo RMS, Indianapolis, IN) were housed in pairs in polypropylene cages on a reverse light cycle (lights off at 0900) in a climate-controlled room with food and water delivered *ad libitum*. At approximately P60, rodents were food restricted to maintain 85-95% of their free feeding weight (weight at time of testing: males, 275-300g; females, 200-230g). All procedures were performed in compliance with

Medical University of South Carolina IACUC protocols. The behavioral training and testing schedule is outlined in Figure 3.1A.

Lever Press Training

Rats were habituated to a reward of 20% sweetened condensed milk (SCM), by giving them free access to 10 ml SCM for two days prior to operant training. Over the course of two phases, rats (P60-70) were trained to lever press in operant chambers (Med Associates, St. Albans, VT) for SCM delivered to a central feeding well via a pump-activated syringe. Phase 1 (2-5 days; 30 min sessions) began with one lever (left or right, pseudo-randomly assigned) reinforced with 45 µl SCM on an FR1 schedule. Upon meeting criteria (50 presses for 2 consecutive days), the presented lever was switched, and rats were tested to criteria before moving on to phase 2. Phase 2 (6-7 days; 60-minute sessions) consisted of 90 trials separated by 35s. Each session began with an illuminated house light and 2s later, the left or right lever extended in a pseudo-random order. When pressed, the lever retracted, and 45 µl SCM was delivered on 75% of trials. If a lever was not pressed within 20s, it retracted, and the trial was recorded as an omission. Following completion of two consecutive days of training with less than 10 omissions per session, the time to omission was reduced to 10s. When rats met criteria again, the lever reward probability reduced to 50%. When rats met criteria a third time, a side preference test was performed as previously described (Brady and Floresco, 2015; Braunscheidel et al., 2017). Briefly, for each of 60 trials, both levers extended simultaneously and were reinforced on an FR1

schedule. A trial concluded when two presses occurred, which resulted in lever retraction for 20s. The preferred side was defined as the side that a rat pressed first most often across trials. Rodents then began training in the probabilistic discounting task.

Probabilistic Discounting

Figure 3.1B illustrates the probabilistic discounting procedure used to assess risk/reward decision making in rodents (St. Onge and Floresco, 2009; Brady and Floresco, 2015). This two-lever choice task consists of a “safe” lever that delivered a small reward (30 µl SCM) 100% of the time and a “risky” lever that delivered a large reward (90 µl SCM) with varying probability of reinforcement. The risky lever was assigned to the non-preferred lever position as determined by the side preference test. Each session consisted of 90 trials separated into 5 blocks and each block started with 8 forced-choice trials that set

the probability of reinforcement for the following 10 free-choice trials. The probability of obtaining a large reward was varied from low-to-high ("ascending") or high-to-low ("descending") with the following probabilities: 100%, 50%, 25%, 12.5%, 6.25% (Figure 3.1C). Each trial lasted 35s and began with an illuminated house light and 2 s later levers extended into the chamber. A press on either lever

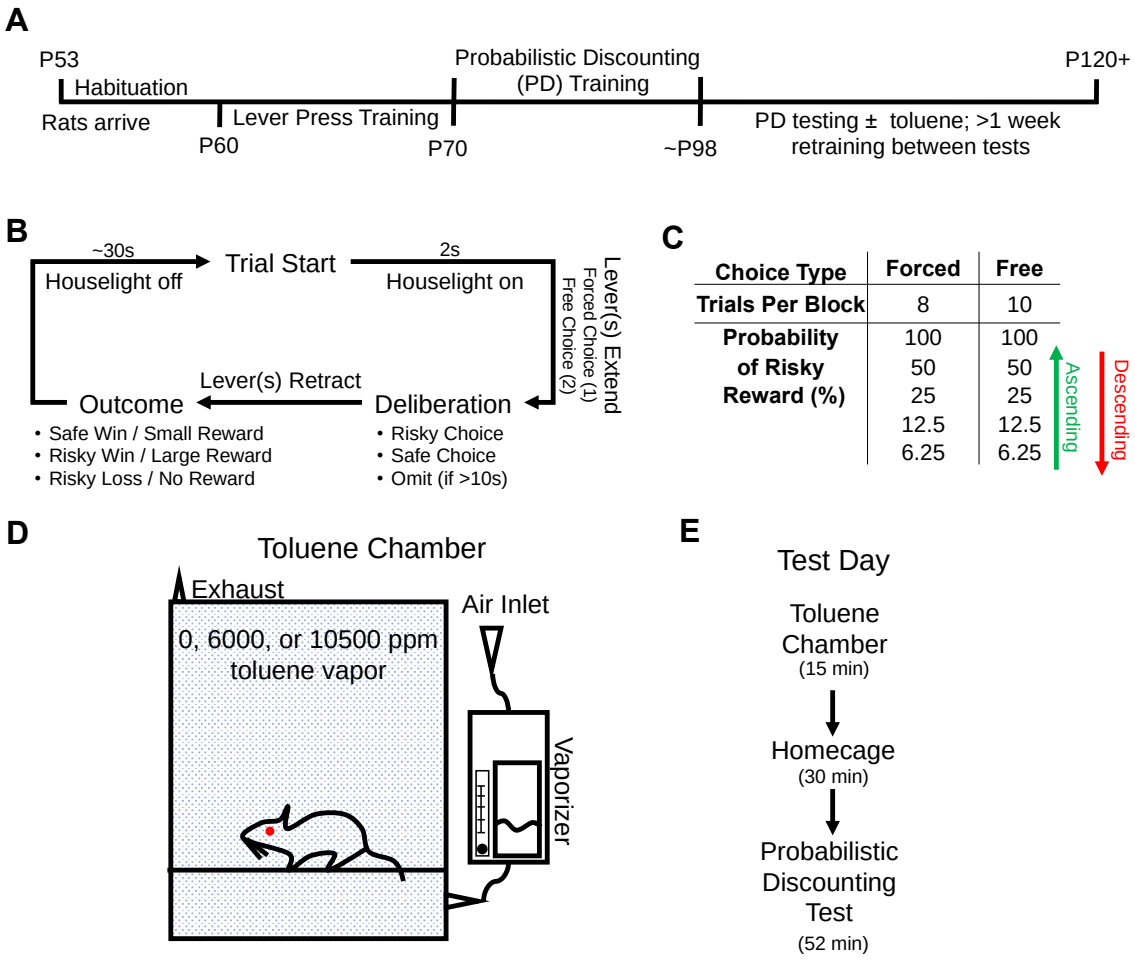


Figure 3.1. Probabilistic discounting training and test design. (A) Behavioral timeline and corresponding rat age. (B) Flow chart detailing a single trial of the probabilistic discounting task. (C) Breakdown of the 10 different behavioral blocks within the probabilistic discounting task. Odds were presented either in ascending or descending order. (D) Toluene inhalation chamber schematic. (E) Test day progression.

caused both of them retract and turned the house light off. On rewarded trials, reward was delivered to the central feeding well. These “wins” were paired with a discriminative cue: a flashing light above the food well to indicate whether the reward was small or large (safe win: 2 pulses, 0.35 s pulse width, 0.5 Hz; risky win: 5 pulses, 0.35 s pulse width, 0.5 Hz). On non-reinforced “loss” trials, no cue light was provided. If a lever was not pressed in 10s, it was recorded as an omission and the houselights were extinguished for 25s. Following ~20 days of training (5-6 days per week), rats exhibited stable responding (two-way ANOVA on three consecutive testing days yields no block x day interaction or main effect of day, $p > 0.1$) and were tested for probabilistic discounting following exposure to air or toluene vapor.

Toluene Exposure

For acute intoxication studies, rodents underwent two days of probabilistic discounting with testing occurring 30 min after a brief (15 min) exposure to an air-filled chamber (30x30x30cm, 4 L/min, Figure 3.1D-E). On toluene test days, the vapor chambers were filled with toluene vapor at concentrations relevant to human solvent abuse (Brouette and Anton, 2001; Bukowski, 2001; Gmaz et al., 2012) via a sevoflurane vaporizer (Penlon Limited; flow rate 4L/min) as previously described (Wayman & Woodward 2017). Toluene vapor concentrations within the chamber were intermittently monitored with a portable toluene gas detector (DOD Technologies, Cary, IL). To establish a dose-effect, each rat was first tested following exposure to 10,500 ppm toluene, given two weeks off, retrained to

baseline performance, and tested again following exposure to 6,000 ppm toluene. At these doses, rats exhibit lethargy after ~10 minutes of exposure and were nearly immobile after 15 minutes. Rats fully regained ambulation following approximately 15 minutes of recovery in the home cage (unpublished observations).

A subset of rats were exposed to a binge-like regimen of, twice daily, 15 minute exposures to10,500 ppm toluene (12 male, 12 female) or air (12 male, 12 female) during adolescence (P39-P43) as described previously (Braunscheidel, et al, 2017). Rats were then tested with the descending odds probabilistic discounting task during adulthood. Rodents tested on the ascending odds version were not treated with air exposures during adolescence.

Fiber Photometry

A subset of 11 rodents underwent stereotaxic surgery prior to lever press training. Deep anesthesia was achieved via an isoflurane vaporizer (Penlon Ltd, 1L/min, 5% induction, 2-3% maintenance) and 300 nl of AAV1-CaMKII-GCaMP6f (Addgene, Watertown MA) was injected into the prelimbic portion of the mPFC (AP: +2.95; ML: -0.6; DV: -2.85 mm). A custom-made fiber optic ferrule (400 µm diameter patch cord in a 2.5 mm ferrule, ThorLabs, Newton NJ) was implanted at these coordinates and secured in place using dental cement (Land Dental Mfg., Wheeling, IL). Post-mortem inspection revealed that two rats lacked viral expression under the fiber optic ferrule, and one ferrule terminated in the anterior cingulate cortex, leaving 8 rats with appropriate viral expression and ferrule

placement. Rats recovered in their home cage for 7 days before beginning lever press training. Following stable probabilistic discounting performance (~5-6 weeks), rats were tested in an operant chamber modified to permit fiber access. Rats performed in this chamber for two consecutive days before experimental testing and recording.

In order to test for an effect of our toluene exposure paradigm on baseline mPFC activity, rats were then given one month of home cage recovery and tested a final time in an inactive operant chamber. Probabilistic discounting test days were mimicked in that baseline testing occurred 30 minutes after a 15-minute exposure to air or 10,500 ppm toluene (counter-balanced) with one week off in between testing. Calcium events were identified in MATLAB using the *findpeaks* function with the following filters: MinPeakProminence = 4, MinPeakDistance = 0.25, and MinPeakWidth = 0.5. One rat's headcap dislodged during this test and data from this animal were not included in the analysis baseline mPFC activity.

Data were acquired using custom-built imaging equipment based on that described by the Deisseroth lab (Lerner et al., 2015), with modifications. Illumination was provided by 405 nm and 490 nm fiber collimated LEDs (Thorlabs; 30 µW per channel) connected to a four-port fluorescence mini-cube (Doric Lenses). The combined LED output passed through a 400 µm optical fiber (0.48 NA) pigtailed to a rotary optical swivel (FRJ_1x1_PT, Doric Lenses) and connected to the implanted fiber using a ceramic sleeve or pinch connector (Thorlabs). Emission light was focused onto a photoreceiver (Newport model 2151; DC low setting) and sampled at 6.1 kHz by a RZ5P lock-in digital processor

(TDT) controlled by Synapse software (TDT). Excitation light was sinusoidally modulated at 531 Hz (405 nm) and 211 Hz (490 nm) via software control of an LED light driver (Thorlabs). Real-time demodulated emission signals from the two channels were acquired at a frequency of 0.93084 kHz and stored offline for analysis. Lever press activity and resulting output were collected using TTL inputs to the digital processor. Data were processed using custom written functions in MATLAB (MathWorks) software. The signals for each channel were first fitted to a polynomial versus time curve and then subtracted from one another to calculate the $\Delta F/F$ time series. Video of the test sessions were recorded using a C930e webcam (Logitech) affixed to the top of the operant chamber.

Experimental Design and Statistical Analysis

The primary dependent variable of the probabilistic discounting test was risky lever preference, expressed as proportion risky choice (number risky lever presses / total lever presses) during each of the 5 behavioral blocks separated by likelihood of a rewarded risky lever press. Additional performance variables including omissions, latency to choice, win-stay (number of risky lever presses following a risky win / total number of risky wins), and lose-shift (number of safe lever presses following a risky loss / total number of risky losses) were also recorded. Only free-choice trials were considered in behavioral analyses. Probabilistic discounting data were analyzed with 2-way ANOVA with reward probability and toluene exposure as repeated, within-subject factors using Prism 8 (Graphpad Software San Diego, CA). A Greenhouse-Geisser correction was

applied to account for repeated measures and Dunnett's test corrected for multiple comparisons. Win-stay, lose-shift, and omissions were analyzed with Sidak's multiple comparison test.

Fiber photometry measures in air vs toluene conditions were compared with paired t-tests. Comparisons within treatment (i.e. air or toluene exposure) were Z-normalized with MATLAB (MathWorks) software in order to compare signal before and after risky and safe choices. GCaMP6f activity preceding risky vs safe lever choice during discounting was analyzed with 2-way ANOVA with reward probability and choice as within-subject factors. Sidak's multiple comparison test was used to directly compare activity during each reward probability block. An alpha value of 0.05 was used in all analyses.

RESULTS

Sex Differences in Baseline Probabilistic Discounting

Males and females reached similar levels of baseline probabilistic discounting:

Figure 3.2 summarizes baseline probabilistic discounting behavior in male and female Sprague-Dawley following ~4 weeks of training. Males and females had similar risky lever preference (Fig. 3.2A) in the descending odds (two-way ANOVA, sex x block interaction $F_{(4, 88)} = 1.73$, $p = 0.15$; main effect of sex $F_{(1, 22)} = 2.18$, $p = 0.15$) and ascending odds tests (sex x block interaction $F_{(4, 72)} = 0.22$, $p = 0.93$; main effect of sex $F_{(1, 18)} = 0.26$, $p = 0.62$). Choice data was then sorted on a trial-by-trial basis in order to study the effect of recent outcomes on subsequent choice. Figure 3.2B illustrates the effect of recent positive reinforcement on choice

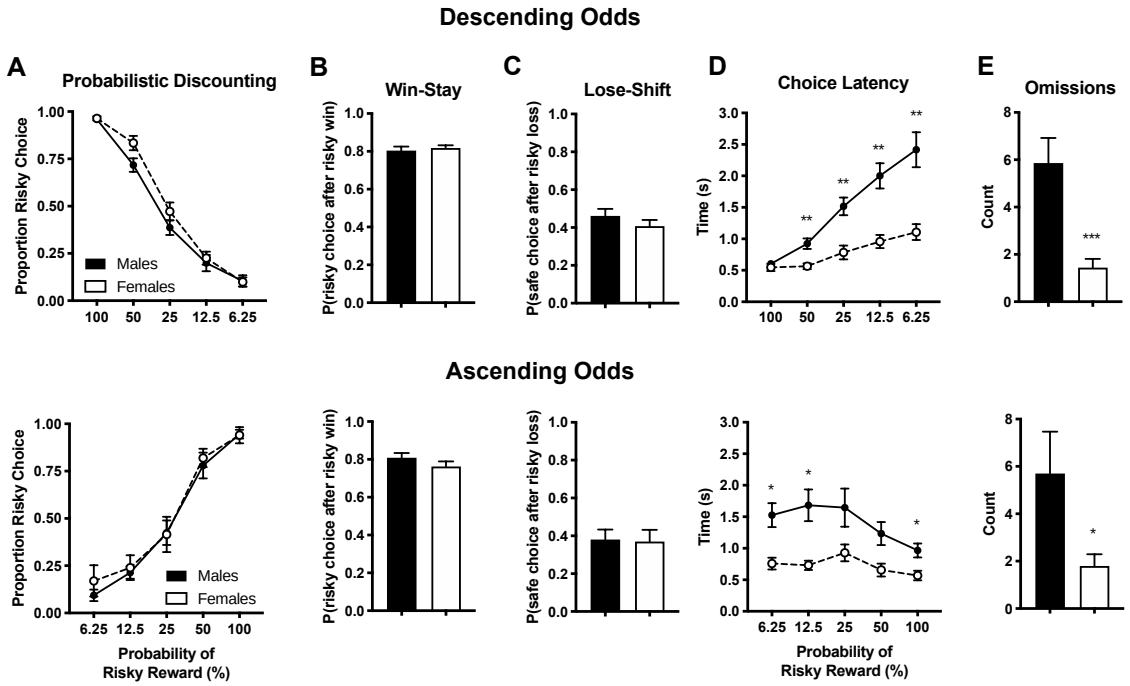


Figure 3.2. Male and female Sprague Dawley rats acquire similar levels of probabilistic discounting. Rats were trained at least twenty days on the probabilistic discounting task with descending odds **(top panels)** or ascending odds **(bottom panels)** until responding stabilized. Performance over the final three days of training were averaged and compared to test for sex differences in baseline task performance. (**A)** Proportion of risky choice within each probability block **(B, C)** Choice strategy across all trials. Win-stay **(B)** indicates choice of risky lever after risky win while lose-shift **(C)** indicates choice of safe lever after risky loss. **(D)** Time to choice selection within each probability block. Omissions across all trials **(E)** indicate no lever press within 10 s time period. Data shown are mean ± sem; descending odds, all n = 12; ascending odds, all n = 10; two-way ANOVA and Sidak's test, *p<0.05, **p<0.01. Student's t-test, *p<0.05, ***p<0.01.

strategy, measured as the probability of choosing the risky lever following a risky win ("win-stay"). Males and females showed similar levels of win-stay behavior in the descending (student's t test, $t_{(22)} = 0.54$, $p = 0.60$) and ascending odds task ($t_{(18)} = 1.26$, $p = 0.22$). Fig. 3.2C details the effect of recent negative feedback sensitivity on choice strategy, measured as the probability choosing the safe lever

following a risky loss ("lose-shift"). Males and females showed similar levels of lose-shift behavior in the descending (student's t test, $t_{(22)} = 1.09$. $p = 0.29$) and ascending odds task ($t_{(18)} = 0.13$, $p = 0.89$).

Choice Latency and Omissions:

Choice latency and omission data may reflect decision speed, impulsivity and/or general motivation to lever press for reward. Females were significantly quicker than males in making a lever selection during probabilistic discounting in the descending odds task (two-way ANOVA, sex x block interaction $F_{(4, 88)} = 11.0$, $p < 0.0001$; main effect of sex $F_{(1, 22)} = 25.6$, $p < 0.0001$). The main effect of sex was also evident in the ascending odds task ($F_{(1, 18)} = 13.2$, $p = 0.0019$) despite the lack of a sex x block interaction ($F_{(4, 72)} = 1.630$, $p = 0.1761$). Females also omitted significantly fewer trials than males during the descending (student's t test, $t_{(22)} = 3.94$. $p = 0.0007$) and ascending odds task ($t_{(18)} = 2.12$, $p = 0.048$). Taken together, these data suggest that females may be more engaged with the probabilistic discounting task than their male counterparts.

Effect of Acute Toluene Inhalation on Probabilistic Discounting

Toluene impairs shifts in choice biases

Figure 3.3 shows the effect of toluene vapor on probabilistic discounting under conditions of descending (top two panels) or ascending odds (bottom two panels). Under control conditions, both males and females trained on the descending version of the task displayed a strong choice preference for the risky

option during the early part of the session, when reward probabilities were relatively high, but preference gradually shifted towards the small certain option as reward probabilities decreased over a session. The opposite profile was observed in rats trained on the ascending version of the task (Fig. 3.3A). When odds of receiving a large reward decreased, acute exposure to toluene vapor 30 min prior to testing increased the proportion of risky choices made by male (two-way ANOVA, drug x block interaction, main effect of drug, $F_{(1.511,\ 16.62)} = 15.7$, $p = 0.0003$) and female rats (two-way ANOVA, main effect of drug, $F_{(1.746,\ 19.20)} = 16.8$, $p < 0.0001$). Dunnett's test revealed a significant effect of toluene dose on this effect, as exposure to 10,500 ppm toluene caused a more pervasive disruption in risk preference than 6,000 ppm toluene in male rats (10,500 ppm vs. baseline, 50%, 25%, 12.5% and 6.25% block: all $q_{(11)} > 2.77$, $p < 0.034$; 6,000 ppm vs. baseline, 50% block, $q_{(11)} = 2.72$, $p = 0.036$). This dose-dependent effect was also evident in females (10,500 ppm vs. baseline, 50%, 25%, 12.5% and 6.25% block: all $q_{(11)} > 3.13$, $p < 0.018$; 6,000 ppm vs. baseline, 25% and 12.5% block, both $q_{(11)} > 3.49$, $p < 0.010$).

In contrast to these results, rats trained on the ascending version of the task and exposed to toluene vapor showed a reduction in the proportion of risky choices when odds of receiving a large reward increased during the task (Fig. 3.3A, bottom panels). This effect was observed in males (two-way ANOVA, main effect of drug, $F_{(1.981,\ 17.83)} = 29.4$, $p < 0.0001$) and females (main effect of drug $F_{(1.1861,\ 16.75)} = 11.1$, $p = 0.0010$). Dunnett's test revealed a significant effect of dose of this effect in male (10,500 ppm vs. baseline, 6.25%, 12.5%, 25%, and 50% block: $q_{(9)} > 3.09$, p

< 0.024) ; 6,000 ppm vs. baseline, all blocks, $q_{(9)} < 1.32$, $p > 0.38$) and in female rats (10,500 ppm vs. baseline, 12.5%, 50% blocks, $q_{(9)} > 2.75$, $p < 0.041$; 6,000 ppm vs. baseline, all blocks, all $q_{(9)} < 1.40$, $p > 0.31$). These findings reveal that the effect of toluene on risk preference is dependent on the order in which the odds of risky reinforcement are presented. Nonetheless, under both conditions, rats demonstrated impaired behavioral flexibility, as they were slower to modify their choice biases in response to changes in probabilities of obtaining the large/risky reward.

Choice Strategy

Choice data were sorted on a trial-by-trial basis in order to study the effect of recent outcomes on subsequent choice (Fig. 3.3B-C). Fig. 3.3B illustrates the effect of toluene on recent positive reinforcement, measured as win-stay behavior. Toluene (10,500 ppm) produced a strong trend for an increase in win-stay behavior vs. baseline in males when odds were presented in descending order (top panels, Dunnett's test, $q_{(11)} = 2.50$, $p = 0.053$). This trend was also observed in females (Dunnett's test, $q_{(11)} = 2.16$, $p = 0.095$). Interestingly, during the ascending phase of the task (bottom panels), 6,000 ppm toluene increased win-stay behavior in female (Dunnett's test, $q_{(9)} = 4.05$, $p = 0.0053$), but not male rats (Dunnett's test, $q_{(9)} = 1.73$, $p = 0.20$).

Fig. 3.3C details the effect of toluene on recent negative feedback sensitivity, measured as lose-shift behavior. Exposure to both doses of toluene vapor decreased lose-shift behavior in male (Dunnett's test, 10,500 ppm vs.

baseline, $q_{(11)}$ = 3.89, p = 0.0047; 6,000 ppm vs. baseline $q_{(11)}$ = 3.12, p = 0.018) and female rats (10,500 ppm vs. baseline $q_{(11)}$ = 3.17, p = 0.016) when odds descended during testing (upper panels). However, when the odds of receiving a large reward increased over the session (bottom panels), exposure to 10,500 ppm toluene enhanced lose-shift behavior in both male (Dunnett's test, $q_{(9)}$ = 4.35 , p = 0.0034) and female rats ($q_{(9)}$ =3.18, p = 0.020). Taken together, these data suggest that impaired flexible decision making induced by toluene exposure is driven by perturbations in negative feedback sensitivity, and that this is exacerbated (or mitigated, depending on task) by increased sensitivity to recently rewarded actions.

Choice Latency and Omissions

In the descending odds version of the task, choice latency (Fig. 3.3D, top panels) decreased following treatment with 10,500 ppm toluene in blocks where reinforcement was unlikely (Dunnett's test, 10,500 ppm vs. baseline, 6.25% block, $q_{(11)}$ = 2.54, p = 0.049) and females (Dunnett's test, 50% block, $q_{(11)}$ = 3.11, p = 0.018, 6.25% block $q_{(11)}$ = 3.69, p = 0.0066). Interestingly, exposure to 6,000 ppm toluene vapor increased choice latency in male rats during blocks when risky reinforcement was guaranteed (Dunnett's test, 100% block, $q_{(11)}$ = 2.62, p = 0.043) (Fig. 3.3D, bottom panels). Toluene had no effect on choice latency in male or female rats in any block of the ascending odds version of the task (male, all $q_{(9)}$ < 2.13 , p > 0.10 ; female, all $q_{(9)}$ < 1.78 , p > 0.18, Fig. 3.2E). Toluene exposure caused a minor, but statistically significant, decrease in the number of omissions

in two instance during the descending odds task (Dunnett's test, males, 10,500 ppm vs. baseline, $q_{(11)} = 2.58$, $p = 0.046$; females, 6,000 ppm vs. baseline, $q_{(11)} = 3.91$, $p = 0.0054$). There were no differences in omissions detected for the ascending odds task (all $q_{(9)} < 1.39$, $p > 0.32$). These data suggest that toluene can decrease choice latency and promote task engagement (decreased omissions), but other factors need to be taken into consideration (sex, duration of task, task-type) when interpreting the effect of toluene on impulsivity and general motivation.

Effect of adolescent toluene exposure on probabilistic discounting in adulthood

Probabilistic discounting training in adults is not significantly affected by adolescent exposure to toluene

Previous studies suggest that toluene abuse during adolescence can impair cognitive performance in adulthood in humans (Dingwall et al., 2011; Scott and Scott, 2014; Yuncu et al., 2015). and rodents (Dick et al., 2014; Furlong et al., 2016; Braunscheidel et al., 2017). Table 3.1 shows that acute exposure to toluene vapor during adolescence did not significantly alter risk preference of adult rats in the descending odds probabilistic discounting task during any of the first four weeks of training (week average). Transient increases in choice latency were observed in toluene-treated rats vs. control (two-way ANOVA, main effect of treatment, males, week 3, $F_{(1,18)} = 8.99$, $p = 0.0077$; females, week 2 $F_{(1,22)} = 7.90$, $p = 0.010$)

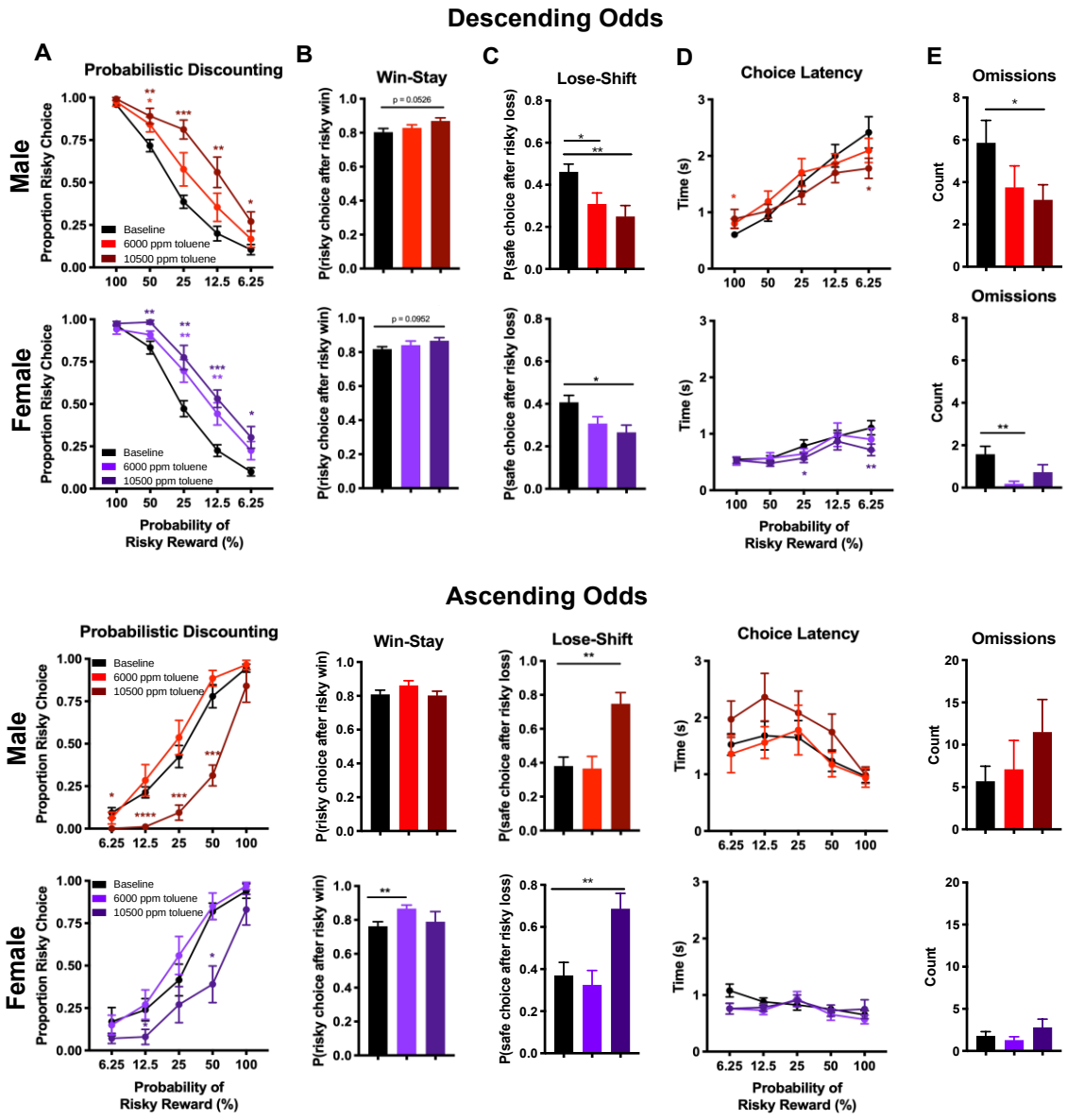


Figure 3.3. Toluene impairs flexible decision making during probabilistic discounting. Male and female rats were trained at least twenty days on the probabilistic discounting task with descending odds **(top panels)** or ascending odds **(bottom panels)** until responding stabilized. (**A)** Proportion of risky choice within each probability block following acute exposure to air or toluene (6,000; 10,500 ppm). **(B, C)** Choice strategy across all trials. Win-stay **(B)** indicates choice of risky lever after risky win while lose-shift **(C)** indicates choice of safe lever after risky loss. **(D)** Time to choice selection within each probability block. Omissions across all trials **(E)** indicate no lever press within 10 s time period. Data shown are mean ± sem; descending odds, all n = 12; ascending odds, all n = 10; two-way ANOVA and Dunnett's test comparing each dose to baseline, *p<0.05, **p<0.01, ***p<0.001, colored to match dose.

but these differences were no longer significant by week 4 (main effect of treatment, males, $F_{(1,18)} = 3.43$, $p = 0.080$; females, $F_{(1, 22)} = 1.66$, $p = 0.21$). Likewise, a transient decrease in lose-shift behavior was detected in male rats in week 3 (Welch's test, $t_{(17.37)} = 2.36$, $p = 0.030$), but this recovered by week 4 ($t_{(17.80)} = 1.35$, $p = 0.20$). Lose-shift behavior also decreased in females during week 4 of training ($t_{(19.53)} = 2.19$, $p = 0.041$). The average number of omissions transiently increased in female rats treated with toluene during adolescence compared to air-treated control (week 1, $t_{(13.13)} = 2.58$, $p = 0.023$, week 2, $t_{(14.09)} = 0.69$, $p = 0.50$).

A history of adolescent toluene exposure does not alter the effects of toluene on decision making in adulthood

To test whether prior exposure to toluene may sensitize or mitigate its effect on probabilistic discounting when animals are exposed again as adults, adolescent-exposed rats were tested during adulthood following an acute exposure to 10,500 and 6,000 ppm toluene (Fig. 3.4A). As shown above, toluene (10,500 ppm) shifted risk preference in male (main effect of drug, $F_{(1.946, 21.41)} = 6.45$, $p = 0.0068$; Dunnett's test, 10,500 ppm vs. baseline, 50% and 25% block, both $q_{(11)} > 3.46$, $p < 0.0012$) and female rats ($F_{(1.847, 20.32)} = 7.43$, $p = 0.0045$; 50%, 25%, 12.5%, 6.25% blocks, all $q_{(11)} > 2.64$, $p < 0.042$). Interestingly, the lower dose of toluene (6,000 ppm) increased risky choice preference exclusively when reinforcement was guaranteed in male (Dunnett's test, 6,000 ppm toluene vs. baseline, 100% block, $q_{(11)} = 4.71$, $p = 0.0012$) and female rats ($q_{(11)} = 2.71$, $p = 0.037$). (Fig. 3.4B) While toluene increased win-stay behavior in this cohort, this

Table 3.1. First four weeks of probabilistic discounting training in adult rats treated 10x with 10,500 ppm toluene vapor during adolescence vs. air treated controls. Proportion risky choice and choice latency were compared over five blocks with the probability of risky reward equal to 100%, 50%, 25%, 12.5%, and 6.25%. Win-stay, lose-shift, and omissions were compared across the entire session.

Male

	Week	1		2		3		4	
	Block	Air	Toluene	Air	Toluene	Air	Toluene	Air	Toluene
Proportion Risky Choice	100%	0.66 ± 0.06	0.62 ± 0.05	0.84 ± 0.02	0.82 ± 0.05	0.89 ± 0.03	0.90 ± 0.03	0.92 ± 0.03	0.96 ± 0.01
	50%	0.65 ± 0.05	0.57 ± 0.06	0.71 ± 0.05	0.72 ± 0.06	0.67 ± 0.05	0.81 ± 0.05	0.73 ± 0.06	0.79 ± 0.05
	25%	0.54 ± 0.07	0.51 ± 0.07	0.46 ± 0.05	0.48 ± 0.07	0.42 ± 0.07	0.46 ± 0.05	0.44 ± 0.06	0.51 ± 0.08
	12.5%	0.45 ± 0.07	0.47 ± 0.05	0.37 ± 0.07	0.34 ± 0.06	0.23 ± 0.06	0.30 ± 0.08	0.22 ± 0.04	0.28 ± 0.05
	6.25%	0.36 ± 0.06	0.40 ± 0.04	0.28 ± 0.06	0.25 ± 0.04	0.15 ± 0.04	0.17 ± 0.04	0.19 ± 0.04	0.16 ± 0.03
Choice Latency (s)	100%	1.04 ± 0.01	1.13 ± 0.09	0.88 ± 0.09	0.90 ± 0.07	0.72 ± 0.05	0.75 ± 0.07	0.65 ± 0.06	0.70 ± 0.08
	50%	1.12 ± 0.08	1.43 ± 0.15	1.15 ± 0.14	1.09 ± 0.10	1.01 ± 0.06	1.15 ± 0.10	0.99 ± 0.14	1.00 ± 0.08
	25%	1.42 ± 0.13	1.69 ± 0.27	1.75 ± 0.17	1.75 0.14	1.46 ± 0.17	1.99 0.17	1.32 ± 0.08	1.67 ± 0.16
	12.50%	1.75 ± 0.14	1.97 ± 0.22	1.65 ± 0.15	1.96 ± 0.19	2.01 ± 0.15	2.64 ± 0.17	1.50 ± 0.08	2.07 ± 0.24
	6.25%	1.74 ± 0.14	2.25 ± 0.33	1.79 ± 0.20	2.37 ± 0.33	2.07 ± 0.13	2.91 ± 0.30	2.01 ± 0.18	2.36 ± 0.22
	Win-Stay	0.58 ± 0.06	0.53 ± 0.05	0.61 ± 0.05	0.62 ± 0.06	0.60 ± 0.06	0.64 ± 0.06	0.61 ± 0.05	0.69 ± 0.06
	Lose-Shift	0.41 ± 0.04	0.45 ± 0.05	0.46 ± 0.05	0.46 ± 0.04	0.49 ± 0.05	0.33 ± 0.04#	0.49 ± 0.04	0.41 ± 0.05
	Omissions	4.7 ± 1.29	5.1 ± 1.88	4.6 ± 2.32	4.0 ± 1.06	7.4 ± 1.36	11 ± 1.51	5.4 ± 1.13	6.9 ± 1.15

Female

	Week	1		2		3		4	
	Block	Air	Toluene	Air	Toluene	Air	Toluene	Air	Toluene
Proportion Risky Choice	100%	0.57 ± 0.05	0.58 ± 0.05	0.82 ± 0.04	0.79 ± 0.05	0.91 ± 0.03	0.90 ± 0.03	0.97 ± 0.01	0.96 ± 0.02
	50%	0.50 ± 0.04	0.57 ± 0.05	0.68 ± 0.05	0.63 ± 0.05	0.73 ± 0.05	0.76 ± 0.04	0.79 ± 0.03	0.83 ± 0.03
	25%	0.44 ± 0.03	0.51 ± 0.05	0.53 ± 0.05	0.46 ± 0.05	0.51 ± 0.05	0.51 ± 0.04	0.47 ± 0.05	0.51 ± 0.05
	12.5%	0.37 ± 0.04	0.41 ± 0.06	0.34 ± 0.05	0.35 ± 0.04	0.29 ± 0.03	0.37 ± 0.06	0.25 ± 0.04	0.33 ± 0.06
	6.25%	0.29 ± 0.03	0.36 ± 0.05	0.24 ± 0.04	0.28 ± 0.04	0.16 ± 0.02	0.26 ± 0.04	0.15 ± 0.03	0.24 ± 0.06
Choice Latency (s)	100%	0.89 ± 0.08	1.07 ± 0.09	0.58 ± 0.05	0.78 ± 0.08	0.52 ± 0.05	0.69 ± 0.07	0.51 ± 0.05	0.62 ± 0.08
	50%	0.90 ± 0.07	0.99 ± 0.10	0.60 ± 0.06	0.81 ± 0.07	0.58 ± 0.06	0.73 ± 0.08	0.63 ± 0.08	0.70 ± 0.08
	25%	0.87 ± 0.07	1.01 ± 0.08	0.70 ± 0.06	0.95 ± 0.08	0.76 ± 0.06	0.97 ± 0.10	0.77 ± 0.08	0.88 ± 0.08
	12.50%	0.92 ± 0.06	1.13 ± 0.12	0.76 ± 0.05	1.09 ± 0.09*	0.97 ± 0.10	1.07 ± 0.08	0.96 ± 0.09	1.18 ± 0.07
	6.25%	0.97 ± 0.07	1.20 ± 0.11	0.98 ± 0.09	1.20 ± 0.10	1.19 ± 0.14	1.36 ± 0.11	1.22 ± 0.14	1.31 ± 0.06
	Win-Stay	0.44 ± 0.04	0.45 ± 0.05	0.61 ± 0.05	0.53 ± 0.06	0.60 ± 0.06	0.60 ± 0.05	0.68 ± 0.05	0.72 ± 0.03
	Lose-Shift	0.52 ± 0.03	0.46 ± 0.04	0.48 ± 0.04	0.52 ± 0.04	0.45 ± 0.03	0.43 ± 0.03	0.44 ± 0.03	0.37 ± 0.02#
	Omissions	0.12 ± 0.04	0.45 ± 0.12#	0.62 ± 0.20	1.0 ± 0.52	1.5 ± 0.44	2.3 ± 0.69	1.4 ± 0.35	1.6 ± 0.42

2-way ANOVA with Geissner-Greenhouse correction:
main effect of drug —— p<0.05.
Sidak's multiple comparison:*p<0.05
Student's t-test with Welch's correction: #p<0.05
male/air n = 10, male/toluene n = 10; female/air n = 12, female/toluene n = 12

was in response to the lower dose of toluene (Dunnett's test, 6,000 ppm toluene vs. baseline, male, $q_{(11)} = 2.48$, $p = 0.055$; female, $q_{(11)}$ 3.63 = , $p = 0.0074$). Like toluene-naïve rats, acute 10,500 ppm toluene exposure decreased lose-shift behavior (Fig. 3.4C) in this cohort (Dunnett's test, male, $q_{(11)} = 3.06$, $p = 0.020$; female, $q_{(11)} = 4.06$, $p = 0.0035$). Choice latency (Fig. 3.4D) decreased when reinforcement was not guaranteed in male (Dunnett's test, 10,500 ppm vs. baseline, 12.5 % block, $q_{(11)} = 3.23$, $p = 0.015$; 6,000 ppm, 6.25% block, $q_{(11)} = 3.54$, $p = 0.0085$) and female rats (10,500 ppm vs. baseline, 50% block, $q_{(11)} = 3.27$, $p = 0.014$; 12.5% block, $q_{(11)} = 3.26$, $p = 0.014$, 6.25% block $q_{(11)} = 3.79$, $p = 0.0056$). Interestingly, 10,500 ppm toluene increased choice latency in male rats when risky reinforcement was guaranteed (Dunnett's test, vs. baseline, 100% block, $q_{(11)} = 2.95$, $p = 0.024$). Omissions (Fig. 3.4E) were reduced in male (10,500 ppm, $q_{(11)} = 3.25$, $p = 0.0144$; 6,000 ppm, $q_{(11)} = 3.09$, $p = 0.019$), but not female rats (all $q_{(11)} < 1.82$, $p > 0.16$).

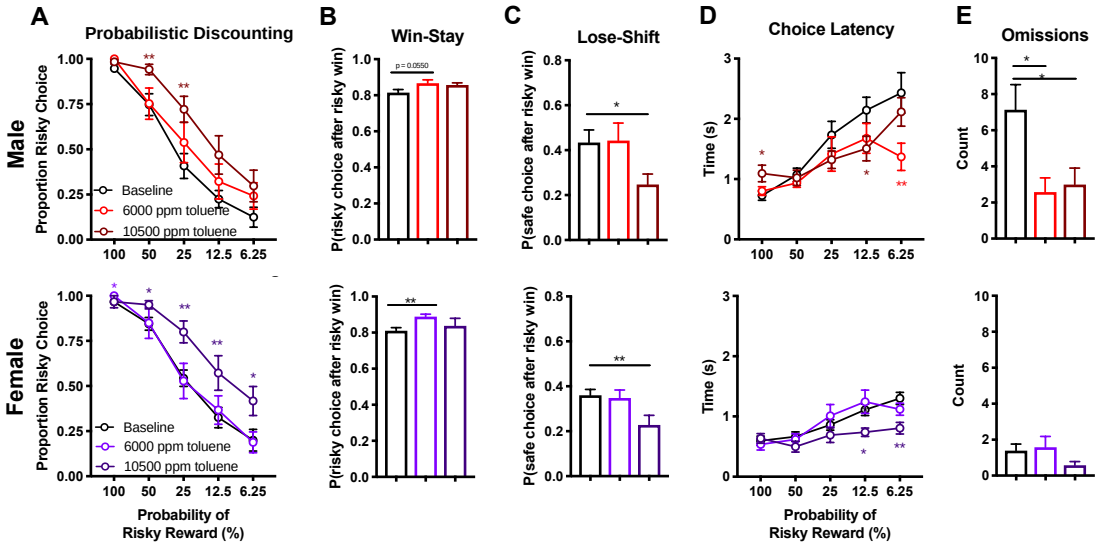


Figure 3.4. Toluene impairs behavioral flexibility in the probabilistic discounting task in adult rats treated with toluene vapor during adolescence. Adolescent rats were treated with a 10,500 ppm toluene for 15 min, twice daily, over five days followed by home cage recovery until adulthood. Adult male and female rats were trained at least twenty days on the probabilistic discounting task with descending odds until responding stabilized. **(A)** Proportion of risky choice within each probability block following acute exposure to air or toluene (6,000; 10,500 ppm). **(B, C)** Choice strategy across all trials. Win-stay **(B)** indicates choice of risky lever after risky win while lose-shift **(C)** indicates choice of safe lever after risky loss. **(D)** Time to choice selection within each probability block. Omissions across all trials **(E)** indicate no lever press within 10 s time period. Data shown are mean ± sem; all n = 12; two-way ANOVA and Dunnett's test comparing each dose to baseline, *p<0.05, **p<0.01, colored to match dose).

Effect Of Toluene on mPFC Activity Measured *in Vivo* Using The Genetically Encoded Calcium Sensor GCaMP6f

Baseline Activity

The activity of prelimbic mPFC neurons was measured by fiber photometry in rats injected with AAV1-CaMKII-GCaMP6f and exposed to air or 10,500 ppm toluene and 30 min of home cage recovery (Figure 3.5). Paired t-tests of average

GCaMP6f responses revealed no difference in event frequency ($t_{(6)}$ = 0.221, p = 0.832), event amplitude($t_{(6)}$ = 0.342, p = 0.744), event width($t_{(6)}$ = 1.41, p = 0.209), or inter-event interval ($t_{(6)}$ = 0.112, p = 0.914).

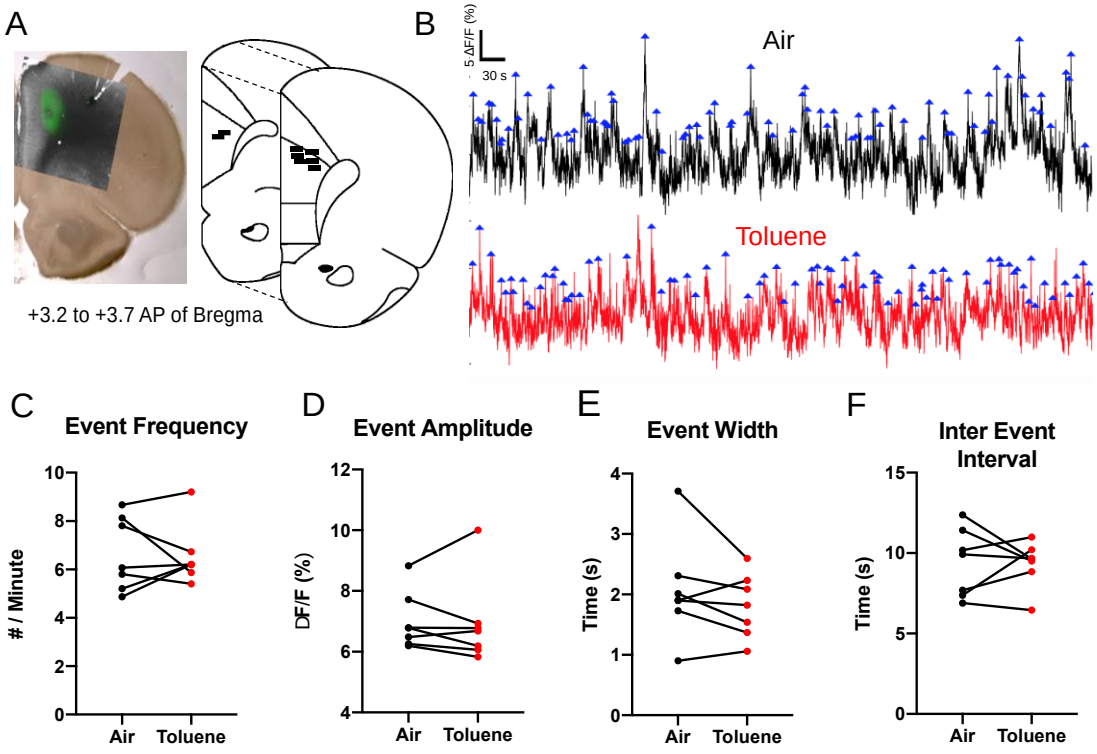


Figure 3.5. Basal prelimbic mPFC calcium activity is unaffected by acute toluene. Activity was measured in prelimbic mPFC neurons with fiber photometry in rats injected with AAV1-CaMKII-GCaMP6f and exposed to air or toluene **(A)** Exemplar viral expression (left) and fiber optic ferrule placement (right, black bars). **(B)** Representative 15 minute photometry recording following air (black) or toluene (red) treatment with detected calcium peak (blue arrows). Paired comparison of **(C)** GCaMP6f event frequency, **(D)** mean amplitude, **(E)** mean width, and **(F)** mean inter event interval in toluene and control conditions in seven rats.

mPFC activity tracks deliberation and outcome during probabilistic discounting

Figure 3.6A illustrates the average GCaMP6f signal across all trials during the probabilistic discounting task in air-treated rats (8 rats, 1081 trials total). Visual inspection of these graphs revealed a peak of activity during deliberation, approximately 1 to 2 s prior to lever press, and decreases in signal during outcome, 1-16 s following lever press. (Figure 3.6B) A one-way ANOVA revealed an effect of choice outcome on GCaMP6f signal ($F_{(1.377, 9.638)} = 37.9$, $p < 0.0001$). This effect was due to differentiation between large/risky wins and small/safe wins (Sidak's post-hoc, risky win vs safe win, $t_{(7)} = 4.27$, $p = 0.011$) as well as losses vs any win (risky loss vs safe win $t_{(7)} = 4.81$, $p = 0.0058$; risky loss vs risky win, $t_{(7)} = 8.001$, $p = 0.0003$). Choice outcome also affected putative consumption as measured by time spent in the food well (Fig. 3.6B, right, one-way ANOVA, $F_{(1.503, 10.52)} = 588$, $p < 0.0001$). This effect was also driven by differentiation between large/risky wins and small/safe wins (Sidak's post-hoc, risky win vs safe win, $t_{(7)} = 13.5$, $p < 0.0001$) as well as losses vs any win (risky loss vs safe win $t_{(7)} = 29.5$, $p < 0.0001$; risky loss vs risky win, $t_{(7)} = 28.5$, $p < 0.0001$).

Z-normalization of data during deliberation (Fig. 3.6C) revealed that activity increased relative to baseline (one-sample t-test vs. baseline: before risky, $t_{(500)} = 6.92$, $p < 0.0001$; before safe, $t_{(522)} = 6.71$, $p < 0.0001$, data not shown.) Traces were then sorted into forced-choice and free-choice trials. In forced-choice trials (Fig. 6C.i), there was a significant interaction between peak activity prior to lever press and behavior block (two-way ANOVA, upcoming choice x behavior block, $F_{(4, 433)} = 5.37$, $p = 0.0030$).

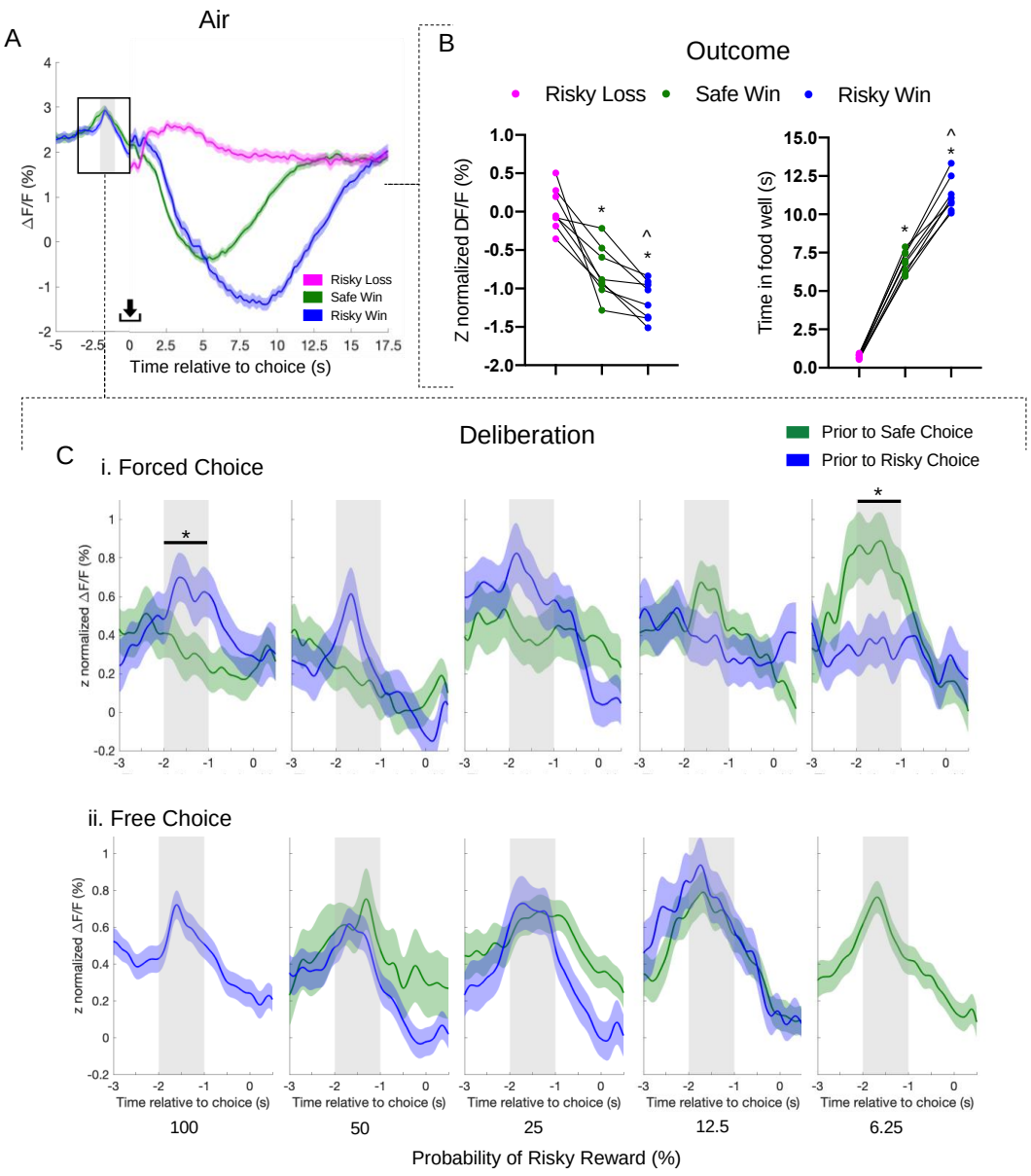


Figure 3.6. PrL mPFC pyramidal activity tracks choice tracks choice selection and outcome during probabilistic discounting. (A) Aggregate calcium response (8 rats; 1081 trials) during entire probabilistic discounting task from air-treated controls. Lines represent average ± sem ΔF/F for safe choice/win (green), risky choice/win (blue), risky loss (pink). An increase in calcium activity was detected during deliberation, ~1.5 s prior to choice (shaded column throughout figure). (B) Average Z-normalized ΔF/F% (left) and consumption time (right) during choice outcome for each rat with paired comparison: safe win vs risky win, ^p<0.05; risky loss vs safe win or risky win, *p<0.05. (C) Traces during deliberation were sorted on probability of rewarded

risky choice (i.e. 100%, 50%, 25%, 12.5%, and 6.25%) and choice type (i.e. free and forced); aggregate Z-normalized mean ± sem ΔF/F with average peaks summarized (right). Average calcium response during forced-choice (C.i, 443 traces, 8 rats) and free-choice (C.ii, 560 traces, 8 rats). Summary traces exclude instances with < 10 traces across < 3 rats. Sidak's multiple comparison of mean value, safe vs risky choice, *p<0.05. Arrow indicates moment of lever press.

This was due to a relatively greater increase prior to risky choice during the 100% block (Sidak's test, $t_{(433)} = 2.73$, $p = 0.033$) and prior to safe choice during the 6.25% block ($t_{(433)} = 3.06$, $p = 0.012$). The interaction between activity prior to lever press and behavior block was not present during free-choice trials (Fig 3.6C.ii, $F_{(4, 569)} = 0.550$, $p = 0.70$).

Toluene disrupts mPFC activity during deliberation and outcome

Figure 3.7A shows the average fluorescence changes across all trials during the probabilistic discounting task on toluene test days (8 rats, 1081 trials each). Like air-treated control days, these graphs show a peak of activity during deliberation and troughs during outcome. (Figure 3.7B) Further, A one-way ANOVA revealed an effect of choice outcome on GCaMP6f signal ($F_{(1.698, 11.88)} = 16.5$, $p = 0.0005$). However, unlike controls, there was no differentiation between large/risky wins and small/safe wins (Sidak's post-hoc, risky win vs safe win, $t_{(7)} = 0.05$, $p > 0.9999$). There was a distinction between GCaMP6f signal following losses vs wins (risky loss vs safe win $t_{(7)} = 4.56$, $p = 0.0078$; risky loss vs risky win, $t_{(7)} = 4.48$, $p = 0.0086$).

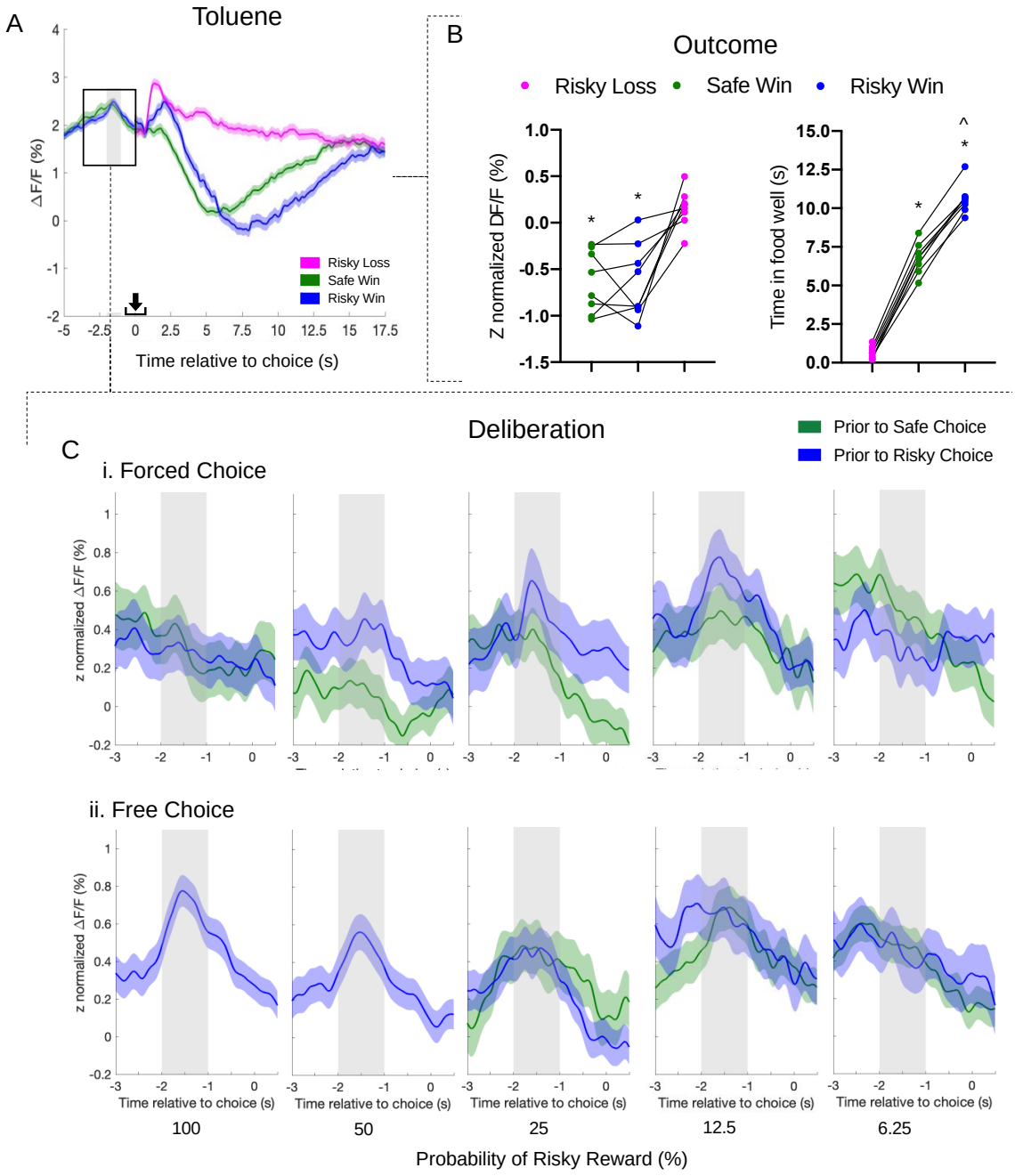


Figure 3.7. Toluene disrupts PrL mPFC pyramidal activity responsible for predicting preferred choice and encoding consumption during probabilistic discounting. **(A)** Aggregate calcium response (8 rats; 1081 trials) during entire probabilistic discounting task on toluene treated test days. Lines represent average ± sem ΔF/F for safe choice/win (green), risky choice/win (blue), risky loss (pink). An increase in calcium activity was detected during deliberation, ~1.5 s prior to choice (shaded column throughout figure). **(B)**

Average Z-normalized ΔF/F% (left) and consumption time (right) during choice outcome for each rat with paired comparison: safe win vs risky win, ^p<0.05; risky loss vs safe win or risky win, *p<0.05. **(C)** Traces during deliberation were sorted on probability of rewarded risky choice (i.e. 100%, 50%, 25%, 12.5%, and 6.25%) and choice type (i.e. free and forced); aggregate z normalized mean ± sem ΔF/F). Average calcium response during forced-choice (**C.i,** 452 traces, 8 rats) and free-choice (**C.ii,** 566 traces, 8 rats). Summary traces exclude instances with < 10 traces across < 3 rats. Paired t-tests *p <0.05, **p<0.01. Arrows indicate moment of lever press.

Choice outcome also affected putative consumption as measured by time spent in the food well (Fig. 3.7B, right, one-way ANOVA, $F_{(1.811, 12.68)} = 763$, $p < 0.0001$). This effect was also driven by differentiation between large/risky wins and small/safe wins as well as losses vs any win (all $t_{(7)} > 17.9$, $p < 0.0001$). (Figure 3.7B, inset) Toluene also caused a slight, but significant reduction in consumption time (two-way ANOVA, main effect of treatment, ($F_{(1,21)} = 4.43$, $p = 0.048$), an effect driven by consumption during large/risky wins (Sidak's post hoc, $t_{(21)} = 2.83$, $p = 0.030$) and not small safe wins ($t_{(21)} = 0.318$, $p = 0.99$) or risky losses ($t_{(21)} = 0.501$, $p = 0.96$).

Z-normalization of data during deliberation (Fig. 3.7C) revealed that mPFC activity increased relative to baseline (one-sample t-test vs 0% ΔF/F : before risky, $t_{(424)} = 6.24$, $p < 0.0001$; before safe, $t_{(611)} = 10.7$ $p < 0.0001$, data not shown.) Traces were then sorted into forced-choice and free-choice trials. Unlike control conditions, there was no interaction between activity prior to lever press and behavior block during forced-choice trials following toluene exposure (Fig. 3.7C.i, two-way ANOVA, $F_{(4, 442)} = 1.48$, $p = 0.21$). Similar to control conditions, this

interaction did not exist during free-choice trials (Fig. 3.6C.ii, $F_{(4, 574)} = 0.264$, $p = 0.90$).

Acute exposure to 10,500 ppm toluene vapor 30 min prior to testing disrupted descending odds probabilistic discounting in rats expressing GCaMP6f in the mPFC (Fig. 3.8A-E). This included increased risky choice preference (Fig. 3.8A, two-way ANOVA, main effect of drug, $F_{(1, 7)} = 11.6$, $p = 0.011$) and reduced lose-shift behavior (Fig. 8C, paired t-test, $t_{(7)} = 2.52$, $p = 0.040$). Finally, we directly compared signal during deliberation (Fig. 8G) and outcome (Fig. 3.8H). mPFC activity increased in toluene exposed animals compared to air prior to a safe lever press (Fig. 3.8G; paired t-test, $t_{(7)} = 2.38$, $p = 0.049$) but not a risky lever press ($t_{(7)} = 1.15$, $p = 0.29$). Toluene exposure resulted in a smaller mPFC activity trough compared to air following safe wins (Fig. 3.8H; $t_{(7)} = 2.80$, $p = 0.027$) and risky wins ($t_{(7)} = 3.96$, $p = 0.0055$), but not risky losses ($t_{(7)} = 0.536$, $p = 0.61$).

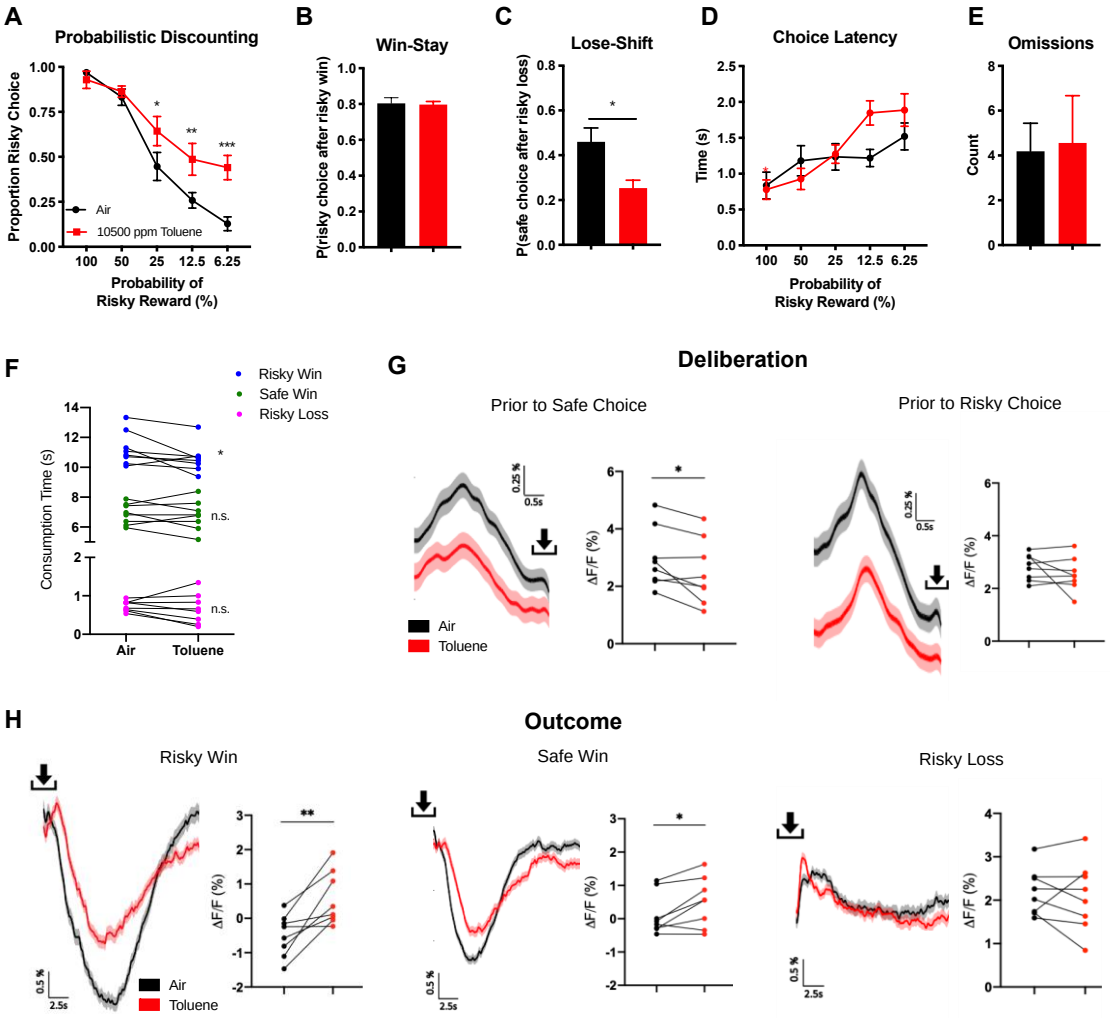


Figure 3.8. Probabilistic discounting during fiber photometry recordings following acute air or toluene exposure. Fiber-tethered rodents expressing GCaMP6f performed the probabilistic discounting task following exposure to toluene and air. **(A)** Proportion of risky choice within each probability block following acute exposure to air or 10,500 ppm toluene. **(B, C)** Choice strategy across all trials. Win-stay **(B)** indicates choice of risky lever after risky win while lose-shift **(C)** indicates choice of safe lever after risky loss. **(D)** Time to choice selection within each probability block. Omissions across all trials **(E)** indicate no lever press within 10 s time period. (F) Paired comparison of consumption time on air vs toluene treatment days across all outcomes. **(G)** Mean ΔF/F% +/- sem for all 8 rats on air vs toluene treatment days during deliberation: prior to safe choice (air, 501 traces; toluene, 425 traces) and prior to risky choice (air, 523 traces; toluene, 610 traces) with paired comparison summaries (right). **(H)** Mean ΔF/F% +/- sem during choice outcome: during a safe win (air, 501 traces; toluene, 425 traces), during a risky win (air, 264 traces; toluene, 282 traces), and during risky loss (air, 276 traces; toluene, 330 traces) with paired

comparison summaries (right). Data shown are mean ± sem; n = 8; 2-way ANOVAs with Sidak's post hoc, n.s. = not significant, *p<0.05, **p<0.01, *p<0.001)

DISCUSSION

Acute toluene impairs probabilistic discounting

In order to understand how the abused inhalant toluene affects risk/reward decision making, we exposed animals highly trained in the probabilistic discounting task to air or two levels of toluene vapor. We found a dose-dependent effect of toluene on risky choice preference in both male and female rats. Toluene increased risky choice preference when the initial expected value of the risky lever was high (descending odds task), but decreased risky choice preference when the initial expected value of the risky lever was low (ascending odds task). These effects were driven primarily by a perturbation in sensitivity to negative feedback, although a reduction in positive feedback sensitivity was also observed. We interpret these findings as a toluene-induced failure to update expected values, resulting in behavioral inflexibility as the task progressed and reward probabilities changed.

The alterations in choice and response latencies induced by toluene are similar to those induced by of amphetamine (St. Onge and Floresco, 2009; St. Onge et al., 2010), one of only two known drugs for which toluene serves as a discriminative stimulus (Bowen, 2006). As first suggested by St. Onge and colleagues, enhanced mPFC dopamine signaling might be the cause of amphetamine-induced discounting impairments since (1) increasing dopamine

perturbs complex cognitive functions mediated by the mPFC (2) pharmacologically silencing the mPFC caused the same procedural-dependent effects, and (3) mPFC dopamine levels track reward rates during probabilistic discounting (St. Onge and Floresco, 2010; St. Onge et al., 2010, 2012). Interfering with this dopamine signal might disrupt the ability to accurately encode reward value and/or act on choice valuation, resulting in more static patterns of choice. A similar mechanism should be considered for toluene, since it is known to increase extracellular levels of dopamine in the mPFC (Gerasimov et al., 2003; Koga et al., 2008) and as described in these studies, alters mPFC activity during probabilistic discounting. One caveat is that pharmacological activation of dopamine D2, but not D1, receptors in the mPFC only serve to decrease risky choice preference during probabilistic discounting. (St. Onge et al., 2011). Interestingly however, simultaneous stimulation of mPFC D2 receptors while pharmacologically silencing the basolateral amygdala causes the same procedural-dependent effects on probabilistic discounting as acute toluene exposure (Jenni et al., 2017). Future studies on the effect of toluene on complex cognitive behaviors should take this circuit into consideration.

The increase in task engagement (i.e. decreased latency and omissions) following toluene exposure was not universal across sex or task type (ascending or descending). Toluene's effects on these measures is likely more to do with differences in the task and how these differences affect each sex than a general impairment in performance. In fact, inactivating the mPFC causes a slight increase in latency during safe-optimal trials of descending odds probabilistic discounting

(St. Onge and Floresco, 2010). Furthermore, omissions during probabilistic discounting are unaffected by mPFC inactivation or disconnection of mPFC-nucleus accumbens circuitry (St Onge et al., 2012). On the other hand, treatment with the adrenergic auto receptor antagonist yohimbine (Montes et al., 2015) has been shown to produce an effect on choice behavior during probabilistic discounting that is similar to that of toluene reported here, while at the same time reducing response latencies. Thus, although not currently known, toluene may simultaneously impair shifts in choice bias and enhance task engagement via alterations in catecholaminergic signaling.

Effect of adolescent toluene exposure on future probabilistic discounting

Drug abuse during adolescence can lead to future cognitive impairments, substance abuse issues, and mood disorders. In fact, rodents show increased risk preference during probabilistic discounting following chronic adolescent alcohol exposure, despite a protracted abstinence (Boutros et al., 2015). Unlike ethanol, we found that adult rats with a history of toluene exposure during adolescence performed the risk task similarly to air-treated controls. Some transient increases in choice latency were observed during training that could be the result of instrumental learning deficits observed despite abstinence from toluene (Dick et al., 2014; Braunscheidel et al., 2017). Another non-mutually exclusive possibility is that increased anxiety during toluene withdrawal (Bowen et al., 2018) led to increased indecisiveness, and thus deliberation time. This notion agrees with the increased choice latency observed following acute stress or infusions of

corticotropin releasing factor effort-discounting tasks (Shafiei et al., 2012; Bryce and Floresco, 2016). Finally, while rats received multiple toluene exposures during adolescence, the experience was over a relatively brief time period (5 days). A more extensive exposure period like that employed by Boutros and colleagues (2015) might cause a greater effect on the measured parameters.

In this study, an acute challenge with 10,5000 ppm toluene in adult rats with a history of toluene exposure during adolescence produced similar alterations in probabilistic discounting, lose-shift behavior, and choice latency as those only exposed to toluene as adults. However, we did not observe any increase in win-stay behavior in the combined adolescent/adult exposed animals. Interestingly, the lower dose of toluene failed to produce an appreciable change in discounting but did increase win-stay behavior in both male and female rats. This combination of sensitization to the reward-promoting effects of toluene at lower doses with simultaneous desensitization to cognitive impairments could contribute to inhalant relapse despite protracted abstinence.

mPFC calcium activity encodes information about upcoming choice and outcome

Neuronal activity within the mPFC is critical for tasks involving behavioral flexibility (Birrell and Brown, 2000; Marquis et al., 2007; Floresco et al., 2008) including probabilistic discounting (St. Onge and Floresco, 2010). In the present study, we used in vivo fiber photometry to investigate the importance of mPFC activity on probabilistic discounting with temporal specificity and to provide

physiological evidence for a mPFC-dependent mechanism of toluene-induced deficits on probabilistic discounting. We observed increases in mPFC activity prior to any free-choice press, independent of the actual expected value of that press. This is consistent with previous reports showing that the firing rate of mPFC neurons increases prior to an action that is intended to generate reward (Sul et al., 2010). Data from forced-choice trials indicate that this signal does not simply predict any action that might generate reward. Rather, increases in mPFC activity appeared to flexibly shift from predicting forced risky choice to forced safe choice throughout the session. Since this pattern reflects the theoretical optimal choice to maximize expected outcome (risky early when risky reward probabilities are high, safe late when risky reward probabilities are low) we interpret this as a "preferred action" signal. This interpretation is similar to the recently described "outcome-prediction" activity discovered in mPFC neurons that fire specifically once an attentional set-shift is completed (Del Arco et al., 2017).

In this study, we found a prominent decrease in mPFC calcium activity during rewarded outcome that was correlated with reward magnitude in air-treated animals. At first take, these data seem in stark contrast to many reports of increased mPFC electrical activity during reward outcome and consumption (McCoy and Platt, 2005; Horst and Laubach, 2013; Petykó et al., 2015; Del Arco et al., 2017). However, these studies refer to a very short time scale following reward delivery (0 – 2 seconds) compared to the duration of reward consumption during which we recorded mPFC calcium transients (0 – 20 seconds). Increases in mPFC spiking is tightly linked with initiation of consumption behavior (i.e.

licking), activity which desensitizes over time (Horst and Laubach, 2013). Further, a majority of mPFC activity during outcome switches from excitatory to inhibitory around 1.5 seconds post reward delivery (Del Arco et al., 2017). Combined, these factors could explain the sustained dip in mPFC calcium activity we observed during reward consumption and might represent portions of the brain's executive functions "going offline" while engaging in a habitual or automatic behavior. It is clear however that this is not a global effect as Passecker and colleagues recently identified two dorsal mPFC ensembles that actively relay information about the value of the current reward, and influence upcoming choice (Passecker et al., 2019).

Effect of toluene on mPFC activity during probabilistic discounting

Toluene disrupted flexible risk/reward decision making in a manner similar to pharmacological inactivation of the mPFC (St. Onge and Floresco, 2010). As such, it is possible that the effects of toluene were driven by a general suppression of PFC in neural activity. Yet, when we measured baseline mPFC calcium activity in animals treated with the same toluene exposure protocol as during behavioral tests, we failed to detect any significant differences in magnitude or frequency of spontaneous calcium transients compared to air-treated controls. Thus, although it is clear that toluene can disrupt mPFC function, this does not appear to result from a global reduction in neural activity, but rather, may be mediated by disruptions in task-dependent patterns of mPFC activity.

mPFC activity increased during epochs prior to lever selection (i.e. deliberation). Acute administration of toluene blunted this increase, an effect that was driven by activity specifically prior to an upcoming safe, but not risky choice when collapsed across all trials. However, whereas increases in mPFC activity shift from pre-risky to pre-safe press during forced-choice trials in air treated controls, activity in toluene-treated animals display a slightly different activity profile: (1) activity prior to either lever press did not increase in the first probability block and (2) the shift in activity from pre-risky to pre-safe was disrupted. Further, toluene-treated animals still show strong increases prior to safe lever presses in free-choice trials. Taken together, these results suggest a toluene-induced failure to recognize changes in relative value of both levers as the task progresses rather than a nonspecific reduction in signaling preceding a low-risk decision.

As with air-treated controls, toluene exposed rats showed a sustained dip in mPFC calcium activity across all rewarded trials. This dip was mitigated by toluene during both safe and risky wins, suggesting reduced mPFC silencing during consumption. Further, mPFC activity during a safe win was indistinguishable from a risky win following toluene treatment. While this could be explained by a general deficit in reward magnitude discrimination, toluene-treated animals did show appropriate discrimination (i.e. large reward preference) when the risky lever was reinforced 100% of the time. It is more likely that the deficit reflects impaired function of a subpopulation of neurons responsible for updating current outcome in order to influence future decision, like those recently discovered by Passecker and colleagues (2019). One such subpopulation might be deep-

layer mPFC neurons projecting to the nucleus accumbens core, that are hypo-excitable following toluene treatment (Wayman and Woodward, 2017). Alterations in the activity of these neurons by toluene could explain the drug's effect on mPFC activity during choice outcome.

Sex differences in baseline probabilistic discounting

Sex differences have been reported in other preclinical models of decision making involving risk (for review, see Orsini and Setlow, 2017). In these studies, we found that male and female rats acquired similar levels of probabilistic discounting, largely consistent with a recent report (Westbrook et al., 2018). Males and females were similarly sensitive to recent positive and negative feedback as measured by win-stay and lose-shift behavior, respectively. Females made much quicker decisions and fewer omissions than males. It is possible that females were hungrier than males during task performance, leading higher relative value of the food reward and thus greater task engagement. However, the food-restriction protocol whereby animals are limited based on their individual free-feeding weight should control for this possibility. The sex-differences in task-engagement are in stark contrast to probabilistic discounting under risk of footstock, where females omit significantly more than males (Orsini et al., 2016). This indicates that females and males utilize different strategies regarding the decision to engage a risky situation when the punishment is neutral (reward omission) compared to aversive (foot shock. These sex differences should be taken into consideration when testing the effects of other experimental factors on probabilistic discounting.

CONCLUSION

The studies herein describe the effect of abuse-levels of toluene vapor exposure on risk/reward decision making and strongly implicate mPFC dysfunction as a source of impaired flexible adjustments in choice biases. This deficit might be caused by perturbed task-related activity in sub-populations of projection specific mPFC neurons (e.g. BLA-projecting D2+ or nucleus accumbens core-projecting deep-layer pyramidal neurons) failing to integrate reward magnitude information to inform and/or execute future decisions. Future studies should attempt to identify these populations with molecular and circuit-level specificity and how they are specifically impaired by toluene.

CHAPTER 4: REDUCTION OF CANNABINOID RECEPTOR 1 SIGNALING ALTERS ASPECTS OF RISK/REWARD DECISION MAKING INDEPENDENT OF TOLUENE-MEDIATED DEFICITS.

INTRODUCTION

Abused substances impair risky decision making in humans (Lane et al., 2005; Euser et al., 2011; Buelow and Suhr, 2014) and rodents (St. Onge and Floresco, 2009; Mitchell and Blankenship, 2011). Preclinical studies of the effects of drugs of abuse on decision making have focused primarily on stimulants and alcohol. However, recent studies from our laboratory have shown that toluene, a volatile organic solvent and abused inhalant, also perturbs flexible risk/reward decision making and associated reward-related activity in the prefrontal cortex (PFC) (Braunscheidel et al., 2019).

Interestingly, some of the inhibitory effects of toluene on cell signaling are mediated by enhanced cannabinoid receptor type 1 receptor (CB1R) activity in regions of the brain that are important for decision making (Beckley and Woodward, 2011; Beckley et al., 2016). In this regard, cannabis is the most common illicit intoxicant observed in ambulance attendees involving inhalant misuse (Crossin et al., 2018). Delta9-tetrahydrocannabinol is one of the key psychoactive components of cannabis, and it elicits its effects by acting as a partial agonist on CB1R. Beyond these reports, preclinical studies investigating the interaction between inhalants and endocannabinoids are limited.

Research on the modulation of CB1Rs in the context of cost/benefit decision making are important for developing informed health and safety policies. Relaxed

legislation on cannabis restrictions is leading to greater consumption of delta9-tetrahydrocannabinol-containing products in adults (Carliner et al., 2017). Given the comorbities of cannabis use with substance use disorders, mood disorders, and anxiety disorders (Stinson et al., 2006), it is not surprising that CB1R receptor expression is widespread in regions that are important for decision making such as the prefrontal cortex (Eggan et al., 2010), hippocampus (Davies et al., 2002), and striatum (Julian et al., 2003; Pickel et al., 2004) for review, see (Hu and Mackie, 2015). Enhancing CB1R signaling disrupts higher cognitive functions including risky decision making in humans (Lane and Cherek, 2002; Lane et al., 2005; Anderson et al., 2010). However, these effects have not been reproduced in two separate preclinical models (Ferland et al., 2018; Freels et al., 2020). Here we use a third, well-validated rodent model of risk/reward decision making – the probabilistic discounting task (St. Onge and Floresco, 2009; Braunscheidel et al., 2019) – to test the hypotheses that CB1R silencing is sufficient to modulate risky decision making and that toluene-induced impairments in this behavior depend on CB1R signaling.

As described above, CB1R expression is widespread in the central nervous system, but it is also expressed in periphery (Kulkarni-Narla and Brown, 2000) and involved in the immune response (Cabral et al., 2015). This suggests that the ameliorative potential of CB1R silencing in the above experiments could also reflect off-target effects. Interestingly, the impairments in behavioral flexibility caused by toluene mimic those observed by inactivation of the mPFC, and toluene exposure disrupts mPFC pyramidal activity during contingency updating and

monitoring (St. Onge and Floresco, 2010; Braunscheidel et al., 2019). Further, results from whole cell patch clamp electrophysiology recordings in the mPFC show that ex vivo toluene application reduced AMPA-mediated excitatory signaling via activation of presynaptic CB1Rs (Beckley and Woodward, 2011). While mPFC CB1R signaling is involved in fear and anxiety-related decision making (Draycott et al., 2014; Schneider et al., 2015), the effects on risk/reward decision making have not been explored. To address this gap in the literature, we studied the effect of local pharmacological manipulation of mPFC CB1Rs on probabilistic discounting. Finally, we address hypotheses that toluene-induced impairments depend on mPFC CB1R signaling. The results of these studies provide evidence for the involvement of CB1R signaling during probabilistic discounting that is unrelated to toluene-induced deficits in risk/reward decision making.

MATERIALS AND METHODS

Animals

Fifty-eight male Sprague-Dawley rats (post-natal day (P) 53 on arrival; Envigo RMS, Indianapolis, IN) were housed in pairs in polypropylene cages on a reverse light cycle (lights off at 0900) in a climate-controlled room with food and water delivered *ad libitum*. At approximately P60-70, rats were food restricted to maintain 85-90% of their free feeding weight (weight at time of final testing (300-400 g). Figure 4.1A details the experimental timeline for all rodents. All procedures were performed in compliance with Medical University of South Carolina IACUC protocols.

Surgeries

A subset of 30 animals underwent stereotaxic surgery ~1 week after arrival during which deep anesthesia was achieved via an isoflurane vaporizer (Penlon; 1 L/min, 5% induction, 2–3% maintenance) and bilateral guide cannula (Plastics One) were implanted above the prelimbic mPFC (± 0.6 ML, +2.95 AP, -2.85 DV from Bregma). Microinfusion tips extended 1 mm from the guide cannula for a final injection location of -3.85 DV from Bregma.

Lever Press Training

Lever press training occurred over the course of 1-2 weeks as previously described (Braunscheidel et al 2019). In brief, rats were habituated to a reward of 20% sweetened condensed milk (SCM), by giving them free access to 10 ml SCM for two days prior to operant training. Over the course of two phases, rats (P60-70) were trained to lever press in operant chambers (Med Associates, St. Albans, VT) for SCM delivered to a central feeding well via a pump-activated syringe. Phase 1 (2-5 days; 30 min sessions) began with one lever (left or right, pseudo-randomly assigned) reinforced with 45 µl SCM on an FR1 schedule. Upon meeting criteria (50 presses for 2 consecutive days), the presented lever was switched, and rats were tested to criteria before moving on to phase 2. Phase 2 (6-7 days; 60-minute sessions) consisted of 90 trials separated by 35s. Each session began with an illuminated house light and 2s later, the left or right lever extended in a pseudo-random order. When pressed, the lever retracted, and 45 µl SCM was delivered on 75% of trials. If a lever was not pressed within 20s, it

retracted, and the trial was recorded as an omission. Following completion of two consecutive days with less than 10 omissions per session, the time to omission was reduced to 10s. When rats met criteria again, the lever reward probability reduced to 50%. When rats met criteria a third time, a side preference test was performed. Briefly, for each of 60 trials, both levers extended simultaneously and were reinforced on an FR1 schedule. A trial concluded when two presses occurred, which resulted in lever retraction for 20s. The preferred side was defined as the side that a rat pressed first most often across trials. Rodents then began training in the probabilistic discounting task.

Probabilistic Discounting

A probabilistic discounting procedure was used to assess risk/reward decision making in rodents as previously described (St. Onge and Floresco, 2009; Braunscheidel et al., 2019). This two-lever choice task (Fig. 1B) consists of a "safe" lever that delivered a small reward (30 µl SCM) 100% of the time and a "risky" lever that delivered a large reward (90 µl SCM) with varying probability of reinforcement. The risky lever was assigned to the non-preferred lever position as determined by the side preference test. Each session consisted of 90 trials separated into 5 blocks and each block started with 8 forced-choice trials that set the probability of reinforcement for the following 10 free-choice trials (Fig. 4.1C). The probability of obtaining a large reward was varied from high-to-low with the following probabilities: 100%, 50%, 25%, 12.5%, 6.25%. Each trial lasted 35 s and began with an illuminated house light and 2 s later levers extended into the

chamber. A press on either lever caused both of them retract and turned the house light off. On rewarded trials, reward was delivered to the central feeding well. These “wins” were paired with a discriminative cue: a flashing light above the food well to indicate whether the reward was small or large (safe win: 2 pulses, 0.35 s pulse width, 0.5 Hz; risky win: 5 pulses, 0.35 s pulse width, 0.5 Hz). On non-reinforced “loss” trials, no cue light was provided. If a lever was not pressed in 10 s, it was recorded as an omission and the houselights were extinguished for 25s. Following ~20 days of training (5-6 days per week), rats exhibited stable responding (two-way ANOVA on three consecutive testing days yields no block x day interaction or main effect of day, $p > 0.1$) and were subjected to drug tests.

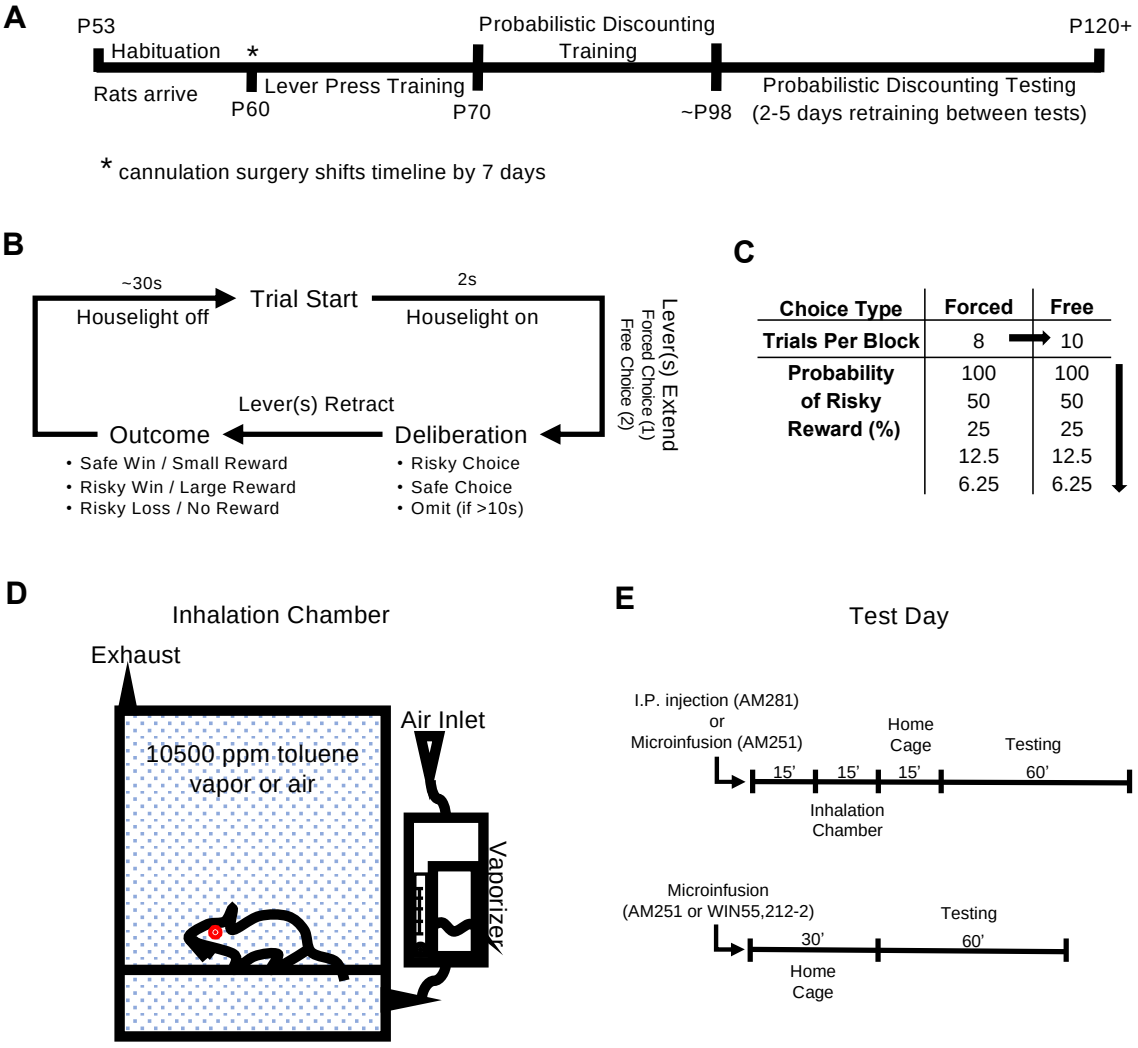


Figure 4.1. Probabilistic discounting training and test design. (A) Experimental timeline and corresponding rat age. **(B)** Flow chart detailing a single trial of the probabilistic discounting task. **(C)** Breakdown of the 10 different probability blocks within the probabilistic discounting task with odds presented in descending order. **(D)** Inhalation chamber schematic. **(E)** Test day progression for the two toluene exposure experiments (top) and two mPFC CB1R modulation experiments (bottom).

Systemic Drug Administration And Inhalation Chamber Treatments

In some studies, rats received an intra-peritoneal (i.p.) injection of CB1R inverse agonist AM251 (2mg/kg) or vehicle (DMSO) 15 min prior to 15 minute gas exposures (air or 10,500 ppm toluene) in a 30x30x30 cm inhalation chamber (Fig. 4.1D, Plas Labs, Lansing, MI). Rats then were then returned to their home cage for 30 min prior to task performance (Fig. 4.1E). Toluene concentrations were achieved via a sevoflurane vaporizer (Penlon Limited; flow rate 4L/min) as previously described (Braunscheidel et al., 2017, 2019; Wayman and Woodward, 2018), and confirmed with a portable toluene gas detector (DOD Technologies, Cary, IL). At this dose, rats exhibit lethargy after ~10 minutes of exposure and were nearly immobile after 15 minutes. Rats fully regained ambulation following approximately 15 minutes of recovery in the home cage (unpublished observations). These four treatments (AM251 + air, AM251 + toluene, vehicle + air, vehicle + toluene) were administered in a within-subject counter balanced design with 2-5 days of retraining between tests such that pre-test day performance was equivalent (two-way ANOVA yields no main effect of day, $p > 0.1$).

Microinjections

For the mPFC CB1R manipulation studies, rats received microinfusions (300 nl over 1 min) of selective CB1R mediators (AM251,5 and 50 ng; WIN55,212-2, 50 ng and 500 ng) or vehicle (3% DMSO, 3% Tween80, PBS) into the prelimbic mPFC 30 min before testing (Fig. 4.1E). These doses were selected as they have been shown to alter fear-related behaviors when microinjected into the mPFC

(Laviolette and Grace, 2006). In both experiments, the three treatments (high dose, low dose, vehicle) were administered in a within-subject counter balanced design with 2-5 days of retraining between tests such that pre-test day performance was equivalent (two-way ANOVA yields no main effect of day, $p > 0.1$).

In a separate cohort, 50 ng AM251 or vehicle was injected 15 min prior to toluene exposure (i.e. 1hr prior to task performance, see Fig. 4.1E). All microinjections were administered using a within-subject, counter-balanced design with 2-5 days of retraining between tests such that pre-test day performance was equivalent (two-way ANOVA yields no main effect of day, $p > 0.1$). This design has been validated elsewhere for addressing the neuropharmacology of the PD task(Stopper et al., 2013).

Statistics

The primary dependent variable of the probabilistic discounting test was risky lever preference, expressed as proportion risky choice (number of risky lever presses/total lever presses) during each of the five probability blocks separated by likelihood of a rewarded risky lever press. Additional performance variables including omissions, latency to choice, win-stay (number of risky lever presses following a risky win / total number of risky wins), and lose-shift (number of safe lever presses following a risky loss / total number of risky losses) were also recorded. Only free-choice trials were considered in behavioral analyses. Choice data obtained from the microinjection experiments were analyzed with a two-way

repeated measures ANOVA with treatment and probability block as factors. Win-stay, lose-shift, and omissions were analyzed with a one-way ANOVA with appropriate multiple comparisons. The systemic AM281 studies were analyzed with a three-way ANOVA with i.p. treatment, inhalation treatment, and probability block as factors. Win-stay, lose-shift, and omissions were analyzed with a 2-way ANOVA with appropriate post hoc multiple comparisons. All statistics and graphing was performed using Prism 8 (Graphpad Software San Diego, CA).

RESULTS

Systemic Reduction In CB1R Activity Alters Probabilistic Discounting Performance Independently From Acute Toluene Effects

Previous studies in our laboratory have shown that acute toluene exposure impairs flexible adjustments in choice biases by rats during probabilistic discounting (Braunscheidel et al., 2019). In this experiment, we sought to determine whether this effect may be modulated by CB1R activity. Figure 4.2A shows risk preference during probabilistic discounting behavior in well-trained Sprague-Dawley rats following four treatments: 2 mg/kg (i.p.) AM281 + air inhalation, vehicle + air inhalation, 2 mg/kg AM281 (i.p.) + toluene inhalation, and vehicle + toluene inhalation. Analysis of the choice data revealed that toluene exposure again impaired shifts in choice biases manifesting as an increase in risky choice under these conditions (main effect of toluene ($F_{(1, 18)} = 14.58$, $p = 0.0013$). Post hoc two-way ANOVA of these data collapsed across all toluene/air treatments revealed that toluene exposure increased risky choice specifically during times of

high uncertainty (50%, 25%, and 12.5% blocks, all $t_{(38)} > 2.86$, $p < 0.024$) and not during blocks where there was relatively little uncertainty of obtaining the larger reward (100% and 6.25% blocks, both $t_{(38)} < 1.78$, $p > 0.33$). However, there was no interaction between the factors (three-way ANOVA, toluene x AM281 x probability block, $F_{(4, 72)} = 0.85$, $p = 0.50$) or any combination of two factors (toluene x AM281 $F_{(1, 18)} = 0.12$, $p = 0.74$; toluene x probability block $F_{(4, 72)} = 1.96$, $p = 0.11$; AM281 x probability block $F_{(4, 72)} = 0.55$, $p = 0.70$). Furthermore, AM281 did not appear to alter choice during probabilistic discounting (main effect, $F_{(1, 18)} = 0.0020$, $p = 0.97$).

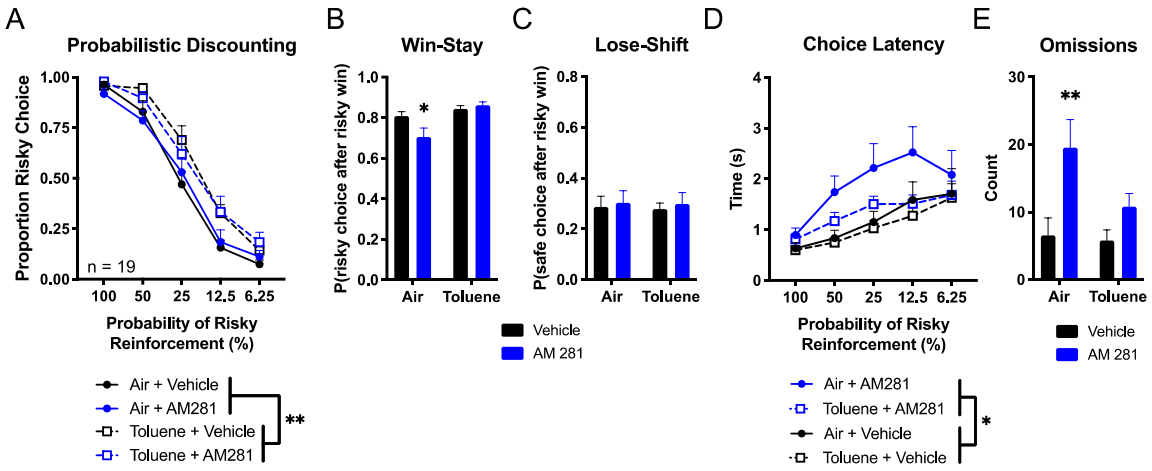


Figure 4.2. Systemic CB1R inverse agonism alters probabilistic discounting performance independently from acute toluene effects. Well-trained rats were treated with a combination of i.p. injections (2mg/kg AM281 or vehicle) and vapor exposure (toluene or air) prior to task performance. **(A)** Proportion of risky choice within each probability block across treatments. **(B, C)** Choice strategies employed across all trials. Win-stay **(B)** indicates choice of risky lever after risky win while lose-shift **(C)** indicates choice of safe lever after risky loss. **(D)** Time to choice selection within each probability block. **(E)** Omissions across all trials indicate no lever press within the 10 s trial period. Data shown are mean + sem; all n = 19; three-way ANOVA main effects, *p < 0.05, **p < 0.01; Tukey's post hoc, *p < 0.05, **p < 0.01.

Subsequent trial-by-trial analysis of the choice data were conducted to examine how the outcomes of risky choices influenced subsequent choice. Figure 4.2B illustrates the effect of recent positive reinforcement on choice strategy, measured as the probability of choosing the risky lever following a risky win ("win-stay"). Two-way ANOVA revealed an increase in win-stay behavior in toluene treated animals, (main effect, $F_{(1, 18)} = 13.18$, $p = 0.0020$). Notably, the analysis also revealed as a significant interaction between toluene and AM281 treatments ($F_{(1, 18)} = 7.00$, $p = 0.017$). This effect was driven in part by a reduction in win-stay behavior by AM281 relatively to vehicle treatment in air treated animals (Tukey's post hoc, AM281 vs vehicle, $q_{(14)} = 4.48$, $p = 0.025$). Thus, even though CB1R inhibition did not alter overall choice levels, it did appear to reduce the influence that recently rewarded risky choices exerted over subsequent choices. In contrast, the effect of recent negative feedback sensitivity on choice strategy did not differ across treatment conditions, measured as the probability choosing the safe lever following a risky loss ("lose-shift"; all $F_{(1, 18)} < 0.28$, $p > 0.60$; Fig 4.2C). Taken together, the fact that AM281 did not alter the effects of toluene on in probabilistic discounting suggests that the alterations in decision making induced by this inhalant are likely not mediated by increased systemic CB1R activation.

Choice latency and omission data may reflect decision speed, impulsivity and/or general motivation to lever press for reward. Analysis of the choice data partitioned across blocks yielded no interaction between the factors (three-way ANOVA, toluene x AM281 x risk preference, $F_{(4, 72)} = 0.42$, $p = 0.79$) or any combination of two factors (toluene x AM281 $F_{(1, 18)} = 1.37$, $p = 0.26$; toluene x risk,

$F_{(4, 72)}$ = 1.07, p = 0.38; AM281 x risk, $F_{(4, 72)}$ = 1.10 , p =0.38) on choice latency (Fig 4.2D). On the other hand, whereas toluene did not impact choice latency (main effect, $F_{(1, 18)}$ = 2.45, p = 0.14), it was affected by AM281 (main effect, $F_{(1, 18)}$ = 7.36, p = 0.014). Post hoc two-way ANOVA of these data collapsed across all AM281/vehicle treatments reveals that these differences are during times of high uncertainty (50%, 25%, and 12.5% blocks, all $t_{(38)} > 3.0$, $p < 0.015$) and not when the potential outcome of a risky choice was more certain (100% and 6.25% blocks, both $t_{(38)} < 1.24$, $p > 0.70$. AM281-treated animals also omitted more than their vehicle-treated counterparts (Fig. 4.2E, two-way ANOVA, main effect of AM281, $F_{(1, 18)}$ = 13.20, p = 0.0020). Interestingly, AM281 increased omissions following air treatment (Tukey's post hoc, AM281 vs vehicle, $q_{(18)}$ = 5.77, p = 0.0036), but not after toluene exposure. Taken together, these data suggest that AM281 causes task disengagement or delayed decision making speeds, especially during times of increased difficulty or uncertainty.

Toluene Induced Deficits In Probabilistic Discounting Are Not Prevented By Reduced mPFC CB1R Activity

In the next set of experiments, we specifically targeted mPFC CB1Rs via a bilateral microinfusion of AM251 into the prelimbic cortex. In keeping with the findings following systemic administration of this compound, intra-PFC infusion of AM251 did not alter risk preference in toluene treated animals compared to vehicle + toluene positive controls (Fig. 4.3A, two-way ANOVA, treatment x probability block, $F_{(8, 64)}$ = 0.70, p = 0.69; main effect of treatment, $F_{(2, 16)}$ = 2.15, p = 0.15;

Sidak's post hoc comparing AM251 + toluene to vehicle + toluene, all $t_{(64)} < 0.93$, $p > 0.73$). With respect to reward and negative feedback sensitivity, inspection of the data presented in Figure 4.3B suggest that toluene again increased win-stay behavior relative to air-treatment. However, a one-way ANOVA of these data reveals this effect only approached statistical significance ($F_{(2,16)} = 3.04$, $p = 0.076$.), although post-hoc pairwise comparisons indicate that trend was driven by the trend in vehicle + toluene treatment vs vehicle + air (Tukey's post hoc, $q_{(16)} = 3.48$, $p = 0.063$; both other $q_{(16)} < 2.01$, $p > 0.35$). Figure 4.3C shows the significant effect of these treatments on lose shift behavior (one-way ANOVA, $F_{(2,16)} = 10.58$, $p = 0.0012$). Surprisingly, toluene decreased this metric compared to the vehicle + air control, irrespective of whether rats were treated with AM251 (Tukey's post hoc, both $q_{(16)} > 5.09$, $p < 0.0064$), and AM251 did not alter the effect of toluene ($q_{(18)} = 9.45$, $p = 0.76$). Finally, there was no effect of these treatments on choice latency (Fig. 4.3D, two-way ANOVA, probability block x treatment, $F_{(8, 64)} = 1.17$, $p = 0.33$; main effect of treatment, $F_{(2, 16)} = 1.10$, $p = 0.36$) or omissions (Fig. 4.2E, one-way ANOVA, $F_{(2, 16)} = 0.78$, $p = 0.48$). These data suggest that reducing CB1R activity within the prefrontal cortex does not block toluene-induced deficits in probabilistic discounting.

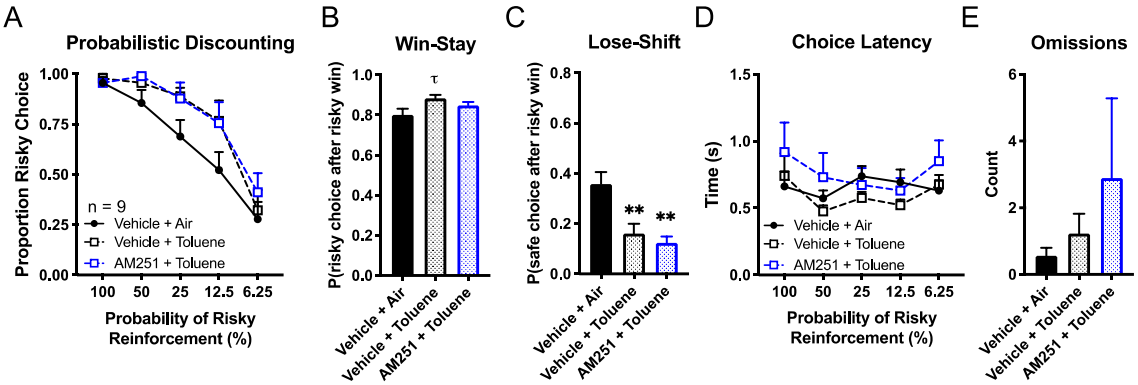


Figure 4.3. Toluene-induced impairments in probabilistic discounting does not depend on mPFC CB1R signaling. Well-trained rats were given the following treatments prior to task performance across three test days: vehicle mPFC microinjection + air exposure, vehicle mPFC microinjection + toluene, or 50 ng AM251 mPFC microinjection + toluene. **(A)** Proportion of risky choice within each probability block across treatments. **(B, C)** Choice strategies employed across all trials. Win-stay **(B)** indicates choice of risky lever after risky win while lose-shift **(C)** indicates choice of safe lever after risky loss. **(D)** Time to choice selection within each probability block. **(E)** Omissions across all trials indicate no lever press within the 10 s trial period. Data shown are mean + sem; all n = 9; *p < 0.05; Tukey's post hoc, τp = 0.063, *p < 0.05, **p < 0.01.

Effect Of mPFC CB1R Modulation On Probabilistic Discounting

Results from these experiments warranted further exploration of how mPFC CB1R signaling may modulate probabilistic discounting. To address this, we microinfused different doses of AM251 or vehicle directly into the prelimbic cortex prior to probabilistic discounting test sessions (Fig. 4.4). This treatment did not alter risk preference (Fig. 4.4A, two-way ANOVA, probability block x AM251, $F_{(8, 112)} = 0.41$, $p = 0.91$; main effect of AM251, $F_{(2,28)} = 1.20$, $p = 0.32$). However, despite the lack of effect of these treatments on overall choice, intra-mPFC infusion of the high dose of AM251 did decrease win-stay behavior (Fig. 4.4B, one-way ANOVA, $F_{(2, 28)} = 4.00$, $p = 0.030$ and Dunnett's post hoc, 50 ng AM251 vs vehicle, $q_{(28)} = 2.78$, $p = 0.018$), consistent with its effect when administered systemically.

Lose-shift behavior was unaffected by mPFC AM251 (Fig. 4.4C, one-way ANOVA, $F_{(2, 28)} = 0.60$, $p = 0.55$) as was choice latency (Fig. 4.4D, two-way ANOVA, probability block x AM251, $F_{(8, 112)} = 0.76$, $p = 0.64$; main effect of AM251, $F_{(2,28)} = 0.027$, $p = 0.97$) and omissions (Fig. 4.4E, one-way ANOVA, $F_{(2, 28)} = 3.11$, $p = 0.060$). These data suggest that mPFC CB1R signaling contributes to promoting win-stay behavior during probabilistic discounting. However, intra-PFC infusions of this compound did not recapitulate the actions of systemic treatment on omissions and choice latency, suggesting these effects were likely mediated by reducing CB1R activity in other brain regions

We then tested how intra-mPFC infusions of a CB1R agonist affected task performance, using two doses of WIN55,212-2 (50 ng and 500 ng). These treatments also did not alter risk preference (Fig. 4.4F, two-way ANOVA, probability block x treatment, $F_{(8, 112)} = 0.79$, $p = 0.61$; main effect of treatment, $F_{(2, 28)} = 0.48$, $p = 0.62$). CB1R stimulation also did not affect win-stay (Fig. 4.4G, one-way ANOVA, $F_{(2, 28)} = 1.40$, $p = 0.26$), lose-shift (Fig. 4.4H, $F_{(2, 28)} = 0.47$, $p = 0.63$), choice latency (Fig. 4.4I, two-way ANOVA, probability block x treatment, $F_{(8, 112)} = 0.53$, $p = 0.83$; main effect of treatment $F_{(2, 28)}$, $p = 0.56$), or omissions (Fig. 4.4J, one-way ANOVA, $F_{(2, 28)} = 0.78$, $p = 0.47$). Thus, activation of CB1R receptors in the mPFC does not appear to alter risk/reward decision making or other motivational measures.

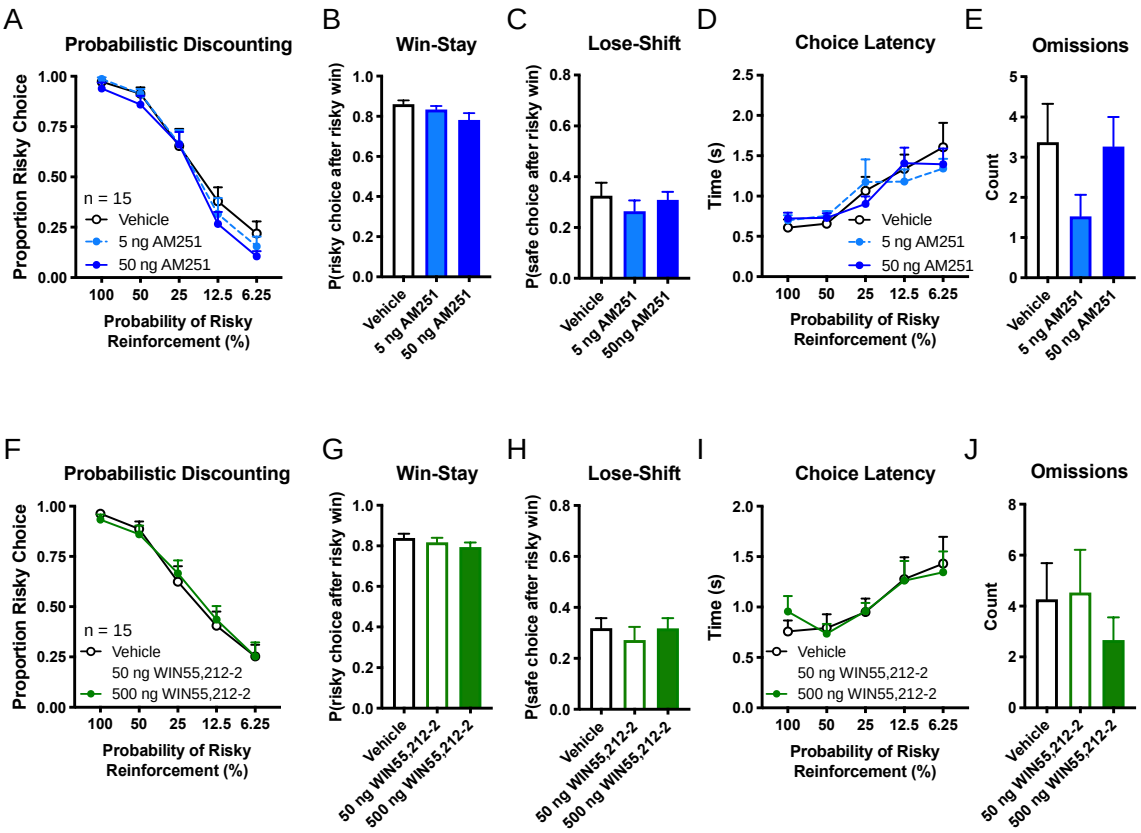


Figure 4.4. Effects of mPFC CB1R manipulation on probabilistic discounting. CB1R inverse agonist AM251 (5 ng, 50 ng) or vehicle was bilaterally microinjected into the mPFC of well-trained rats prior to task performance. **(A)** Proportion of risky choice within each probability block across treatments. **(B, C)** Choice strategies employed across all trials. Win-stay **(B)** indicates choice of risky lever after risky win while lose-shift **(C)** indicates choice of safe lever after risky loss. **(D)** Time to choice selection within each probability block. **(E)** Omissions across all trials indicate no lever press within the 10 s trial period. **(F-I)** In a separate cohort of animals, CB1R agonist WIN55, 212-2 (50 ng, 500 ng) or vehicle was bilaterally microinjected into the mPFC of well-trained rats prior to task performance**.** Data shown are mean + sem; all n = 15; Dunnett's post hoc, *p < 0.05.

DISCUSSION

Toluene-Induced Impairments In Probabilistic Discounting Do Not Depend On Systemic Or mPFC CB1R Inverse Agonism.

Previous studies in our laboratory have shown that acute toluene exposure impairs flexible risk/reward decision making during probabilistic discounting

(Braunscheidel et al., 2019). Given that toluene impairs cellular activity via CB1R signaling (Beckley and Woodward, 2011; Beckley et al., 2016) and systemic CB1R treatment mitigates choice perseveration in rats (Hill et al., 2006), we first examined whether toluene-induced impairments in probabilistic discounting are mediated by enhanced endocannabinoid signaling. To test this, we treated rats systemically or intra-mPFC perfusion of a CB1R inverse agonist followed by toluene vapor. Replicating our previous findings, toluene vapor increased risk preference during a probabilistic discounting task when reward probabilities progress from high to low over a session (Braunscheidel et al., 2019). Counter to our hypothesis however, this effect was not mitigated by co-treatment with a systemic injection or mPFC infusion of a CB1R inverse agonist.

An analysis of trial-by-trial choice strategies suggests that reducing CB1R activity either systemically or within the mPFC mitigated the expected toluene-induced increases in win-stay behavior, a marker of sensitivity to recent positive reinforcement. This effect was independent of toluene treatment however, as a reduction in win-stay was observed in animals in the absence of toluene. There were also no interactions between toluene and CB1R treatment on lose-shift behavior, a marker of sensitivity to recent negative reinforcement. Likewise, no interaction between toluene and CB1R treatment were detected in choice latency or number of omissions, markers of decision impulsivity, processing speeds and/or task engagement.

Results from these experiments suggest that toluene-induced impairments in probabilistic discounting do not depend on enhanced endocannabinoid signaling

in the mPFC. One alternative possibility is that toluene directly inhibits NMDA receptors (Cruz et al., 2000; Beckley and Woodward, 2011), which may in turn perturb mPFC functions that facilitate flexible decision making (St. Onge and Floresco, 2010; Braunscheidel et al., 2019). Alternatively, the mPFC modulates probabilistic discounting via functional descending projections to the basolateral amygdala, as disruption of this circuitry induces similar effects on probabilistic discounting as bilateral mPFC inactivation (St Onge et al., 2012; Jenni et al., 2017). Thus, it is possible that that toluene acts on CB1R expressing mPFC terminals in the BLA. This is a particularly interesting idea as we have shown that toluene causes endocannabinoid-dependent inhibition BLA neurons receiving input from the mPFC (see *Chapter 5: Toluene-Induced Alterations In Basolateral Amygdala Physiology*). Future studies are required to identify a mechanism for the toluene-induced disruption of behavioral flexibility during risk/reward decision making.

Effect Of Systemic CB1R Inverse Agonism On Probabilistic Discounting

Systemic AM281 administration did not affect risky choice during probabilistic discounting. This finding is consistent with a set of reports showing that systemic administration of the CB1R inverse agonist rimonabant does not change risky decision making in the rodent gambling task (Ferland et al., 2018) or probabilistic discounting under threat of shock (Freels et al., 2020). However, the AM281 treatments were not completely without effect, as an analysis of trial-by-trial choice strategies suggests that AM281 reduced the effect of recent positive enforcement on upcoming choice selection, without affecting the influence of non-

rewarded choices on action selection. The lack of effect of reducing CB1R on flexible choice is perhaps surprising, given that treatment with AM281 reduces perseverative errors during strategy set shifting (Hill et al., 2006) which may be thought of as a failure in lose-shift decision making. However, the optimal choice following a loss trial varies drastically between the two tasks. For example, returning to a choice on the risky lever that is not rewarded during the 50% probability block in this task is still the theoretically optimal selection, whereas perseverating during a simple strategy shift is always sub-optimal. Moreover, the effects of AM281 on set-shifting in the Hill et al. study were tested after a single shift, whereas in the present study, rats were well-trained to adjust choice biases as reward probabilities changed. These critical differences in task design are important when interpreting the effect of systemic CB1R inverse agonism on choice behavior. Nevertheless, it is possible that CB1R activity may play a more prominent role in modulating flexible action selection when outcomes are deterministic, rather than probabilistic.

Systemic injection of a CB1R inverse agonist increased choice latency and omissions, which may reflect a reduction in task engagement and/or reduced executive processing speeds. This idea is in keeping with the observation that that the CB1 receptor antagonist rimonabant decreases premature responding in the five-choice serial reaction time test (Pattij et al., 2007; Wiskerke et al., 2011) and reduced motivation to obtain food and drug rewards absent reward consumption (Solinas and Goldberg, 2005). Alternatively, these results might reflect a generalized reduction in food motivation caused by reduced CB1R activity

(Freedland et al., 2000; Thornton-Jones et al., 2005; McReynolds et al., 2016). However, this would not explain why increases in choice latency in this study emerged exclusively in the middle of the task, during times of high difficulty (50% - 12.5% probability blocks) while being absent in the beginning and end of the task, when choices are clear (100% and 6.25% probability blocks). Furthermore, reducing food motivation with pre-feeding does alter choice during probabilistic discounting (St. Onge and Floresco 2009), whereas AM281 did not affect this measure. Moreover, treatment with this drug did not alter choice latency and omissions on a rodent gambling task (Ferland, 2018, Gueye et al., 2016). Notably, in that assay, probabilities associated with four different rewards remain static over the session, whereas in the probabilistic discounting task used here, probabilities of obtaining the larger reward are volatile. The extra cognitive effort of monitoring a changing probabilistic environment might be better suited to elicit latency effects caused by CB1R inverse agonism. Further, reductions in intake caused by CB1R inverse agonism did not extend to water consumption (Verty et al., 2004; Gardner and Mallet, 2006), which may be more comparable to the liquid reinforcer used in these studies. Another alternative hypothesis is that this manipulation causes a generalized motor deficit. Although this was not measured in the current set of experiments, it would be surprising given that CB1R inverse agonism typically does not impact locomotion (Freedland et al., 2000; Verty et al., 2004; Gardner and Mallet, 2006; McReynolds et al., 2016).

Freels et al (2020) did not observe changes in choice latency following systemic treatment with a CB1R inverse agonist in a risky decision making task,

where choice of a larger reward was associated by increasing probabilities of foot shock. Divergence of findings between probabilistic discounting with and without physical punishment are not uncommon, as the tasks employ overlapping, but not identical neurocircuitry (Orsini et al., 2015; Winstanley and Floresco, 2016). Further, differences exist in the specificity and mechanism of action of rimonabant, the CB1R inverse agonist used by Freels et al, and AM281, the compound used in these studies (Pertwee, 2006). However, and in line with our results, systemic enhancement of CB1R activity decreased choice latency during discounting (Freels et al., 2020). Taken together, these results suggest an important role of CB1Rs in regulating decision making speed and/or task engagement during probabilistic discounting.

Future studies are required to identify the locus of the CB1R-mediated choice latency and omission deficits observed here. Of particular interest might be mediating inactivation of the nucleus accumbens, given the unique expression profile of CB1Rs in this region (Pickel et al., 2004) and that pharmacological inactivation of the nucleus accumbens (or a mPFC-accumbens disconnection) also increases choice latency without affecting overall risk preference (Stopper and Floresco, 2011; St Onge et al., 2012).

Modulation Of mPFC CB1R Signaling during Probabilistic Discounting

The alterations in probabilistic discounting caused by systemic administration of CB1R inverse agonist warrant further exploration. CB1Rs are abundantly expressed in the PFC (Marsicano and Lutz, 1999; Eggan et al., 2010)

and mPFC CB1R inverse agonism impairs certain fear-related memories in rodents (Laviolette and Grace, 2006; Lin et al., 2009; Kuhnert et al., 2013). Moreover, the mPFC plays a critical role in facilitating flexible adjustments in choice biases (St. Onge and Floresco, 2010). Yet, very little is known about how diminishing mPFC CB1R signaling may affect appetitive decision making. Here we found that mPFC CB1R inverse agonism is sufficient to mediate the observed reduction in positive reinforcement on biasing future choice seen in the systemic CB1R applications, although this effect was not sufficient to cause significant changes in risky choice. Nevertheless, the finding that these treatments blunted the impact that rewarded risky choices have on subsequent choice suggests that targeting CB1R may be a potential strategy for mitigating the effects of maladaptive pleasure seeking (e.g. gambling addiction treatment, substance use relapse prevention, OCD treatment) .

Unlike silencing mPFC CB1R signaling, stimulating mPFC CB1R signaling did not affect any of the task parameters measured during probabilistic discounting. These results are a bit surprising given that acute administration of CB1R partial agonist delta9-tetrahydrocannabinol impairs behavioral flexibility (Lane and Cherek, 2002; Anderson et al., 2010) increases gambling risk preference, and decreases lose-shift behavior in humans.(Lane et al., 2005) Furthermore, enhancing mPFC CB1R signaling via increased receptor expression impairs behavioral flexibility in a reversal learning task (Klugmann et al., 2011) and direct agonism impairs memory recall and extinction learning in rodents (Kuhnert et al., 2013) However, in line with our results, a recent preclinical study showed

that systemic treatment with a CB1R agonist did not alter risk preference during a rodent gambling task, although it may promote more appropriate responding in risk preferring individuals (Gueye et al., 2016; Ferland et al., 2018). Ferland and colleagues argue that their task perhaps did not engage the circuitry impaired by delta9-tetrahydrocannabinol administration due to the static probabilities used in their task. While the studies herein certainly would engage such circuitry, it appears that enhanced mPFC CB1R activity is not sufficient to drive abnormal risk preference. Future studies should consider the potential effects of systemic CB1R agonism on probabilistic discounting to address this hypothesis.

CONCLUSION

The studies herein investigated an endocannabinoid-mediated mechanism for the behavioral flexibility deficits caused by acute administration of the abused inhalant toluene. We found that systemic or local mPFC CB1R inhibition did not mitigate the impairments caused by toluene. Follow up experiments suggest that mPFC CB1R signaling is necessary for normal integration of recent positive reinforcement on future decisions, and that non-mPFC CB1R signaling is important for maintaining decision making speeds and task engagement during risky decision making. To our knowledge these studies are the first to investigate the role of mPFC CB1R inverse agonism in the context of risk/reward decision making and could help inform future studies on disorders marked by maladaptive pleasure seeking.

CHAPTER 5: TOLUENE-INDUCED ALTERATIONS IN BASOLATERAL AMYGDALA PHYSIOLOGY

INTRODUCTION

The amygdala plays a crucial role in regulating emotions and the response to fear and anxiety (Andrewes and Jenkins, 2019) as well as aspects of alcohol use disorder. For example, the amygdala mediates alcohol seeking behaviors (De Guglielmo et al., 2016), alcohol cue reactivity in addicts (Claus et al., 2011), and anxiety-like behaviors during withdrawal from chronic alcohol use (Menzaghi et al., 1994; Diaz et al., 2011). The focus of this chapter is on the basolateral amygdala (BLA), a region involved in reinstatement of alcohol seeking behavior and whose physiology is uniquely affected by chronic alcohol exposure (Läck et al., 2007; Marinelli et al., 2010; Keistler et al., 2017; Chesworth and Corbit, 2018).

Alcohol is an organic solvent that acts as a central nervous system depressant via actions on glutamatergic receptors and GABAA receptors, among others (Möykkynen and Korpi, 2012; Olsen and Liang, 2017). An interaction between alcohol and the organic solvent, toluene, has been shown in rodents (Pryor et al., 1985) as well as human addicts (Marín-Navarrete et al., 2016) suggesting common underlying neurobiology. In fact, toluene and alcohol have a similar chemical profile, overlapping pharmacology, as well as abuse potential; for review, see (Beckley and Woodward, 2013). Despite this, only one study exists on the effect of volatile organic solvents on the amygdala where Perit and colleagues (2012) noted increased c-Fos immunoreactivity, a proxy for cellular activity, in the rat amygdala following a brief exposure to abused concentrations of toluene vapor.

Here we extend these findings by using whole-cell patch-clamp electrophysiology to investigate the actions of toluene on BLA neuronal activity.

As the BLA contains a biochemically and physiological diverse population of neurons, it is perhaps not surprising that specific BLA subcircuits mediate discrete behaviors (Wassum et al., 2016). For instance, the medial prefrontal cortex (mPFC)-BLA pathway is important for executive control during appetitive risk/reward decision making, fear extinction (Park and Chung, 2020), as well as escalation of alcohol intake in rodents (Gioia et al., 2016, 2017). While previous reports have investigated circuit specific effects of toluene on the neurophysiology of mPFC projecting VTA dopamine neurons (Beckley et al., 2013; Wayman and Woodward, 2017, 2018), the studies herein are the first to report on the BLA neurons that receive input from the mPFC.

Previous studies from this laboratory show that some of toluene's inhibitory effects on glutamatergic transmission are mediated by a CB1 receptor-dependent mechanism (Beckley and Woodward, 2011; Beckley et al., 2016). CB1 receptors are expressed pre-synaptically in the BLA, and activation of CB1R, Gi-coupled signaling can reduce neurotransmitter release (Melis et al., 2004; Hu and Mackie, 2015). Interestingly, alcohol inhibits glutamatergic function in the BLA via a presynaptic CB1 receptor-mediated mechanism (Läck et al., 2007; Robinson et al., 2016). The following studies test the hypothesis that toluene affects mPFC-BLA neurocircuitry via a CB1 receptor dependent mechanism.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (P77-P86 on arrival, Envigo RMS) were housed in pairs in polypropylene cages on a reverse light cycle (lights off at 09:00) in a climate-controlled room with food and water delivered ad libitum. For current clamp experiments, animals remained in their homecage until testing at age P105 - P125.

Surgery

For voltage clamp experiments, animals underwent stereotaxic surgery one week after arrival for viral infusion of channelrhodopsin-2. Deep anesthesia was achieved via an isoflurane vaporizer (Penlon; 1 L/min, 5% induction, 2–3% maintenance) and 300 nl of AAV2-hSyn-ChR2(H134R)-EYFP (AddGene) was injected into the prelimbic portion of the mPFC (AP: ± 2.95; ML: ± 0.6; DV: ± 2.85 mm). Rodents were given 3-7 weeks of recovery to allow for channelrhodopsin-2 expression in mPFC in terminals of the BLA before recording at age P115 - P145.

Preparation of Brain Slices

As previously described (Wayman and Woodward, 2017), brain tissue was rapidly removed and placed in an ice-cold sucrose solution that contained (in mM): sucrose (200), KCl (1.9), NaH_2PO_4 (1.4), $CaCl_2$ (0.5), $MgCl_2$ (6), glucose (10), ascorbic acid (0.4), and $NaHCO_3$ (25); osmolarity 305–315mOsm. This solution was bubbled with 95% O_2/5% CO_2 to maintain physiological pH. Sections

containing the BLA were cut coronally into 300 µm slices using a Leica VT1000 vibrating microtome with a double walled chamber through which cooled solution (2–4°C) circulated (Isotemp 3006, Fisher Scientific). Slices were transferred to a warmed chamber (32–34°C) containing a carbogen-bubbled aCSF solution containing (in mM): NaCl (125), KCl (2.5), NaH_2PO_4 (1.4), $CaCl_2$ (2), $MgCl_2$ (1.3), glucose (10), ascorbic acid (0.4), and $NaHCO_3$ (25); osmolarity 290–300 mOsm for 30 min, and then kept at room temperature in carbogen-bubbled aCSF for at least 45 min before recordings.

Ex vivo electrophysiology

Brain slices were transferred to the recording chamber and perfused with oxygenated aCSF at a flow rate of 1.5 ml/min. The temperature was maintained at and heated 34°C during the course of the recordings with in-line and bath heaters (Warner Instruments). A horizontal pipette puller (P-97 Sutter Instrument) was used to pull recording pipettes constructed from thin-walled borosilicate capillary glass tubing (I.D. 1.0 mm, O.D. 1.50 mm; Sutter Instruments). Pipettes were filled with an internal solution containing (in mM) the following: K-gluconate (120), HEPES (10), KCl (10), $MgCl_2$ (2), Na_2ATP (2), NaGTP (0.3), EGTA (1), a pH 7.3-7.4, osmolarity of 285–295 mOsm, and had resistances ranging from 3 to 4 MΩ. Principal BLA neurons were visually identified using an Axioskop FS2 microscope according to landmarks illustrated in a rat brain atlas (Paxinos and Watson, 2005). Following the formation of a gigaohm seal, light suction was applied to break through the cell membrane and achieve whole-cell access. Neurons with an

access resistance greater than 20 megaohm were not used for analysis. Recorded events were acquired with an Axon MultiClamp 700A (Molecular Devices), filtered at 4 kHz and digitized at a sampling rate of 10 kHz with an Instrutech ITC-18 analog-digital converter (HEKA Instruments) controlled by AxographX software (Axograph Scientific) running on a Macintosh G4 computer (Apple).

Intrinsic Excitability

In order to study intrinsic excitability of BLA neurons, the resting membrane potential of BLA neuron was recorded under current clamp and then adjusted to ~-70 mV for electrophysiological assessments of intrinsic excitability. A current ramp (0 to 500 pA over 1 s) was performed on each cell to determine rheobase. Then, action potentials were elicited using a 1 s pulse of current (rheobase + 50 pA) at 0.1 Hz for 15 minutes (2 min baseline, 8 min treatment, 5 min washout). Internal resistance was calculated by measuring the voltage deflection in response to a 50 ms, 30 pA hyperpolarizing pulse given prior to each current pulse. Traces in which internal resistance deviated more than 25% from baseline were excluded from analysis. Recordings were analyzed offline for the number of spikes and action potential characteristics in response to each current step using AxographX software (Axograph, Sydney, Aus).

Glutamatergic Synaptic Transmission

Using a separate cohort of animals expressing channelrhodopsin-2 in mPFC neurons, voltage clamp experiments were performed to measure the effects

of bath applied toluene on light-evoked synaptic glutamate transmission in BLA neurons. For these experiments, K-gluconate and KCl internal solutions were replaced with CsCl (120 mM). To isolate monosynaptic light-activated AMPA-mediated currents in BLA neurons, the extracellular recording solution also contained 250 nM tetrodotoxin (American Radiolabeled Chemicals, Inc.), 500 uM 4-aminopyridine (Sigma), and 50 uM AP5 (Tocris). In some experiments, 0.75 µM AM 281 (Tocris) was also included in the bath solution to inhibit CB1 receptors. Following breakthrough, EPSCs were induced by photostimulation of channelrhodopsin-expressing mPFC terminals in the BLA via a 470 nm LED (LEDD1B, Thor Labs). Light output from the 40X microscope objective ranged from 1.1 - 2.45 mW and generated EPSCs ranging from 100 - 400 pA. Traces were obtained from a paired pulses of photostimulation (1-5 ms with 150 ms inter pulse interval) and collected at a regular interval of 0.05 Hz for 15 min (2 min baseline, 8 min treatment, 5 min washout). This protocol allowed us to detect toluene-induced changes in EPSC amplitude as well as alterations in paired-pulse plasticity that would implicate changes in presynaptic vesicular release probability (Gioia et al., 2016).

In all experiments, baseline values were collected until responses were stable (~1-5 minutes before recordings began). For toluene treatments, a known volume of HPLC grade toluene (Sigma-Aldrich, Saint Louis, MO) was added to pre-gassed aCSF for a final concentration of 3 or 0.3 mM toluene. Solutions were then immediately perfused into recording bath using Teflon tubing to minimize solvent loss. To control for loss of oxygen in the pre-gassed toluene solution, sham

recordings were conducted where slices were exposed to pre-gassed aCSF without toluene. Previous studies in our laboratory monitored the loss of toluene from experimental recording solutions and found that the concentration of toluene 15 min after dilution was 77.9±15% (mean±SEM) of baseline value obtained at 0 min (Cruz et al, 1998). Following this initial rapid loss because of volatility, toluene concentrations in recording solutions were relatively constant. Concentrations of toluene reported in the results section are not corrected for this loss. Once toluene was applied to a slice, no subsequent cells were recorded from that slice.

All procedures were performed in compliance with Medical University of South Carolina IACUC protocols in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Statistics

Data were analyzed with Prism 8 software (Graphpad Inc., San Diego, CA) using a mixed effects model with treatment and time as factors. Values during and following toluene exposure were compared to baseline with Dunnett's post-hoc test. For secondary measures of intrinsic excitability, averaged responses in the last minute of treatment and washout were compared to baseline (one-way ANOVA, Dunnett's post hoc)

RESULTS

Toluene Increases Intrinsic BLA Neuronal Activity

In order to test the effect of toluene on the intrinsic firing properties of BLA neurons, action potentials (APs) were evoked by direct current injection (rheobase + 50 pA). Figure 5.1A shows representative spike trains and first action potentials during baseline, treatment, and washout of aCSF sham (black, top) or 3.0 mM toluene (red, bottom). Figure 5.1B illustrates that 3.0 mM toluene increased firing (% of baseline) by the end of eight minutes of bath application (mixed effects model, time x treatment interaction, $F_{(89, 1419)} = 2.26$, $p < 0.0001$; main effect of toluene, $F_{(1, 16)} = 5.33$, $p = 0.044$, Dunnett's post hoc, several points within minutes 9 to 11, $q_{(1419)} > 3.32$, $p < 0.047$). This effect reversed soon after washout of the toluene solution (all points from minute 12 to 15, $q_{(1419)} < 3.05$, $p > 0.092$) and was not observed during toluene-free sham recordings (all points, $q_{(1419)} < 2.1$, $p > 0.71$).

Figure 5.1C details the average values of several measures of cell excitability including number of action potentials (i) resting membrane potential (ii), latency to first action potential (iii), AP amplitude (iv), AP rise time (v), AP decay (vi), AP half width (vii), inter-spike interval (vii), and fast afterhyperpolarization potential (ix) during the final minute of baseline, treatment and washout. Toluene (3 mM) increased the total number of action potentials (Fig. 5.1C.i, one-way ANOVA, $F(2,20) = 7.39$, $p = 0.0036$) during treatment (Dunnett's test, treatment vs. baseline, $q_{(20)} = 3.83$, $p = 0.0020$), an effect that normalized following washout (washout vs. baseline, $q_{(20)} = 2.22$, $p = 0.070$). The increased excitability caused

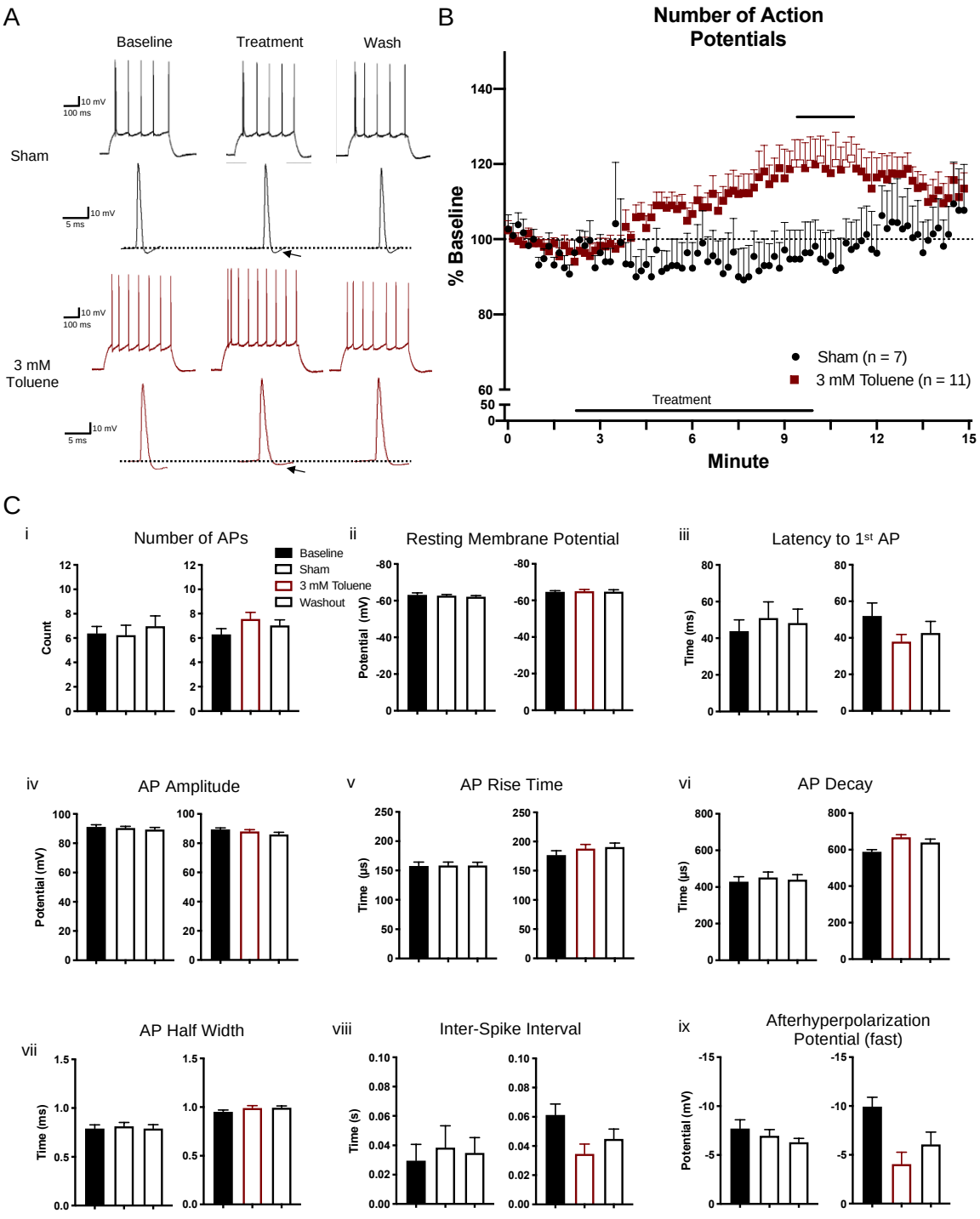


Figure 5.1. Toluene increases intrinsic excitability of BLA principle neurons. A) Representative spike trains evoked by direct current injection in BLA principal neurons under baseline, treatment, and washout of sham aCSF (black, top) or 3 mM toluene (bottom, red). Traces shown below are the first action potential (AP) of each recording aligned at membrane potential inflection point (dotted line) to highlight changes in after-hyperpolarization potential (arrows). **B)** Time course of number of evoked APs during testing (mean + sem,

expressed as percent of baseline, dotted line). Values significantly different from baseline are marked with unfilled data points (mixed effects model, Dunnett's *post hoc,* p <0.05, see *). **C)** Summary of the effects of 3 mM toluene and sham on number of spikes **(i)**, resting membrane potential **(ii)**, latency to first action potential **(iii**), AP amplitude **(iv),** AP rise time **(v),** AP decay **(vi)**, AP half width **(vii),** inter event interval **(vii)**, and fast afterhyperpolarization potential **(ix).** Data are expressed as mean + sem; toluene n = 11 cells / 4 animals, aCSF sham n = 7 cells / 3 animals; one-way ANOVA with Dunnett's post hoc, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

by toluene was reflected in several secondary measures including decreased latency to fire (vs. baseline, Fig. 5.1C.iii, $q_{(20)} = 4.18$, $p = 0.0009$), decreased AP amplitude (vs. baseline, Fig. 5.1C.iv, vs. baseline, $q_{(20)} = 2.39$, $p = 0.049$), decreased inter-event interval (vs. baseline, Fig. 5.1C.viii, vs baseline, $q_{(20)} = 4.45$, $p = 0.005$), and a smaller fast after-hyperpolarization potential (vs. baseline, Fig 5.1C.ix, $q_{(20)} = 6.58$, $p < 0.0001$, see Fig. 1A, arrows for representative traces). However, in the presence of toluene, AP rise time increased (vs. baseline, Fig. 5.1C.v, vs baseline, $q_{(20)} = 3.27$) and AP decay time increased (vs. baseline, Fig. 5.1C.vi, $q_{(20)} = 4.79$, $p = 0.0002$), which could be expected to decrease overall excitability. Sham aCSF treatment did not affect spike number, resting membrane potential, fire latency, AP rise time, AP decay, AP half width, inter event interval, or fast afterhyperpolarization potential. However, a small, yet statistically significant decrease in AP amplitude was detected during washout (vs. baseline, Fig. 5.1C.iv, $q_{(12)} = 3.38$, $p = 0.0040$) and increase in AP decay during aCSF treatment (vs. baseline, Fig. 5.1C.vi, $q_{(12)} = 2.652$, $p = 0.0382$).

Toluene dose-dependently inhibits excitatory mPFC-BLA signaling in a CB1R-dependent manner

Toluene inhalation (Braunscheidel et al., 2019) deficits in probabilistic discounting caused by interrupting mPFC-BLA signaling (St. Onge et al., 2012; Jenni et al., 2017). Recently, it has been shown that this circuit mediates withdrawal-related neurophysiology to the common organic solvent, alcohol (Gioia and McCool, 2017; Gioia et al., 2017). In the present set of experiments, AMPA-mediated EPSCs in the BLA were evoked by a pair of light pulses (blue lines) on channelrhodopsin-2 expressing mPFC terminals (Fig. 5.2A). This protocol allowed us to detect overall changes in EPSC amplitude as well as paired-pulse plasticity changes caused by toluene. Figure 5.2B shows that treatment with 0.3 mM toluene, 3.0 mM toluene, or 3.0 mM toluene with 0.75 µm AM281 did not alter the paired pulse ratio (EPSC2/EPSC1) over the course of 15 min of testing (mixed effects model: time x treatment $F_{(135, 1613)} = 0.92$, $p = 0.74$; main effect of treatment $F_{(3,36)} = 1.11$, $p = 0.34$). However, as shown in Figure 5.2C, treatment with 3.0 mM toluene decreased the peak amplitude of EPSC 1 (mixed effects model: time x treatment $F_{(135, 1609)} = 1.347$, $p = 0.0064$; main effect of treatment, $F_{(3, 36)} = 5.646$, $p = 0.0028$). This effect required several minutes to occur and did not recover following wash out of the toluene solution (Dunnett's post hoc, 3.0 mM toluene timepoints vs baseline beginning minute ten, $q_{(1609)} > 3.11$, $p < 0.049$). Neither a lower concentration of toluene nor sham aCSF treatment had any effect on EPSC amplitude (0.3 mM toluene vs baseline, all $q_{(1609)} < 2.73$, $p > 0.13$; sham aCSF vs. baseline, all $q_{(1609)} < 2.58$, $p > 0.19$).

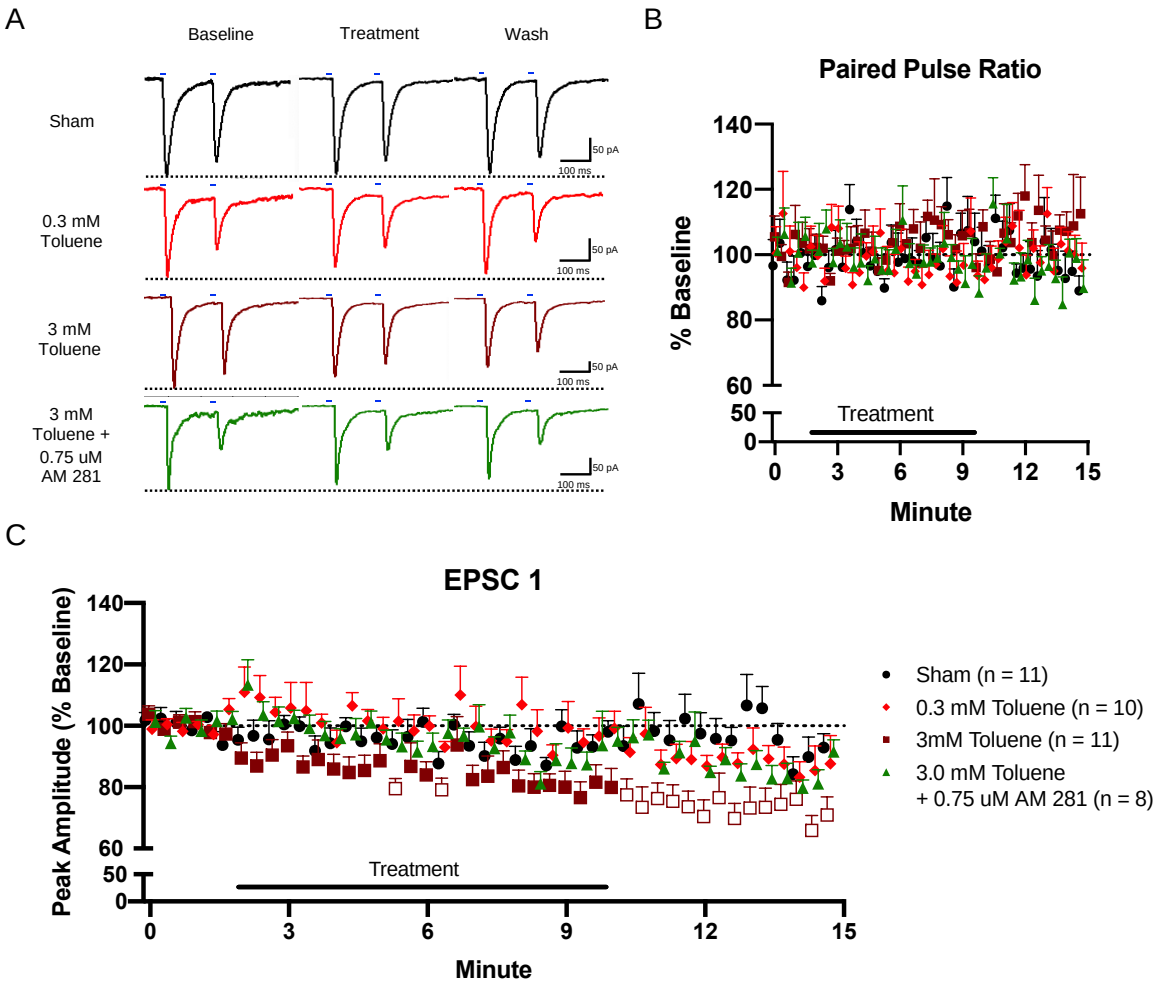


Figure 5.2. Toluene dose-dependently reduces excitatory mPFC-BLA signaling in a CB1R-dependent manner. A) AMPA-mediated EPSCs in BLA principal neurons were evoked by a pair of light pulses (blue lines) applied to channelrhodopsin-2 expressing mPFC terminals. Representative recordings during baseline, treatment and washout of aCSF sham, 0.3 mM toluene, 3.0 mM toluene, and 3.0 mM toluene + 0.75 µM AM 281. Dotted lines mark EPSC 1 amplitude at baseline. **B)** Time course of paired pulse ratio (EPSC 2 / EPSC 1) and **(C)** EPSC 1 peak amplitude during testing. Data are expressed as a percent (mean + sem) of pre-treatment baseline (dotted line); aCSF sham n = 11 cells / 6 animals, 0.3 mM toluene n = 10 cells / 6 animals, 3.0 mM toluene n = 11 / 4 animals, and 3.0 mM toluene + 0.75 µM AM 281 n = 8 cells / 6 animals. Values significantly different from baseline are marked with unfilled data points (mixed effects model, Dunnett's *post hoc,* p <0.05).

However, the inhibition of AMPA EPSCs by 3.0 mM toluene was mitigated by co-application of the CB1R antagonist, AM281 (3.0 mM toluene + 0.75 uM AM281 vs. baseline, all $q_{(1609)} < 2.82$, $p > 0.10$). These findings demonstrate that the toluene-induced reduction in BLA EPSCs evoked from mPFC terminals is both dose- and CB1R-dependent.

DISCUSSION

Intrinsic BLA Excitability

The major finding from this study is that toluene induces opposing effects on excitatory synaptic transmission and intrinsic excitability of principal glutamatergic pyramidal neurons in basolateral amygdala (BLA). The observation that toluene increased the current-evoked firing of BLA neurons is consistent with data in the literature showing that toluene increases c-Fos expression, a proxy for increased neuronal activity, in the BLA (Perit et al., 2012). Several components of the action potential were affected by toluene treatment consistent with increased excitability including decreased latency to fire, decreased inter-spike interval, and a reduced fast after-hyperpolarization potential (AHP). Toluene-mediated increases in neuronal excitability have been reported for some but not all brain regions examined. For instance, toluene increased tonic firing of dopamine neurons within, but not outside of the ventral tegmental area (Riegel and French, 1999c; Riegel et al., 2007). In contrast, nucleus accumbens neurons (Beckley et al., 2016) or deep layer prelimbic mPFC neurons (Beckley and Woodward, 2011) were not affected by acute application of toluene. However, following a single in

vivo exposure to toluene vapor, Wayman et al. (2017) reported that mPFC neurons that project to the nucleus accumbens show sub-region, projection and layer-specific changes in excitability. Core-projecting mPFC neurons in layer 5/6 prelimbic mPFC were hypo-excitable following the toluene exposure while those in layer 2/3 were not affected. In contrast, toluene exposure enhanced firing of core-projecting neurons in both deep and shallow layers of the infralimbic mPFC. Shell projecting neurons in the infralimbic mPFC were hypoexcitable following toluene treatment with no effect seen in shell projecting neurons from layer 2/3 infralimbic mPFC or those from the prelimbic mPFC. Given this surprising degree of selectively, future studies should identify whether there are projection specific changes in BLA neuron excitability following in vitro or in vivo exposure to toluene.

The underlying cause of the increased excitability caused by toluene is not yet known. An interesting possibility is that toluene, via its direct inhibition of large conductance calcium-activated potassium (BK) channels (Del Re et al., 2006) reduced the neuron's relative refractory period. This explanation is consistent with observed decrease in the BK-dependent fast afterhyperpolarization potential (AHP) (Sah and Faber, 2002) and decreased inter-spike interval caused by toluene. Beckley and colleagues also found that toluene dampens the fast component of the AHP in medium spiny neurons in the nucleus accumbens (Beckley et al., 2016), but had no effect on the AHP in mPFC neurons (Beckley and Woodward, 2011). Together with the findings of the present study, these results show that toluene's effects on BK-mediated components of neuronal

excitability are region specific possibly due to differential expression of other BK channel subtypes that may contribute to toluene sensitivity.

A reduction in AHP and inter-spike interval is a likely explanation for driving the observed toluene-induced increases in excitability. However, toluene-mediated reductions in AHP have been identified in the absence of changes in excitability (Beckley et al., 2016). Further, this mechanism does not appear to be responsible for the toluene-induced hyperexcitability of nucleus accumbens core-projecting prelimbic neurons (Wayman and Woodward, 2018), although these studies measured excitability 24 h following toluene inhalation. Homeostatic upregulation of BK channels following the *in vivo* toluene treatment used by Wayman & Woodward could explain the differences between those results and the observations in the current study. More experimentation is required to better clarify the mechanism driving toluene-induced neuronal hyperexcitability in the BLA.

Not all of the observed effects of toluene would be expected to increase excitability. For instance, toluene increased action potential rise time and decay. These effects could be due to toluene's direct inhibition of voltage gated sodium channels (Gauthereau et al., 2005), that might also help explain the decreases in action potential amplitude. Future experimentation is required to address these hypotheses for toluene-induced changes in action potential characteristics recorded from BLA neurons.

Synaptic mPFC-BLA Signaling

Despite its effects on intrinsic activity, toluene is generally regarded as a central nervous system depressant (Bowen et al., 2006) via actions on voltage-gated sodium channels (Gauthereau et al., 2005) NMDA (Cruz et al., 1998, 2000), nicotinic acetylcholine receptors (Bale et al., 2002), $GABA_A$, and glycine receptors (Beckstead et al., 2000, 2001). In line with this notion, we found that 3 mM toluene inhibits AMPA-mediated excitatory post-synaptic currents (EPSCs) in the BLA. Given in vitro volatility losses of 20-25% and that 3% of inhaled toluene reaches the brain (Benignus et al., 1981), this dose equates roughly to 7400 ppm, a concentration similar to that encountered by humans during voluntary solvent inhalation (Brouette and Anton, 2001; Bukowski, 2001). Similar dose-dependent effects of toluene on synaptic neurotransmission have been reported in other addiction-related neurocircuitry (Beckley and Woodward, 2011; Beckley et al., 2013, 2016). Dysregulation of amygdala function is symptomatic of addiction to the most common organic solvent, alcohol (Menzaghi et al., 1994; Marinelli et al., 2010; Diaz et al., 2011; Keistler et al., 2017; Chesworth and Corbit, 2018) and could likely play a crucial role in mediating toluene abuse.

Toluene's inhibitory effect on AMPA signaling progressed slowly over the course of treatment and persisted during washout. This finding agrees with the lack of a direct effect of toluene on recombinant AMPA receptors (Cruz et al., 1998) and suggests a secondary mechanism of action. One possibility is that toluene stimulates endocannabinoid signaling that then inhibits the release of glutamate by binding and activating CB1 receptors on presynaptic glutamate terminals (Melis et al., 2004; Perez-Rosello et al., 2013). In support of this hypothesis, we found

that bath application of the CB1 receptor inverse agonist AM281 blocked the toluene-induced reduction in BLA AMPA EPSCs. This finding is in agreement with results from previous studies from our laboratory showing that toluene-induced decreases in AMPA EPSCs in the nucleus accumbens (Beckley et al., 2016) and mPFC (Beckley and Woodward, 2011) were CB1 receptor-dependent. Beckley and colleagues further defined this mechanism by showing that toluene inhibition of AMPA EPSCs was dependent on release of calcium from intracellular stores, that are necessary for synthesis of the endogenous endocannabinoids.

The present study is the first to show that toluene impairs synaptic signaling in the mPFC-BLA neural circuit, a pathway implicated in the susceptibility to alcohol-seeking behaviors in mice (Gioia et al., 2016, 2017; Gioia and McCool, 2017). The impaired mPFC-BLA signaling observed in the current studies is also interesting in the context of recent work from our lab investigating risk/reward decision making following toluene intoxication (Braunscheidel et al., 2019). Importantly, in that study, behavioral testing occurred thirty minutes following toluene exposure when toluene should have been mostly eliminated from the brain (Gerasimov et al., 2002). The toluene-induced deficits in behavioral flexibility also mimicked those following pharmacological inactivation of the mPFC-BLA pathway (St Onge et al., 2012; Jenni et al., 2017). The persistent nature of the CB1 receptor-mediated suppression of mPFC-BLA excitatory signaling observed in the present study may be a critical factor that underlies the reduction in behavioral flexibility in toluene intoxicated animals. Future studies could examine this idea by

testing whether restoring mPFC-BLA activity using opto-or chemo-genetic approaches can reverse toluene's effects on behavioral flexibility.

The presynaptic mechanism of action proposed here for toluene should impair vesicular release probability as detected by changes in the paired pulse ratio. The paired pulse ratio of BLA AMPA EPSCs was not, however, affected by toluene exposure. While these results could speak to different mechanisms of action between alcohol (Läck et al., 2007) and toluene, it is also possible that the inter-pulse interval used in this study (150 ms) was too large to detect any significant changes in release probability. Läck and colleagues showed that the effects of alcohol on BLA synaptic transmission were most noticeable using inter-pulse intervals of 25 or 50 ms as only modest effects were observed at the 250 ms inter-pulse intervals. It should also be noted that those experiments were conducted following several days of alcohol treatment and not a single in vitro exposure as used in the present study. Additional experiments are thus required to explore a presynaptic mechanism for the toluene-mediated reduction in AMPA EPSCs.

CONCLUSION

To our knowledge, these studies are the first to investigate the effect of inhalants on BLA neurophysiology. Using whole-cell patch clamp electrophysiology, we found that a concentration of toluene that is associated with voluntary solvent abuse transiently increased the excitability of BLA pyramidal neurons. The increase in firing was accompanied by a significant reduction in the

fast AHP potential and decreased inter-spike interval, factors that depend on a toluene-sensitive BK channel. Optical stimulation of mPFC terminals in the BLA revealed that toluene induced a slow-onset, sustained, and CB1R-dependent decrease in AMPA-mediated excitatory signaling. This mechanism may contribute to deficits in executive function that are observed following toluene intoxication in vivo (Braunscheidel et al., 2019).

CHAPTER 6: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Adolescent Inhalant Use: Persistent Cognitive And Morphological Effects

We found that repeated exposure to addictive concentrations of toluene vapor in adolescent rats had lasting effects on specific executive functions during adulthood. Namely, toluene-treated animals took longer to acquire operant- and classical-conditioning to an appetitive reward (Braunscheidel et al., 2017). This effect only pertained to the initial acquisition of these simple behaviors and largely did not extend to more cognitively demanding tasks (reversal learning, within-session strategy-shifting, probabilistic discounting). Additionally, these deficits could not be explained by impaired motivation or latent-inhibition. Two persistent effects of toluene on cognition were observed. Toluene-treated animals extinguished responding to a previously unrewarded cue and changed strategies (when tested between-session) more rapidly than air-treated controls. Decreased perseveration on unrewarded pursuits during complex tasks is a common thread for both of these results and should be considered a potentially persistent phenotype following toluene use during adolescence. Taken together, these results suggest that full cognitive and behavioral recovery in human inhalant abusers is promising, given enough skill training and drug abstinence.

Dendritic spine alterations were not evident in the medial prefrontal cortex following seven weeks of drug abstinence following toluene vapor exposure (Braunscheidel et al., 2017). This result is perhaps not surprising given the modest effect of toluene use during adolescence on the mPFC-dependent behavioral tasks

in adulthood. However, we did observe an increase in immature dendritic spines in the nucleus accumbens of toluene-treated rats. Future studies should explore the cellular and molecular changes driving this immature-like accumbens spine morphology. Special focus should be given to a D2-mediated mechanism given toluene's specificity for D2 medium spiny neurons in the accumbens (Beckley et al., 2016), the high percentage of post-synaptic densities of striatal neurons that contain D2 receptors (Hersch et al., 1995), and the finding that D2 receptors interact with several important post-synaptic scaffolding proteins including actin binding protein filamin A, protein 4.1N and spinophilin (Smith et al., 1999; Li et al., 2000; Binda et al., 2002). Modulation of D2 signaling pathways by toluene could potentially destabilize the cytoarchitecture, leading to the increase in synaptically-immature long-thin spines observed in the present study.

Adult Inhalant Use: Acute Effects On Behavioral Flexibility And Underlying Neurophysiology

The studies herein describe the effects of risk/reward decision making immediately following toluene intoxication. We found that toluene treatment does not affect risk/reward decision making, per se, but rather impairs behavioral flexibility and utilization of recent negative reinforcement on upcoming choice (Braunscheidel et al., 2019). This effect was dose-dependent, sex-independent, and strongly indicative of mPFC dysfunction. Using fiber photometry to monitor mPFC activity during discounting, we found that toluene treatment specifically disrupted activity during the contingency updating portions of the task. Toluene

also reduced the mPFC's ability to distinguish between small and large rewards. We then explored two mechanisms for this effect: impaired mPFC-BLA signaling and increased endocannabinoid signaling.

The toluene-induced deficits in behavioral flexibility mimicked those following pharmacological inactivation of the mPFC-BLA pathway (St Onge et al., 2012; Jenni et al., 2017). In this dissertation, we stimulated mPFC terminals in the BLA and discovered a CB1R-dependent decrease in AMPA-mediated EPSCs during exposure to toluene. This effect developed over several minutes of bath-applied toluene and persisted following aCSF washout. If present in vivo, this time course would help explain the underlying behavioral flexibility deficits detailed above since brain toluene concentrations at the start of behavioral testing (30 minutes following toluene exposure) are likely negligible (Gerasimov et al., 2002). Future studies could examine this idea by testing whether restoring mPFC-BLA activity using opto- or chemo-genetic approaches can reverse toluene's effects on behavioral flexibility.

Some of the inhibitory effects of toluene on neuronal transmission are mediated by activation of CB1R signaling (Beckley and Woodward, 2011; Beckley et al., 2016). Here we found that systemic administration of a CB1R inverse agonist did not mitigate deficits caused by toluene inhalation, but did independently reduce win-stay behavior, increase choice latency, and increase omissions. We next provided evidence that mPFC CB1R inverse agonism did not protect against toluene-induced behavioral inflexibility. This treatment did, however, cause a dose-dependent reduction in win-stay behavior. These studies indicate that toluene-

induced deficits cannot be prevented by systemic or mPFC modulation of CB1Rs, but they do not rule out a potential involvement of CB1Rs in mediating toluene's behavioral effects. For instance, and in light of the CB1R-dependent decrease in BLA neurons innervated by the mPFC discovered in this dissertation, future studies should test if microinjecting CB1R inverse agonists into the BLA mitigates toluene-induced behavioral flexibility. To gain circuit specificity, follow-up studies could use CRISPR-Cas9 gene editing technology to knock out mPFC CB1R expression and test for toluene sensitivity during probabilistic discounting. A second approach could be to selectively delete CB1Rs from glutamatergic terminals in floxed CB1R mice, although this would likely require several control experiments since it uses a different rodent model.

While enhancing CB1R signaling disrupts higher cognitive functions in humans including risky decision making (Lane and Cherek, 2002; Lane et al., 2005; Anderson et al., 2010), these effects have not been reproduced in two separate preclinical models (Ferland et al., 2018; Freels et al., 2020). Our data suggest that mPFC CB1Rs are important for mediating normal behavioral responses to recent positive reinforcement and that systemic CB1R function is necessary for normal decision speeds and task engagement during complex decision making. However, CB1R manipulation did not alter overall risk preference in the probabilistic discounting task. Thus, preclinical modeling of the human condition with regards to risky decision making and the endocannabinoid system remains elusive.

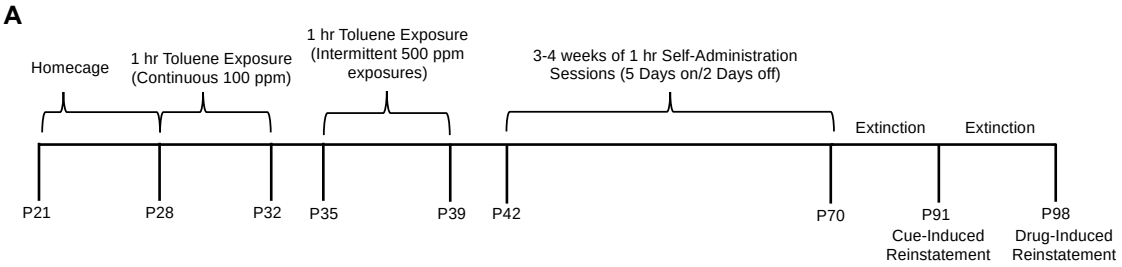
Future Directions: Toluene Self-Administration

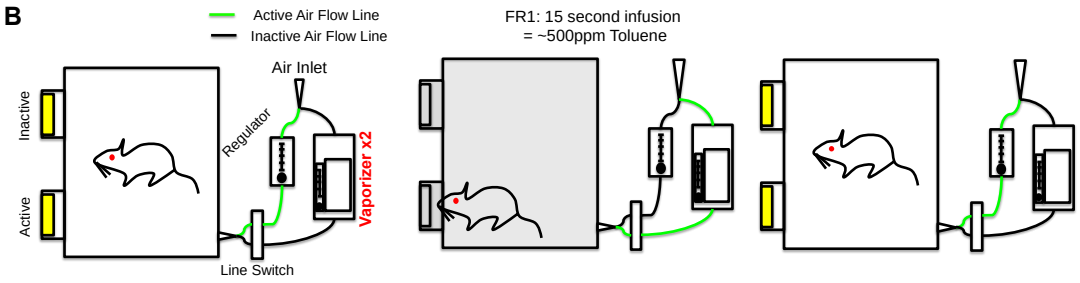
The gold-standard for modelling addiction in the preclinical setting is operant-based self-administration; for review, see (Sanchis-Segura and Spanagel, 2006). In this paradigm, rodents or non-human primates self-administer a drug (orally or intravenously via a chronically implanted catheter) for multiple hours per day over the course of several weeks. This is followed by various drug-cue extinction, reinstatement or relapse trials. The development of a rodent model of inhalant self-administration has lagged behind other drugs of abuse presumably due to the difficulty in controlling inhalant concentrations and overcoming initial aversive effects associated with solvent odor. In the first of two published attempts to model toluene abuse, Weiss and colleagues (1979) trained four squirrel monkeys outfitted with a custom inhalation helmet to lever-press for a toluene vapor. This approach required a significant investment of time and economic resources and has not been repeated. The second study involved a single, 30 min intravenous administration of aqueous toluene in mice (Blokhina et al., 2004). This approach is problematic for studying addiction for several reasons: 1) it does not mimic the inhaled route of administration in humans, 2) as a solvent, toluene can induce significant vein damage (Kulkarni et al., 2015), and 3) it prevents the study of critical aspects of addiction that require multiple administration sessions. These include measures such as acquisition, drug use escalation, withdrawal, extinction, drug-seeking, drug-craving, and relapse among others.

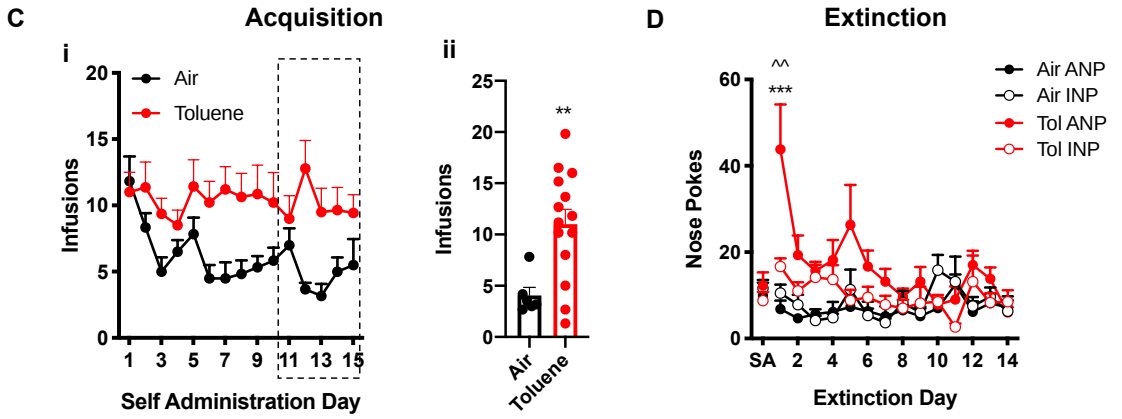
The majority of preclinical studies on toluene used passive exposure paradigms due to the technical difficulties involved in inhalant self-administration

(namely, precise control of the delivery and elimination of a vaporized substance). However, there are several critical advantages to using an inhalation-based model for toluene self-administration: 1) self-administration of toluene vapor has strong face-validity, as inhalation is the preferred route of administration in humans 2) drug intake is voluntary and thus, likely involves critical reward pathways that may be unaffected by noncontingent drug administration (Fernàndez-Castillo et al., 2012; Lominac et al., 2012). 3) longitudinal studies with repeated testing can be conducted over the course of the rodent's lifetime 4) there are no concerns about catheter patency, that is especially important since toluene can cause vein damage (Kulkarni et al., 2015) and 5) there is no need for solubilizing agents (e.g. corn oil), commonly used when administering toluene intraperitoneally (Riegel et al., 2004; Lo et al., 2009; Lin et al., 2010; Chan et al., 2012; Wu et al., 2018).

As an ongoing effort during this dissertation I have helped develop a novel rodent model of self-administration of toluene vapor. Figure 6.1A details the behavioral timeline of this procedure, which includes weeks of habituation to the testing chamber and toluene vapor odor. Operant self-administration sessions in the following weeks are conducted on an FR1 schedule, where a single nose poke into the active port triggers an infusion of toluene vapor paired with conditioned cue for 15 seconds (Fig 6.1B). Promising preliminary data include increased sustained operant responding for toluene vapor, a stereotypical spike in responding on the first day of extinction, as well as cue-induced reinstatement (Fig. 6.1C-E).


A
Homecage
1 hr Toluene Exposure (Continuous 100 ppm)
1 hr Toluene Exposure (Intermittent 500 ppm exposures)
3-4 weeks of 1 hr Self-Administration Sessions (5 Days on/2 Days off)
Extinction
Extinction
P21
P28
P32
P35
P39
P42
P70
P91
Cue-Induced Reinstatement
P98
Drug-Induced Reinstatement


B
Active Air Flow Line
Inactive Air Flow Line
FR1: 15 second infusion = ~500ppm Toluene
Air Inlet
Inactive
Active
Regulator
Vaporizer x2
Line Switch


C
Acquisition
i
Air
Toluene
Infusions
Self Administration Day
ii
Infusions
Air
Toluene
D
Extinction
Air ANP
Air INP
Tol ANP
Tol INP
Nose Pokes
Extinction Day

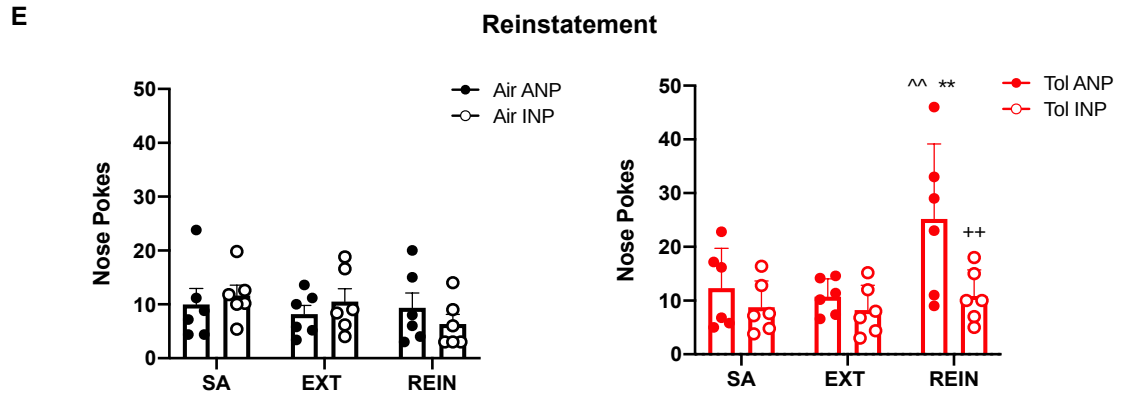

E
Reinstatement
Air ANP
Air INP
Nose Pokes
SA
EXT
REIN
Tol ANP
Tol INP

Figure 6.1 Toluene vapor self-administration. (A) Timeline for acquisition, extinction, and reinstatement of operant responding for toluene vapor. (B) Illustration of the vapor SA apparatus. Active nose pokes triggered a 15 s exposure of air or toluene (Tol) vapor while inactive nose pokes had no consequence. Additional responses during the 15 s drug delivery had no additional consequence. For Air SA and extinction sessions, the toluene vaporizers were replaced with a second air regulator. (C.i) Number of daily infusions with the final week (dotted box) averaged (C.ii); student's t-test **p < 0.05. (D) Number of active and inactive nose pokes rats during the last week of SA (averaged) and extinction training. Tol SA vs Toluene EXT ANP ^^p < 0.01; EXT day 1 Tol ANP vs Tol INP ***p<0.001. (E) Cue-induced reinstatement of responding in rats fully extinguished from toluene SA behavior. Tol ANP comparing REIN to EXT and ^^p<0.01 to SA; ++p<0.01 Tol ANP vs INP paired comparison during REIN. Data shown are daily mean + SEM during reinstatement (REIN), last week average of self-administration (SA) or extinction (EXT); active nose poke (ANP), inactive nose poke (INP); air n = 6, toluene n = 14 (6 during EXT).

If this protocol provides reproducible data, the investigative potential is virtually endless. One promising direction is to use this technique in combination with probabilistic discounting to test if individual differences in risk/reward decision making preclude inhalant abuse susceptibility. Given the effect of toluene on mPFC-BLA described in this dissertation, involvement in C57BL/6J mice's susceptibility to alcohol-taking behaviors (Gioia et al., 2016, 2017; Gioia and McCool, 2017) future studies should monitor this pathway in the context to toluene self-administration. Since the mPFC-BLA does not regulate extinction or reinstatement of alcohol seeking (Keistler et al., 2017), future studies should focus on mPFC-BLA activity during acquisition of toluene intake and test whether manipulation of this circuitry can prevent this initial drug seeking behavior.

Another promising direction follows previous studies from our laboratory that demonstrating that toluene vapor reduces the intrinsic excitability of nucleus

accumbens shell (NAcs) projecting infralimbic (IL) neurons in a layer-dependent fashion (Wayman and Woodward, 2017) and that chemogenetic activation of the IL-NAcs pathway impairs the expression of toluene-induced conditioned place preference (Wayman and Woodward, 2018). These neurons may also be involved in the reduction in cue-induced reinstatement of alcohol seeking behaviors (Keistler et al., 2017). Assessing whether toluene self-administration produces similar effects on the excitability of NAc projecting mPFC neurons and if manipulating this circuit disrupts cue-induced reinstatement to toluene are exciting and important future lines of questioning.

CONCLUDING REMARKS

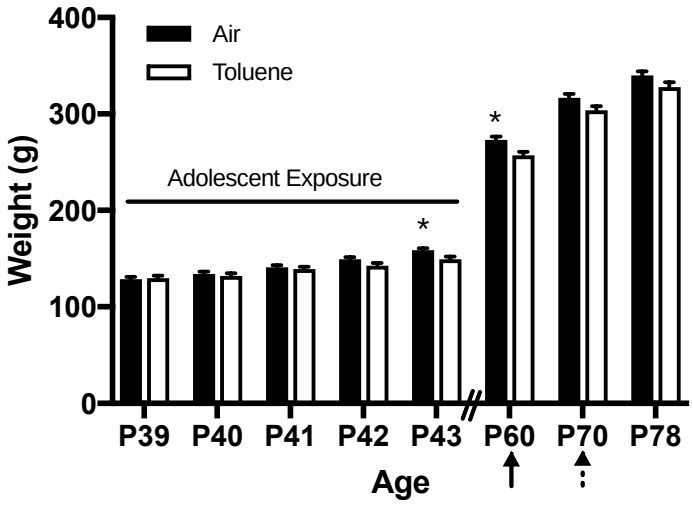
Impaired executive control over behaviors is a hallmark of substance use disorders. Toluene, a commonly-abused yet understudied inhalant, has specific pharmacological actions on major neurotransmitter systems and uniquely alters addiction neurocircuitry. The studies herein add to this growing literature by describing the acute and persistent effects of toluene on complex cognitive behaviors and their underlying neurobiology.

SUPPLEMENTAL TABLES & FIGURES

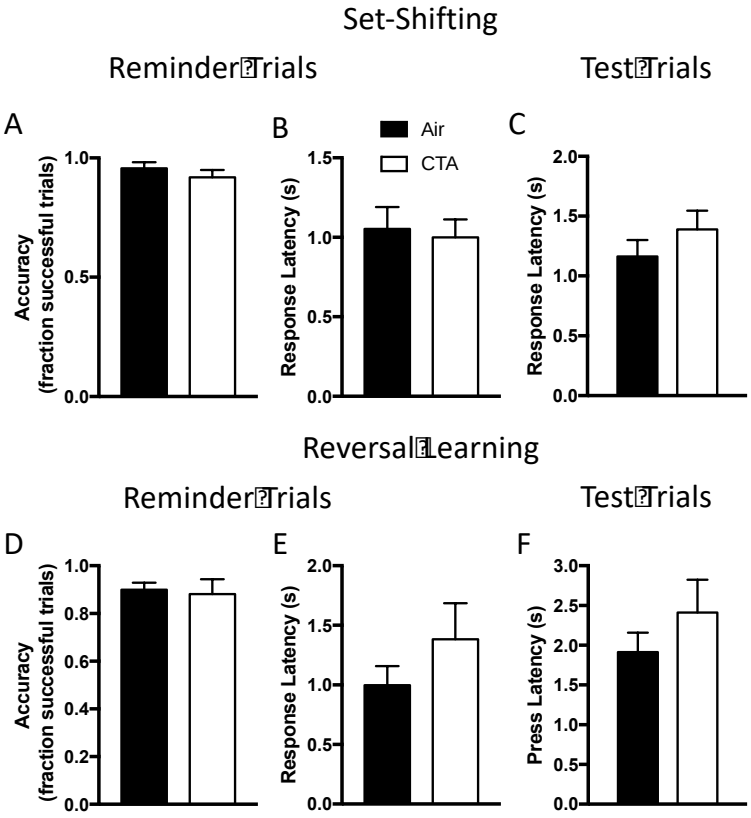
Supplemental Table 2.1. Average number of days to criteria for each training phase (Standard error in parentheses).

Behavioral		Lever Press training				Testing	
Group	Exposure	Phase 1	Phase 2	Phase 3	Visual Cue	Set Shift	Reversal
A	CTA	5.3 (1.0)	2 (0)	2.5 (0.33)	2 (0)	3.3 (0.46)	3.3 (0.18)
B	CTA	4.7 (0.53)	2.1 (0.11)	3.6 (0.75)	2.4 (0.18)	4.3 (1.3)	3.7 (0.18)
A	Air	2.9 (0.40)	2.1 (0.13)	3.9 (0.97)	2.4 (0.18)	3.3 (0.71)	3.6 (0.32)
B	Air	4.1 (0.35)	2 (0)	3 (0.44)	2.4 (0.24)	3.8 (0.78)	3.3 (0.23)

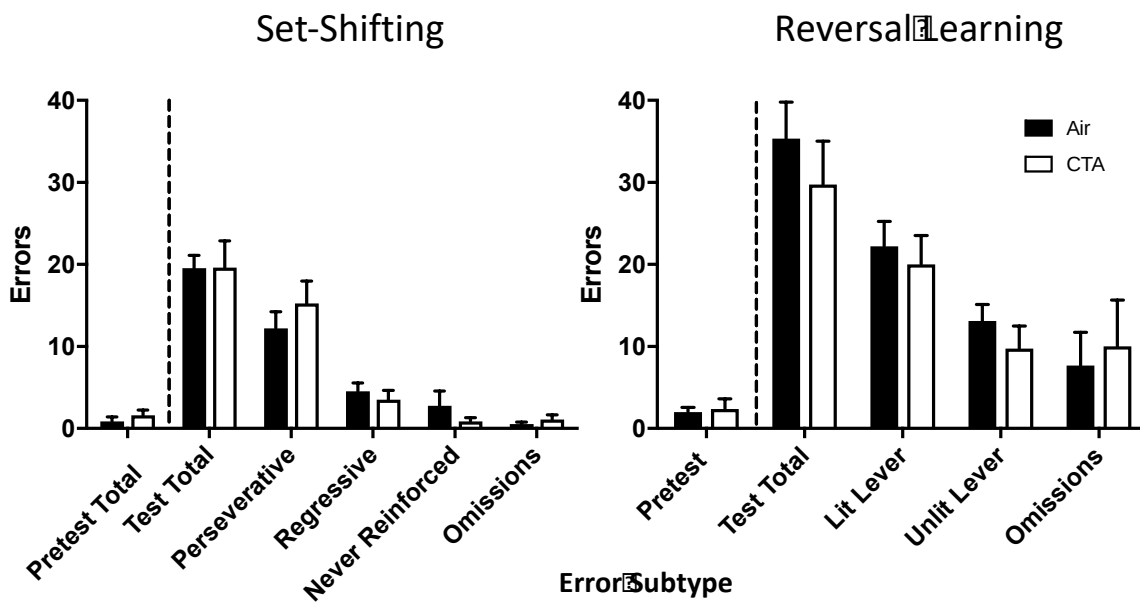
No significant differences between group A and B for either CTA or Air rats. Student's T test with Sidak's post hoc for multiple comparisons. All $t < 1.73$, $p > 0.176$; Group A: air n=8, CTA n=8; Group B air n=9, CTA n=9



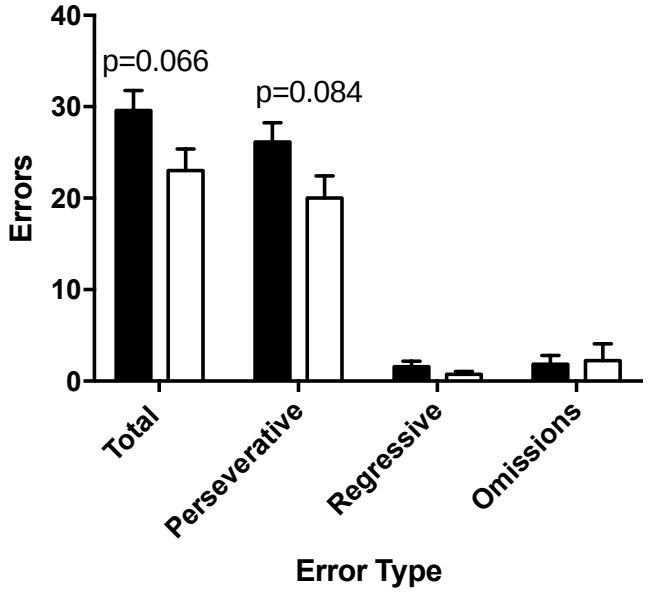
Supplemental Figure 2.1. The effect of binge-like exposure to toluene during adolescence on weight gain. The body weight of toluene exposed rats was significantly less than that of air exposed controls following the fifth day of treatment (P43). This difference persisted until the first day of lever press training (P60, solid arrow). Weights were not significantly different during behavioral flexibility (youngest tested animal was P70, dotted arrow). Data shown are mean +SEM; *p<0.05 student's t test corrected for multiple comparisons with Holm – Sidak method; air n=17, CTA n=18.



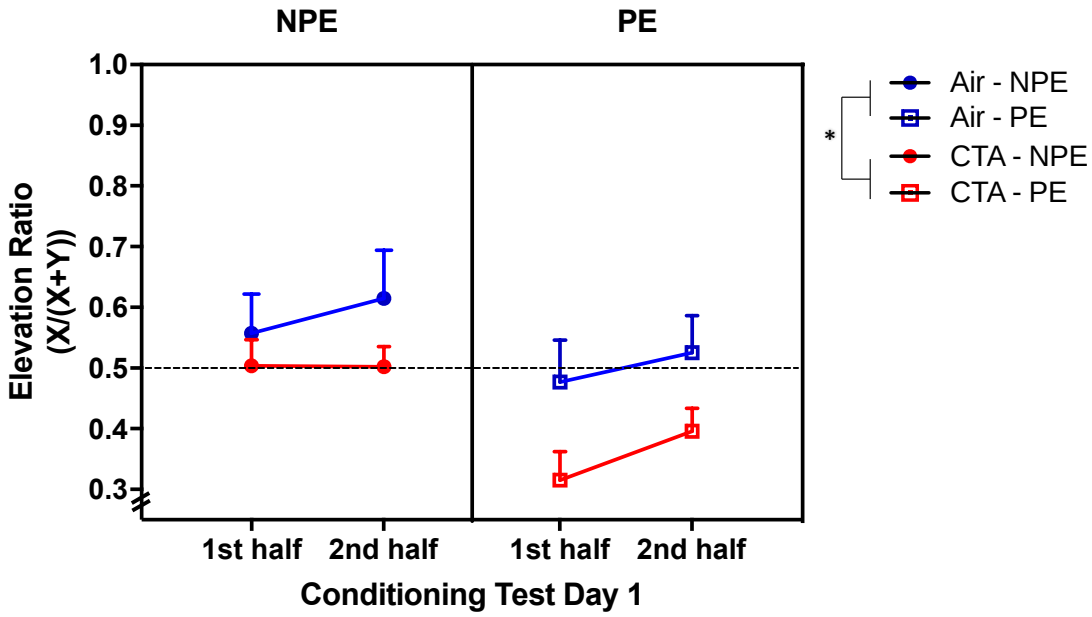
Supplemental Figure 2.2. Response latency and reminder trial performance in within-session testing of behavioral flexibility. There were no differences in accuracy between toluene-exposed and air-treated rats during the reminder trails preceding either within-session behavioral flexibility task. There were no differences in response latency between groups during reminder trials or test trials in either test of behavioral flexibility. Data shown are mean ± SEM; air n=9, CTA n=9.



Supplemental Figure 2.3. Error Subtypes during Within-Session Testing. There were no differences in the number of errors classified by subtype between groups during set-shifting or reversal learning (all *p's* > 0.05) Data shown are mean ± SEM; air n=9, CTA n=9.



Supplementary Figure 2.4. Error Subtypes during Between-Session Set-Shift Task. Data shown are mean ± SEM; air n=8, CTA n=8.



Supplemental Figure 2.5. Task Performance On Day 1 Of Classical Conditioning**.** CTA blunts acquisition of classically conditioned approach behavior without altering latent inhibition within the first day of acquisitioning. CTA and Air treated animals were either cue-naïve (NPE) or pre-exposed (PE) to cue during the training phase. Conditioning was measured as the elevation ratio defined as X/(X+Y) where X is the number of food well entries during the first 30 s of cue and Y is the number of entries 30 s prior to cue onset. CTA treated rats had lower elevation ratios during the first day of testing compared to air treated controls, but there were no drug x cue interactions. Data shown are mean + SEM; random well entry (dotted line); main effect of drug *p<0.05; all n=8.

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