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Developing an Animal Model of Polysubstance Abuse in Adolescence: The Role of NMDA Receptors in Alcohol/Cocaine Reward

Adriana Rebecca Uruena-Agnes
University of South Florida, uruena@mail.usf.edu

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The Role of NMDA receptors in Alcohol/Cocaine Reward

by

Adriana R. Uruena-Agnes

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University of South Florida

Major Professor: Cheryl L. Kirstein, Ph.D.
Toru Shimizu, Ph.D.
Mark Goldman, Ph.D.
Kristen Salomon, Ph.D.
Anne L. Prieto, Ph.D.

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DEDICATION

I dedicate this dissertation to my loving and supportive family, friends, committee members and to my major professor, Dr. Cheryl L. Kirstein. Thank you for the time you invested, directly assisting me with portions of my project. Who else would feel proud to format references (mom), become certified to work in the lab (my husband, Dominick) watch behavioral videos (mom and my husband), make ID cards (Natalya, Kyra and Tristan), clean CPP boxes and floors (my husband) and give me the most amazing professional and personal advice through out this entire process (Kim)? Who else would escort me to lab every weekend and stay for 12-16 hours from 2010 to 2014 (my husband), or keep my babies happy throughout the entire PhD process (dad and mom)? Thank you to my aunts and cousins who were always there to pick up my babies from school because I was running late in the lab.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Repeated exposure to drugs of abuse conditions individuals to anticipate the behavioral consequences of drug use specifically in the presence of a drug-associated context. In rodents, preferences and aversions for alcohol and cocaine have been conditioned; however, the mechanisms underlying the expression of these conditioned effects remain unknown. Given that alcohol and cocaine polysubstance abuse is prevalent in young individuals, with more than 50% of these polysubstance abusers reporting to be under the age of 21, it is important to understand the mechanisms contributing to the behavioral effects of alcohol and cocaine co-dependency. Aim 1 determined if age differentially impacted the effects of repeated alcohol exposure on conditioned cocaine preferences. Adolescent [postnatal day (PND) 30) and adult (PND 60) male Sprague-Dawley rats were administered ethanol (0.5 or 1.75 g/kg, i.p.) immediately before each cocaine conditioned place preference (CPP) session (20 mg/kg, i.p.; 15 minutes). Aim 2, Experiments 1 and 2, identified the role of NMDA receptors within the nucleus accumbens septi (NAcc) in conditioned ethanol/cocaine behavior. Adolescent and adult rats in Experiment 1 were administered the NMDA antagonist MK-801 (0.1 or 0.2 m/kg, i.p.) 30 minutes prior to cocaine conditioning. Adolescent and adult rats within Experiment 2 underwent bilateral cannulation for chronic implantation of the cannulae into the NAcc of both hemispheres. Rats administered 1mM MK-801 or saline into the NAcc prior to cocaine (20.0 mg/kg, i.p.) conditioning, completed additional testing to determine the role of NAcc NMDA receptors in the consolidation, reconsolidation and expression of cocaine conditioned behavior in a drug-induced

reactivation manner. Findings show adolescent and adult rats responded similarly to co-administration of ethanol/cocaine with both ages showing a decrease in the rewarding properties of cocaine. What differed between the age groups were the aversive properties of ethanol, with adolescents being less sensitive to the aversive properties of ethanol and its modulating effects on cocaine reward. A role for the NAcc NMDA receptors was observed in contributing to the modulating effects of ethanol on cocaine reward. Lastly, the reconsolidation of cocaine reward was more sensitive to disruption in adolescent rats, as compared to their adult counterparts. These results suggest an increased vulnerability for adolescents to continue engaging in polysubstance abuse. However, this at-risk age group also appeared to be more responsive to pharmacological treatment in decreasing addictive behavior.

CHAPTER ONE:

ETHANOL AND COCAINE POLYSUBSTANCE ABUSE

Introduction

Drug abuse is a major societal issue for juveniles and is costly to our nation. Healthcare related costs attributed to alcohol and cocaine has exceeded $40 billion annually (Rehm et al., 2009). National estimates in 2011 report 2.5 million drug-related emergency room admissions (Drug Abuse Warning Network: DAWN, 2011). In adults, lifetime prevalence of alcohol and illicit drug use is increasing at an alarming rate. Sixty-nine percent of American 8th, 10th and 12th graders report drinking alcohol at least once in their lifetime (Monitoring the Future, 2012) and half of hospital admissions in this age cohort involve underage drinking (DAWN, 2011). According to the 2012 National Survey on Drug Use and Health (NSDUH), more than 22 million Americans (approximately 14.7% of the United States population) ages 12 and older, report having used an illicit drug at least once within the past year. Of these, nearly 5 % of 8th, 10th and 12th graders report using cocaine at least once in their lifetime (Monitoring the Future, 2012).

Using alcohol and illicit drugs before the age of 18 predicts the likelihood of developing future habitual consumption patterns and lifetime prevalence of substance use disorders (Merikangas et al., 1998; Patton et al., 2007; McCambridge et al., 2011). Adolescent and young adults that consume alcohol weekly often engage in simultaneous illicit drug use, also referred to as polysubstance abuse (Bailey, 1992). Alcohol and cocaine polysubstance abuse is defined as the concomitant use of alcohol and cocaine in close temporal proximity. Individuals engaging in

simultaneous oral alcohol consumption and intranasal cocaine use report a potentiated and increased duration of drug-induced euphoria, as well as an overall sense of well-being (McCance-Katz et al., 1993 and 1998). In addition, alcohol consumption increases with simultaneous cocaine use (Gossop et al., 2006; Barrett et al., 2006). Such patterns of consumption increase the risk of cardiotoxicity, hepatoxicity and lethality, contributing to the prevalence of polysubstance-related hospitalizations (Boyer and Petersen, 1992; Jatlow et al., 1995; McCance-Katz et al., 1998). Increased risk for major health concerns are observed in recent surveys reporting 54% of hospital admissions in people under the age of 20 are due to polysubstance abuse involving alcohol (DAWN, 2012).

Alcohol and cocaine polysubstance use is reported in college students (Barrett et al., 2006). An increase in alcohol consumption is reported when alcohol and cocaine are simultaneously used (Gossop et al., 2006; Barrett et al., 2006). Patterns of consumption are likely secondary to the potentiation and increased duration of drug-induced euphoria, as well as an overall sense of well-being commonly reported in users (McCance-Katz et al., 1993 and 1998).

Individuals engaging in polysubstance use demonstrate an increased risk in developing substance use disorders (Merikangas et al., 1998) and are recognized as one of the largest substance abuse populations served in treatment facilities (Kedia et al., 2007). National concern for drug abuse in our youth as well as polysubstance abuse is increasing, as it is manifested in nation-wide prevention efforts, policies and reform. Advancements in clinical and experimental research aim to elucidate the risk factors and biological mechanisms associated with vulnerability to developing and maintaining addictive behaviors. Despite current efforts, limited research, investigating the mechanisms underlying alcohol/cocaine polysubstance reward is

available. Behavioral animal models of drug abuse and physiological studies suggest ethanol (i.e. alcohol) modulates cocaine reward in a dose-dependent manner, at neuroanatomical structures implicated in drug reward (Lewis and June, 1994; Bunney et al., 2001; Busse and Riley, 2002; Busse et al., 2004). However, it remains unknown whether an interaction or synergistic effect occur at these relevant sites when the two drugs are taken in combination. In addition, the mechanism(s) implicated in alcohol/cocaine reward are unknown. Therefore, interest has begun to focus on the identification of brain structures, neural pathways and cellular mechanisms underlying reinforced habitual alcohol/cocaine polysubstance.

Functional Neuroanatomy: The Mesocorticolimbic Circuit

Animal models are useful in identifying primary neuroanatomical sites and the mechanisms underlying drug reward. Alcohol (i.e. ethanol) and cocaine target multiple sites and different molecular structures to elicit their actions. However, one commonality among all addictive drugs is an increase in dopamine neurotransmission within the mesolimbic and mesocortical pathways, collectively termed the mesocorticolimbic circuit (Grace, 1995; Tzschentske, 2000; Sulzer, 2011). Drug-induced alterations within the mesocorticolimbic circuit usurp normal learning and memory processes to reinforce the acquisition of learned drug reward (Sulzer, 2011).

The mesocorticolimbic circuit consists of the mesolimbic and mesocortical dopamine pathways, originating from the ventral tegmental area (VTA). The identification and localization of dopamine cells within the VTA formally classified this structure as an important contributor to catecholamine neurotransmission within the brain (Oades and Halliday, 1987). The mesolimbic and mesocortical dopamine pathways originate from dopaminergic cell bodies in the VTA,

which dominantly innervate limbic and cortical structures, respectfully, in a topographical arrangement (Dahlstrom and Fuxe, 1964; Fallon and Moore, 1978). Long fiber tracts ascending from the VTA terminate onto the nucleus accumbens septi (NAcc), septum and olfactory systems via the medial forebrain bundle (MFB: Fallon and Moore, 1978). A subset of MFB fibers continues beyond the genu of the corpus callosum innervating the anterior cingulate and medial prefrontal cortex (mPFC: Fallon and Moore, 1978). The mesolimbic and mesocortical pathways do not function autonomously. Instead the pathways interact dynamically and are known collectively as the mesocorticolimbic circuit.

The Ventral Tegmental Area (VTA)

The VTA is heterogenous with distinct dorsal/ventral and medial/lateral afferent pathways (Fallon and Moore, 1978; Oades and Halliday, 1987). Dopaminergic afferents from the VTA originate from distinct nuclei: the nucleus paranigralis, nucleus parabrachialis pigmentosus, nucleus interfascicularis, and the nucleus linearis (Fallon and Moore, 1978; Oades and Halliday, 1987) with varying densities of dopamine cell bodies. The nucleus interfasicularis and linearis contain the densest dopamine cell bodies and are therefore a major source of dopamine output (Oades and Halliday, 1987).

The mesolimbic pathway is regarded as a common pathway for drug reward and reinforcement of learned appetitive behavior (Wise and Bozarth, 1987; Sulzer, 2011). Functional significance of the mesolimbic pathway was first observed in rats trained to lever press for electrical stimulation of the VTA (Olds and Milner, 1954). Such observations depict the critical importance for this pathway in guided-motivated behavior to obtain reward. Since then, similar experimental procedures have shown an accompanied increase of dopamine

concentration within the terminal fields (Florino et al., 1993). Therefore extracellular dopamine is regarded as a correlate of reward and reinforcement. Evidence for this assertion is observed in a series of intracranial self stimulation (ICSS) experiments conducted by Gallistel and colleagues (1978 and 1982), showing the extinction of brain stimulation, while preserving motor behavior, following administration of the dopamine antagonist pimozide. Results showed the essential role of VTA-dopamine in guiding motivated and reinforced behavior.

Enhanced VTA-dopamine release is also suggested to mediate core components of cognition (Westerink, 1995; de Wit et al., 2012). In normal states, novel and reward predicting stimuli increase dopamine concentration within the mesolimbic pathway (Wise, 1981; Rebec, 1998; Philpot and Kirstein, 1999; Wise, 2004). Phasic activity of dopaminergic cell bodies within the VTA stimulates dopamine transients to the NAcc, which are suggested to encode and reinforce stimulus-response behavior regardless of stimulus valence (Ljungberg et al., 1991; Rebec, 1998; Philpot and Kirstein, 1999; Cooper and Knutsun, 2008; Cohen et al., 2012). Recently, evidence obtained from investigations utilizing fast cyclic scan voltammetry, suggests VTA-dopamine neurons also display a prolonged, alternate pattern of dopamine activity, termed "ramps," which are thought to facilitate neural encoding of reinforced behaviors executed to obtain long-term goals (Howe et al., 2013). Therefore, learning and memory occur and future behavior is predicted based on the reinforcing properties of the stimulus. In all, the functional repertoire of the VTA is extended beyond the perceived reward, reward prediction and simple reinstatement, to include a mechanism underlying guided behavior aimed to obtain short- and long-term goals.

Deficits in dopamine neurotransmission and clearance are associated with psychopathology, including schizophrenia, Parkinson's Disease and drug addiction. Clearance

and regulation of synthesized and released dopamine is essential in maintaining homeostasis within mesolimbic and mesocortical pathways. Cellular mechanisms modulate dopamine neurotransmission and reinforced behavior. Extracellular dopamine may bind to somatodendritic presynaptic dopamine receptor subtype 2 (D2) autoreceptors, providing a feedback mechanism regulating dopamine synthesis, vesicular storage and future release (Grace, 2000). Activation of "dopamine-release inhibiting autoreceptors" restores steady-state levels of dopamine and neuronal activity at target sites (Grace, 2000). Systemic administration of the D2 receptor agonist, quinpirole, decreases dopamine stimulated behavior (Abrahao et al., 2012). Such findings further support the role of the VTA in mediating learning and memory processes, reinforced behavior and execution of organized and controlled behavioral output.

Vesicular glutamate transporters (VGLUT) located on VTA terminals have recently been identified *in vivo* and in cultured dopamine neuronal preparations (Berube-Carriere et al., 2009; Dal Bo et al., 2004). VLGUT subtype 2 (VGLUT2) expression in VTA axon terminals concentrate and release glutamate in addition to dopamine (Fremeau et al., 2004; Takamori, 2006). VGLUT2 also appears to regulate storage and quantal release of dopamine in basal, stimulated and drug-induced states (Wu et al., 2007; Descarries et al., 2008; Hnasko et al., 2010; Stuber et al., 2010). Optogenetic stimulation of VTA dopamine neurons results in glutamatergic-induced excitatory postsynaptic currents at N-Methyl-D-aspartate (NMDA) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate postsynaptic receptor sites in the NAcc shell but not in the core (Tecuapetla et al., 2010). The functional significance of VGLUT2 is observed in conditional knock out models, demonstrating a depression of extracellular dopamine and reduced responsiveness to the locomotor-stimulating effects of

cocaine in mice (Stuber et al., 2010). Regulation of the mesolimbic pathway by VGLUT2 suggests new mechanisms by which the mesolimbic pathway operates.

Non-dopaminergic cell bodies are identified within the VTA (Oades and Halliday, 1987; Swanson, 1982), particularly in "the tail of the VTA" also referred to as the rostromedial tegmental nucleus (Ciccarelli et al., 2012). This subregion (i.e. tVTA/RMTg) is a major modulatory site of dopamine influence on target structures (Bourdy and Barrot, 2012), as gamma-aminobutyric acid (GABA) neurons within this region inhibit principal dopamine neuronal activity ultimately decreasing dopamine neurotransmission (Ciccarelli et al., 2012). Together, VGLUT2 modulates VTA-DA neurotransmission, D2 autoreceptors and GABA modulated neuronal excitability, restoring mesocorticolimbic function.

The Nucleus Accumbens Septi (NAcc)

The NAcc is located in the ventral region of the striatum and is a primary target site for the mesolimbic dopamine pathway (Dahlstrom and Fuxe, 1964; Fallon and Moore, 1978). The NAcc consists largely of medium spiny neurons, which utilize the major inhibitory neurotransmitter GABA in signal transduction (Kita et al., 2004). Accumbal neurons are quiescent due to a prominent hyperpolarized resting potential state (Wilson, 1993). Stimulation of NAcc-GABA release is contingent upon coordinated synaptic input from VTA-dopamine , VTA-glutamate and mPFC-glutamate neurotransmission (Rebec, 2006; Gu, 2010). Hippocampal and amygdalar innervation of the NAcc permits direct association with the limbic system (Berendse et al., 1992). Accumbal afferent pathways directly innervate the VTA, substantia nigra, ventral pallidum and indirectly innervate the PFC via the mediodorsal thalamus (i.e.

pallidothalamic; Deniau et al., 1994). Activation of NAcc-GABA projections is implicated in motivated behavior and drug reward (Wise and Bozarth, 1987).

VTA-dopaminergic afferents, innervating the medium spiny neurons within the NAcc are dense and extensive, as fibers terminate onto multiple neurons simultaneously (Totterdale and Smith, 1989). Afferent trajectories innervate the NAcc in a region-specific and topographic arrangement within the NAcc. The NAcc is divided into two compartments, the core and shell based on neuroanatomical connections, neurochemistry and functionality (Fallon and Moore, 1978; Berendse et al., 1992; Pennartz et al., 1994; Groenewegen et al., 1999; Di Chiara, 2002;). The NAcc core is considered an extension of the extrapyramidal system of the basal ganglia with efferent projections to the substantia nigra pars reticulata, key in motor execution (Pennartz et al., 1994). Conversely, the NAcc shell is the "rim" or "periphery" of ventral striatum (Fallon and Moore, 1978), with afferent projections from the amygdala and efferent fibers extending to the VTA and substantia nigra pars compacta (Pennartz et al., 1994). In addition, the high density of enkephalin-reactive immunolabeling of opioid receptors in the shell but not the core of the NAcc provides further evidence of functional heterogeneity. While the core is implicated in motor execution, the shell is implicated in drug reward and adaptive behavior (i.e. reinforcement).

In addition to the co-release of VTA-dopamine and VTA-glutamate, the NAcc receives glutamate input from cortex, particularly mPFC and also from the amygdala, hippocampus, and thalamus (Sesack and Pickle, 1989; Groenewegen et al., 1996; for review see Tzschentke, 2000). Furthermore, a subset of VTA-glutamate neurons projects to the NAcc (Hnasko et al., 2010). Glutamate and dopamine afferents converge and terminate in close proximity onto dendrites of accumbal neurons (Cavada and Goldman-Rakic, 1989; Groenewegen et al., 1999; Totterdale and

Smith, 1989). Therefore, the NAcc can be considered a junction site for glutamate and dopamine interaction.

The Medial Prefrontal Cortex (mPFC)

Glutamate is an amino acid known to promote or "excite" neuronal activity at target sites by decreasing the threshold for neuronal depolarization, as observed in iontophoretically applied glutamate on spinal neurons, retinal cells and neurons within the brain (Curtis et al., 1959; Curtis and Watkins, 1961; Curtis and Davis, 1962). The mPFC has been regarded as an essential component of the motive circuit facilitating reinforcement and reward (Pierce and Kalivas, 1997). Electrical and self-stimulation of the mPFC stimulates VTA-dopamine neurotransmission and extracellular concentrations of dopamine within the NAcc (Gariano and Groves, 1988; Taber and Fibiger, 1995; You et al., 1998), an effect blocked by the glutamate antagonist kynurenic acid directly administered into the VTA (You et al., 1998).

A large quantity of glutamate terminals within the NAcc and VTA originate from the pyramidal cells located in the mPFC (Fuxe et al., 1972). In addition, the mPFC receives dopamine from the VTA (Morari et al., 1998; Mora et al., 1976a) and glutamate input from the hippocampus, amygdala and mediodorsal thalamus (Tzschentke, 2000). Medial PFC function is sensitive to dopamine receptor binding, as dopamine agonists and D2 receptor antagonists decrease mPFC firing rates and attenuate self-stimulation behavior in a dose-dependent manner (Mora et al., 1976a; Mora et al., 1976b; Montes et al., 2005). Activation of reciprocal and synchronous connectivity between the mPFC and other glutamate systems has been implicated in synaptic plasticity, facilitating learning and memory (Ahn et al., 2013). The direct and indirect

modulation of mPFC-glutamate on mesocortical and mesolimbic dopamine systems is referred to as the mesocorticolimbic circuit (Tzschentske, 2000).

The mPFC can be divided into 4 subregions: medial precentral, anterior cingulate, prelimbic and infralimbic regions, based on neuroanatomical, histochemical and functional distinctions (Berendse et al., 1992). The prelimbic and infralimbic subregions are directly linked with the limbic system. The prelimbic cortex shares reciprocal connections with the NAcc core. Generalized projections from the prelimbic cortex throughout the entire NAcc have been observed, however, staining was greatest at the site of the NAcc core (Berendse et al., 1992). In turn, the NAcc core indirectly innervates the prelimbic cortex via striato-pallidal-thalamic tracts (Deniau et al., 1994). The infralimbic cortex selectively innervates the NAcc shell, which is integral in drug reward and reinforcement (Wise and Bozarth, 1987; Sesack and Pickle, 1909; Groenewegen et al., 199; Rebec, 2005).

In all, a dynamic interplay appears to occur within the mesocorticolimbic circuit, regulating cognition and expression of motivated behavior. Functional heterogeneity has been shown to be present at all terminal fields: the VTA, NAcc and PFC. Moreover, neurochemical heterogeneity appears to be present in the VTA. The NAcc has been referred to as the "limbic-motor interface" (Deniau et al., 1994; Rebec and Sun, 2005) and a site critical for neuroplasticity associated with the maladaptive learning theory of drug addiction (Wolf et al., 2005).

Neuropsychopharmacology

Ethanol. Alcohol (i.e. ethanol)-mediated effects on GABA, NMDA, opioid, serotonergic, cannabinoid, cholinergic and dopaminergic activity are observed and have been extensively reviewed (Lovinger et al., 1989; Marinelli et al., 2003; Krystal et al., 2003; Lindholm

et al., 2007; Jarjour et al., 2009; Kumar et al., 2009; Wee and Koob, 2010; Jerlhag et al., 2012; Sari, 2013). Due to its widespread effects, ethanol may be considered as a broad-spectrum drug of abuse, as it directly and indirectly modulates the neurophysiology of the mesocorticolimbic circuit by affecting different molecular targets. Acute ethanol exposure increased dopamine release at VTA terminals (Weiss et al., 1993; Imperato and Di Chiara, 1986) via disinhibition GABA interneurons within the VTA (Tateno and Robinson, 2011). The result was a net increase in neuronal excitability (Gessa et al., 1985; Brodie et al., 1990; Löf et al., 2007), independent from reuptake inhibition (Yim and Gonzales, 2000). Ethanol attenuated NMDA receptor function in a dose-dependent manner and augmented the GABA response (Lovinger et al., 1989). It is likely the reinforcing properties of ethanol are due to a delicate balance and interaction between NMDA and GABA receptor function (Hodge and Cox, 1998), as ethanol binds to NMDA receptors with high affinity and an increase in glutamate release in the NAcc is observed during early stages of ethanol withdrawal (Krystal et al., 2003). Importantly, glutamate receptor sensitivity is increased and primes for subsequent exposure, perhaps contributing to the persistency of ethanol-craving and seeking behavior (Nestler, 2011). Cannabinoid (Onaivi, 2008), opioid (Lindholm et al., 2007; Jarjour et al., 2009) and cholinergic (Jerlhag et al., 2012) neural systems also play a role and may further contribute to ethanol-induced reward and addictive behaviors. Nitric oxide (Gerlach et al., 2001) and histone methylation mediate ethanols' oxidative stress response and regulate downstream gene expression, respectively, possibly contributing to ethanol-induced neurodegeneration and epigenetic modulation of neural sensitivity to future drug experience and exposure (Nestler, 2011). Despite the widespread interplay of neurotransmitter systems following ethanol exposure, the interaction between

dopamine and NMDA receptors may mediate learning and memory processes, impacting neuroplasticity and the persistency of drug-seeking behavior.

Cocaine. Cocaine is a psychostimulant and sympathomimetic that increases general arousal, facilitates cognitive acuity, and increases physical endurance. Such effects are rapid and are subjectively reported as pleasurable. However it is the experienced "euphoria" or "high" that supports repeated use. Cocaine onset, duration of action and reinforcing properties are dependent on the route of administration (Volkow et al., 2000). For instance, intravenous infusion of cocaine hydrochloride affects the brain within seconds (Mejias-Aponte and Kiyatkin, 2012). However smoked cocaine is reported to have a more intense and rapid onset of the well known "high" compared with intranasal (i.e. snorting) and intravenous administration (Volkow et al., 2000). The rapid effects of smoking cocaine are short lived and may reflect the increased likelihood of continuous use. Regardless of the route of administration, both acute and repeated administration of cocaine have enduring effects within the brain thought to usurp normal information processing of motivating and rewarding stimuli. As a result, organized and motivated behavioral output appears to be modified to promote cocaine-seeking behavior, increasing the propensity to relapse.

Cocaine exerts its effects by altering catecholamine transmission within the mesocorticolimbic pathway. Cocaine alters neuronal membrane excitability by acting on voltage-gated sodium and potassium channels and presents high affinity binding for all catecholamine transporters including: norepinephrine, serotonin and dopamine (Ritz, 1987). Such actions account for the acute effects of cocaine centrally and peripherally.

Despite the widespread effect of cocaine on neuronal responsiveness, its impact on mesocorticolimbic dopamine neurotransmission has been in the forefront of scientific

investigation in attempt to elucidate the mechanisms underlying its addictive properties. In 1960, Whitby and colleagues were the first to demonstrate cocaine-induced catecholamine reuptake inhibition. Catecholamine transporter molecules, specifically the dopamine transporter (DAT) were later identified as the mechanism mediating cocaine's action on reuptake inhibition within striatum (Ritz et al., 1987; Thomsen et al., 2009). The reinforcing properties of cocaine were directly associated with binding affinity for the DAT, rather than the route of administration (Volkow et al., 2000). Such observations suggested the rewarding properties of cocaine are independent of the pharmacokinetic profile of the drug. Rather it is the pharmacodynamics profile of cocaine that mediates drug reinforcement and addiction.

The reinforcing properties of cocaine have been directly associated with VTA-dopamine innervation of the NAcc. Supporting evidence for this is observed in "time-locked" alterations in NAcc firing patterns prior to and in response to cocaine self-administration in rats (Carelli et al., 1993). Following cocaine self-administration, NAcc neurons either increase or decrease in activity, while a subset of neurons increase in excitability immediately prior to lever pressing (Carelli et al., 1993; for review see Carelli, 2002). An increase in extracellular dopamine, predicting cocaine reward has also been observed (Shultz, 1998; Philpot and Kirstein, 1999). Therefore, it appears the NAcc plays a more advanced role in cocaine reinforcement, as an apparent reward expectancy state has been shown. A recent procedure demonstrating simultaneous *in vivo* microdialysis sampling from the NAcc and electrophysiological recordings from the VTA demonstrate cocaine-induced attenuation of VTA neuronal firing and an increase in dopamine concentration (Panin et al., 2012). Methodological considerations are key, as drug-induced changes in VTA neurophysiological responses appear to be dependent on whether rats are awake or anesthetized during sampling (Koulchitsky et al., 2012). Specifically, systemic

cocaine decreased VTA-dopamine neuronal firing activity in anesthetized rats, while an increase in firing burst activity was observed in awake rats following cocaine administration. Despite such complexity, these studies demonstrate a cognitive component for drug-induced VTA neuronal activity implicated in reward learning and in the acquisition of learned behavior resulting from drug reinforcement (Thomas et al., 2008).

Recent research suggested an interactive role between dopamine and glutamate in ethanol and cocaine addiction. Advancements in experimental methodologies have demonstrated co-release of glutamate and dopamine from the VTA neurons projecting to the NAcc shell, VGLUT2-mediated function in dopamine availability and cocaine locomotor activity (Stuber et al., 2010; Gu, 2010). In addition, there are enduring alterations in the neurophysiological responses (Mejias-Aponte and Kiyatkin, 2012; Vanderschuren and Kalivas, 2000; Rebec and Sun, 2005; Wolf and Ferrario, 2010), neurochemistry (Hurd et al., 1989; Williams and Steketee, 2005; Philpot and Kirstein 1999; Badanich et al, 2006) and morphology (Bock et al., 2013) of neurons within the mesocorticolimbic circuit. New theories highlighting the importance of drug context and neuroplasticity in the neural circuitry involved in the expression of addictive behaviors have been proposed (For reviews see Kalivas 2008; Wolf and Ferrario, 2010). Altered excitability of downstream targets have been suggested to be associated with the induction of transcription factors contributing to modified gene expression and the persistency of drug addictive behaviors (Nestler, 2011). Specifically, long-term neuroadaptations in glutamate and DA terminals in the NAcc appear to contribute to maladaptive learning between the drug and dopamine-associated stimuli, increasing vulnerability to relapse behavior in human addicts (Vanderschuren and Kalivas, 2000; Robinson and Berridge, 2008; Wolf and Ferrario, 2010; Nestler, 2011).

Ethanol and Cocaine Co-administration. Ethanol/cocaine polysubstance abuse is defined as the co-administration of ethanol and cocaine in close temporal proximity. Individuals engaging in simultaneous oral ethanol consumption and intranasal cocaine administration report a potentiated and increased duration of drug-induced euphoria, as well as an overall sense of well-being (McCance-Katz et al., 1993 and 1998). Increased risk of cardiotoxicity, hepatoxicity and lethality following ethanol/cocaine polysubstance use is documented and may contribute to the prevalence of hospitalizations (Boyer and Petersen, 1992; Jatlow et al., 1995; McCance-Katz et al., 1998).

Concurrent ethanol/cocaine polysubstance use results in the transesterification of cocaine, synthesizing the psychoactive metabolite cocaethylene (Boyer and Petersen, 1992). The metabolism of cocaine into cocaethylene is only possible in the presence of ethanol therefore; cocaethylene plasma concentration is an indicator of ethanol/cocaine polysubstance use. Cocaethylene has pharmacological, behavioral and neurological profiles similar to those of cocaine. Cocaethylene binds to DAT inhibiting dopamine reuptake (Hearn et al, 1991). Cocaethylene in itself is rewarding, as self-administration, sensitization and place preference for the metabolite has been observed (Jatlow et al, 1995; Schechter, 1995; Prinssen et al., 1996). Utilization of *in vivo* electrophysiology and *in vivo* microdialysis techniques demonstrated cocaethylene-induced increases in VTA-dopamine neuronal responsiveness and dopamine concentration, respectively, following microinfusion, intravenous or intraperitoneal administration of the metabolite (Bradberry et al., 1993; Iyer et al., 1995; Bunney et al., 2001). Behavioral paradigms such as conditioned place preference have shown utility in the investigation of ethanol/cocaine polysubstance reward (Busse and Riley, 2002 and 2003; Busse et al., 2004). However, since drug initiation and the development of habitual polysubstance

abuse is commonly observed in younger populations, it is important to identify age-dependent differences in ethanol/cocaine reward.

Animal Models of Polysubstance Abuse

Conditioned Place Preference

Conditioned place preference (CPP) is an animal model of drug abuse, capable of assessing the reinforcing and affective properties of drugs (i.e. rewarding and aversive), as well as the learned associations between the drug experience and the drug-paired environment (Tzschentke, 1998; Bardo and Bevins, 2000; Sorg, 2012). CPP operates under the basic principle of excitatory classical Pavlovian conditioning. In the CPP paradigm, a drug or drug combination (unconditioned stimulus, UCS) is repeatedly paired with a specific context (neutral, conditioned stimulus, CS+), while saline is paired with a distinct context (CS-). Following repeated drug-context pairings, a post-conditioning test is employed and the amount of time spent in the drug-paired context is assessed. An increase in the amount of time spent in the drug-paired context is defined as a CPP and suggests the drug/drug combination is rewarding. Conversely, the animal spending significantly less time in the CS+ paired chamber following conditioning is indicative of a conditioned place aversion (CPA).

Cocaine CPP is reliably observed across a range of doses and developmental time periods in rats (for reviews see Tzschentke, 1998; Bardo and Bevins, 2000; Tirelli et al., 2003; Sorg, 2012). Interestingly, ethanol place preferences in rats are strictly dependent on the dose administered, length of conditioning trial and treatment age of the rat (Busse and Riley, 2002; Philpot et al., 2003; Busse et al., 2004; Morales et al., 2012; for review see Tzschentke, 2007). Specifically, ethanol CPP has been observed with low and moderate doses during preadolescence

(postnatal day (PND) 25) and late adolescence (PND 45), respectively (Philpot et al., 2003). However ethanol-induced CPA has been observed in adult rats and the degree of aversion increases as a function of dose with 2.0 g/kg inducing the greatest response (Philpot et al., 2003). However, low and moderate doses of ethanol (0.5, 1.0, 1.5 g/kg) have also been reported to neither induce place preference nor aversion (Busse and Riley, 2002; Busse et al., 2004, but see Morales et al., 2012 for 1.5 g/kg CPP). Inconsistent findings suggest methodological considerations (Bardo et al., 1995), including dose and age-dependent sensitivity to ethanol (Philpot et al., 2003). In addition ethanol has widespread impact on the brain, targeting multiple neurotransmitter systems simultaneously (Green and Grant, 1998; Kostowski and Bienkowski, 1999), contributing to its complexity on conditioned behavior (Verendeev and Riley, 2013).

Co-administration of ethanol and cocaine may produce synergistic or interactive effects, reinforcing habitual polysubstance abuse. Animal models utilizing the CPP paradigm suggest ethanol plays a modulatory role on cocaine reward. Rats administered low dose ethanol (0.5 g/kg) and cocaine (5.0 mg/kg) in combination but not in isolation expressed CPP (Busse et al., 2004). However, increasing doses of ethanol decreased cocaine reward (Busse and Riley, 2002). The dose-dependent expression of ethanol/cocaine reward observed in CPP experiments suggests ethanol modulated cocaine reward, however it remains unknown as to whether ethanol decreased the rewarding properties of cocaine or if ethanol increased the aversive, secondary properties of cocaine in drug treated rats. In attempt to elucidate the modulatory role of ethanol on cocaine reward, Busse and colleagues conducted a series of conditioned taste aversion (CTA) experiments and assessed the impact of ethanol on taste aversions to escalating doses of cocaine (20.0, 30.0 and 40.0 mg/kg; 2005). Data demonstrated ethanol-induced attenuation of cocaine (20.0 mg/kg) taste aversions, while having no impact on taste aversions with higher cocaine

doses. In all, the rewarding properties of the low dose ethanol/cocaine combination result from enhanced excitability of dopaminergic cell bodies in the VTA (Bunney et al., 2001), while higher doses that are associated with a decline in drug reward appear to be attributed to additional mechanisms within the mesocorticolimbic pathway. Supporting evidence is the attenuation of cocaine CPP expression in a reactivation-dependent manner following administration of the NMDA receptor antagonist, MK-801 (Brown et al., 2008). Results demonstrated that NMDA receptor activation was necessary for the expression of cocaine reward. Findings suggest ethanols' modulating effect on cocaine reward may take place at the NMDA receptor site. Such interpretations support an interaction rather than a synergistic effect following the ethanol/cocaine polysubstance combination.

Mechanisms in Polysubstance Reward

Previous findings suggest a dose-dependent modulatory effect of ethanol on cocaine reward. However age-dependent effects of ethanol on cocaine reward and the underlying mechanism(s) driving this response remain unknown. Since ethanol blocks NMDA receptor function (Lovinger et al., 1989) and cocaine place preferences are attenuated following treatment with the NMDA receptor antagonist, MK-801 (Brown, Lee and Sorg, 2008; for review see Sorg, 2012), it is possible that increasing doses of ethanol modulate cocaine reward by its actions on NMDA receptors. Since adolescence is a critical time period of brain development associated with unique sensitivity to the rewarding properties of drugs (Philpot et al., 2003; Badanich et al., 2006; Badanich, et al., 2008; Maldonado, et al., 2010; for review see Spear, 2000), the dose-dependent modulatory effects of ethanol on cocaine reward may be absent in adolescents, likely due to age-dependent differences in NMDA receptor function.

Experimental Aims and Hypothesis

Epidemiological data demonstrate a trend towards increasing prevalence of ethanol/cocaine polysubstance abuse and associated incidences of hospitalizations in all age populations, including adolescence (Kedia et al., 2007; DAWN, 2009). Adolescents report engaging in ethanol/cocaine polysubstance abuse, a valid predictor of habitual consumption patterns and diagnosis of substance use disorders later in life (Bailey et al., 1992; Merikangas et al., 1998; Monitoring the Future, 2012). Despite the age-dependent risk factor, even less is known about the long-term effects of simultaneous ethanol and cocaine polysubstance abuse during adolescence. Presently, there are no investigations elucidating the mechanisms of ethanol/cocaine polysubstance reward and abuse in adolescence.

The overall objective of the conducted studies was to develop an effective developmental animal model of polysubstance abuse and identify associated mechanisms driving ethanol/cocaine polysubstance reward. Aim 1 determined age-specific sensitivities of ethanol on cocaine reward. Aim 2 investigated the role for NAcc-NMDA receptors as a potential mechanism ethanol/cocaine polysubstance reward across adolescence. Lastly, the involvement of NMDA receptors following acute and repeated ethanol/cocaine polysubstance abuse across different phases of drug abuse was assessed in Aim 3. A biased CPP procedure was used to measure polysubstance reward across developmental time periods. Male adolescent (PND 30) and adult (PND 60) sprague-dawley rats were conditioned to increasing doses of EtOH (0.5 and 1.75 g/kg, i.p.) or cocaine (20.0 mg/kg, i.p.) in isolation or in combination. In order to assess the role of NMDA receptors in ethanol/cocaine polysubstance abuse, MK-801 was administered systemically and directly into the NAcc of adolescent or adult rats. It was hypothesized that age-

dependent differences in the expression of place preferences for the ethanol/cocaine combination would be observed. In addition, repeated NMDA receptor antagonism was expected to protect against the expression of ethanol/cocaine polysubstance reward following a cocaine challenge. Lastly, NMDA receptor function was anticipated to play a role in the varying phases of CPP acquisition and expression. Overall, findings showed that the CPP paradigm is a sensitive developmental animal behavioral model of polysubstance reward. Ethanol decreased cocaine reward in an age and dose-specific manner, an effect contributed by NMDA receptors in the NAcc.

CHAPTER TWO:

THE ROLE FOR NMDA RECEPTORS IN ETHANOL AND COCAINE POLYSUBSTANCE ABUSE ACROSS DEVELOPMENT

Aim 1: Dose-Dependent Effects of Ethanol on Cocaine Reward Across Adolescence

The present experiment was conducted to assess the modulating effects of escalating ethanol doses (0.5 g/kg or 1.75 g/kg) on cocaine (20.0 mg/kg) reward in adolescent (postnatal day (PND) 30) and adult (PND 60) rats.

Methods

Subjects. Seventy-four adolescent (PND 30) and seventy-nine adult (PND 60) male rats, weighing between 100 and 370g, respectively, were used for experimentation. Breeding pairs derived from Harlan Industries (Indianapolis, IN) established breeding colonies within the University of South Florida. Dates of births for litters were recorded. The day of birth was designated as PND 0. On the following day (PND 1), litters were sexed and culled to no less than 7 and no more than 10 pups per litter. All preweanling rats remained untouched (except for routine cage maintenance) with their respective dams in a temperature- and humidity-controlled environment on a 12 hour light-dark cycle with lights turned on at 0700 and off at 1900 hours. On PND 21, rats were randomly selected for experimentation. In order to prevent the potential confound of litter effects (Spear and File, 1996), no more than one pup per litter was assigned to a given experimental condition. Remaining pups were used for additional laboratory

experiments or were euthanized. At the time of weaning, all scheduled rats were pair-housed with same-sex littermates. Food and water were available *ad libitum* in the home cage. The maintenance and treatment of all rats was conducted under the strict guidelines of the Institutional Animal Care and Use Committee and the National Institutes of Health.

Drugs and solutions. Cocaine hydrochloride (Sigma Aldrich Co., St. Louis, MO) was dissolved in pharmaceutical grade sterile saline (0.9%) to a concentration of 1.0 mg/ml and administered intraperitoneally (i.p.) at a dose of 20.0 mg/kg. Ninety-five percent (95%) ethanol was prepared into a 17% v/v solution by dilution with 0.9% pharmaceutical grade sterile saline for administration at doses 0.5 or 1.75 g/kg. Regardless of ethanol dose, adolescent (PND 30) and adult (PND 60) rats were co-administered 0.5 or 1.75 g/kg ethanol (or saline equivalent) followed by 20.0 mg/kg cocaine (or saline equivalent) within 30 seconds from each other, in this order. Control groups were administered two saline vehicle injections, a volume equivalent to appropriate drug dose. The doses and timing of drug injections were chosen based on previous research demonstrating dose and age-dependent sensitivity to ethanol (Philpot et al., 2003; Busse et al., 2004), reliable cocaine place preference expression across development (Badanich and et al., 2006; Balda et al., 2006; Spyraki et al., 1982; Brown et al., 2008; for reviews see Bardo et al., 1995; Tzschentke, 1998; Tirelli et al., 2003).

Place preference testing apparatus. The place preference testing apparatus consisted of an acrylic, 2-way compartment shuttle box. Each compartment (21 x 24.5 x 20.5 cm) was distinguished by visual and tactile cues. Specifically, one compartment consisted of horizontal black and white striped (2.54 cm) walls paired with 100-grit brown sandpaper flooring. The second compartment contained black and white vertical striped (2.54 cm) walls paired with wire mesh flooring. A two rather than a three-compartment shuttle box (containing a

neutral zone), was used to eliminate the potential confound of age-related differences in novelty-induced exploration in a place preference paradigm (Philpot et al., 2003; Stansfield and Kirstein, 2006) and to increase the effect size of drug conditioning within rats (for review see Bardo et al., 1995). Initial preconditioning (Baseline) and postconditioning preference (Test) assessments occurred in the testing apparatus. Drug conditioning was conducted in the compartment-specific conditioning apparatus.

Place preference conditioning apparatus. Following assessment of initial place preferences, all rats underwent drug conditioning in the compartment-specific conditioning apparatus. Rats were exposed to an acrylic box comprised of either horizontal or vertical black and white stripes (2.54 cm) paired with 100-grit brown sandpaper flooring or wire-mesh flooring, respectively. The dimensions of each box were consistent to each other and to the testing apparatus described above. Therefore, the visual and tactile cues were presented identically to those in the place preference testing apparatus for drug conditioning. Post-conditioning place preferences were conducted in the place preference testing apparatus.

Conditioning procedure. All rats underwent a handling, pre-conditioning assessment (baseline), drug conditioning, and post-conditioning (test) phase. Procedures are illustrated in Figure 1.

Two days prior to Baseline, adolescent (PND 28 and 29) and adult (PND 58 and 59) rats were transported to the laboratory, weighed and underwent a brief 5-minute handling session. All rats will were handled in a fashion aimed to decrease stress due to experimenter handling and to decrease the possibility of stress-induced behavioral responsivity to psychostimulants (Marinelli and Piazza, 2002; Maldonado and Kirstein, 2005).

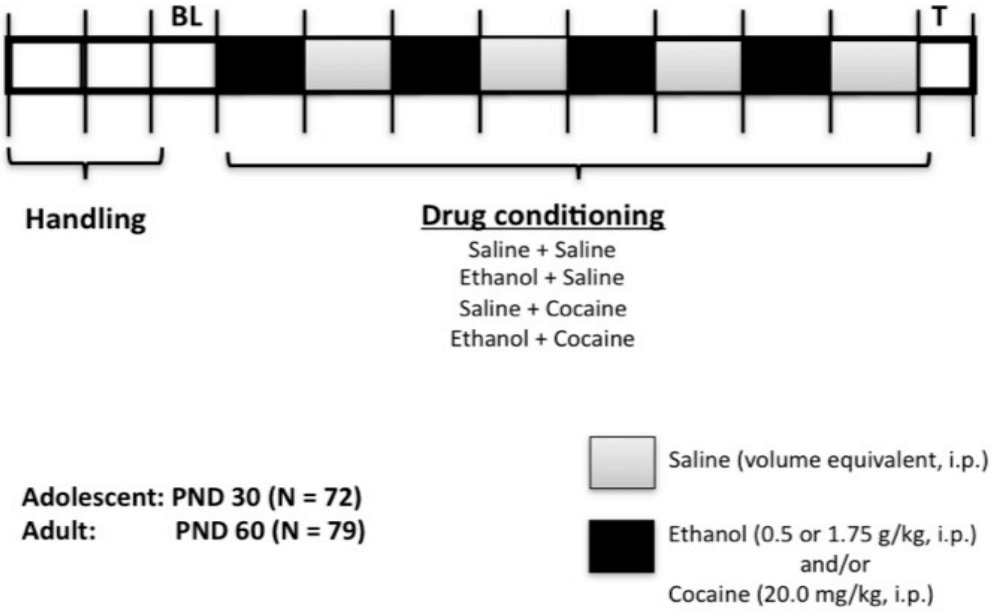

Figure 1. Aim 1 Timeline. Schematic representation of the experimental timeline. Adolescent (PND 30; N = 72) and adult (PND 60; N = 79) underwent a two-day handling procedure, followed by a pre-conditioning assessment Baseline (BL), drug conditioning phase and a post-conditioning assessment Test (T).

Baseline (Day 1). On Day 1, adolescent (PND 30) and adult (PND 60) male rats were transported to the laboratory, weighed and completed a pre-conditioning place preference assessment (i.e. baseline) in the place preference testing apparatus to determine initial place preferences. Baseline preference scores for a particular compartment was used in the application of a "biased" CPP design, a valid model of drug conditioning and reward (Calcagnetti and Schechter, 1993). Specifically, drug-naïve rats were placed in the center of the place preference testing apparatus for 15 minutes (900 seconds). The amount of time (seconds) spent in each compartment was recorded and scored with the behavioral tracking software, Noldus Ethovision®. Detection parameters were set to initiate behavioral tracking and data recording upon movement. Time parameters were set terminate tracking following 15 minutes from the

time of first movement. Rats were removed from the testing apparatus, placed back into the homecage and transported to the colony room. Noldus Ethovision® stored the following dependent measurements: time (seconds) spent in either side of the apparatus, frequency of midpoint crossings (i.e. locomotion), and frequency of rearing behavior. Detection parameters determined placement of the rat in the testing apparatus based on center of gravity. Specifically, the rat was considered to have entered a compartment when the head, both forepaws and upper midsection of the body crossed the midpoint (i.e. center of the apparatus). Typically, when the head, forepaws and center of the body crossed the midpoint, the rat continued to advance and became committed to approaching compartment. An individual place preference index was assessed for each rat. The place preference index was defined as the amount of time (seconds) spent in the least preferred compartment (Bardo et al., 1995). The least-preferred compartment served as the drug-paired conditioning compartment during drug conditioning.

Drug conditioning. Adolescent (PND 31) and adult (PND 61) rats were separated into either the 0.5 g/kg EtOH or 1.75 g/kg ethanol Dose groups. Adolescent (PND 31) and adult (PND 61) rats within each of the Dose conditions, were further separated into one of the following 4 treatment groups: saline-saline; ethanol-saline; saline-cocaine; ethanol-cocaine. Therefore, a total of 75 (PND 31 = 34; PND 61 = 41) and 76 rats (PND 31 = 38; PND 61 = 38) were scheduled into the 0.5 g/kg and 1.75 g/kg ethanol dose condition, respectively. Adolescent and adult rats within the 0.5 g/kg condition were further grouped into the following 4 treatment groups (N represent group totals for adolescent and adult rats, respectively): saline-saline (N = 9; N = 11), ethanol-saline (N = 7; N = 9); saline-cocaine (N = 10; N = 10); ethanol-cocaine (N = 8; N = 11). Rats scheduled to receive 1.75 g/kg ethanol treatment or saline equivalent were grouped identically to the 0.5 g/kg ethanol Dose condition. Specifically, adolescent and adult

rats were scheduled into either the saline-saline (N = 12; N = 11), ethanol-saline (N = 10; N = 8), saline-cocaine (N = 9; N = 9), ethanol-cocaine (N = 7; N = 10), respectively. Each rat regardless of Dose condition or drug treatment group, received 2 injections: ethanol or saline, immediately followed by saline or cocaine. This process was referred to as co-administration and was aimed to closely mimic simultaneous polysubstance abuse. The saline-saline control groups in both Dose conditions received an equivalent volume of saline for both injections.

All rats were transported daily to the laboratory and weighed throughout the drug-conditioning phase. Drug conditioning took place in the place preference drug conditioning apparatus on Days 2-9. On Days 2, 4, 6, and 8 drug-treated rats received ethanol (0.5 or 1.75 g/kg, i.p.) or cocaine (20.0 mg/kg, i.p.) in isolation (ethanol-saline or saline-cocaine, respectively) or in combination (ethanol-cocaine) and were placed in the least-preferred (drug-paired) compartment for 15 minutes. On alternating days, rats were administered two injections of saline and placed in the preferred (i.e. vehicle-paired) compartment for 15 minutes. Rats scheduled in the saline-saline drug-treatment groups were administered two injections of saline daily (Days 2-9) and placed in the respective compartment. Therefore, a 15 minute drug conditioning trial occurred over an 8-day time span. All rats were exposed to 4 drug and 4 saline pairings and placed into the drug-paired and vehicle-paired compartments, respectively. The conditioning apparatus was cleaned with Clidox and isopropyl alcohol between trials to disinfect and neutralize odors.

Test (Day 10). On Day 10, twenty-four hours following the last day of drug conditioning, all rats were transported to the laboratory, weighed and placed into the place preference testing apparatus in a drug-free state to assess drug-induced post-conditioning place preferences. All procedures were conducted identical to the Baseline session (see above). In

brief, rats were placed into the center of the two-compartment, place preference testing apparatus in a drug-free state and were able to freely explore both the drug-paired and the vehicle-paired compartments. Rats were tracked using Noldus Ethovision® behavioral tracking software. The amount of time spent in each compartment was recorded.

Data Analysis

The amount of time (seconds) spent in each compartment on baseline and Test were recorded and analyzed. A Univariate ANOVA examining the amount of time (seconds) spent in the least-preferred (drug-paired) compartment was conducted to determine initial age-dependent differences in behavior prior to experimentation. All adolescent (N = 72) and adult (N = 79) rats in both Dose conditions were included in the analysis. Adolescent rats spent less time in the least-preferred (drug-paired) compartment during the 15 minute Baseline assessment, as compared to adult rats [$F(1, 152) = 18.773$, P = 0.000] (Figure 2).

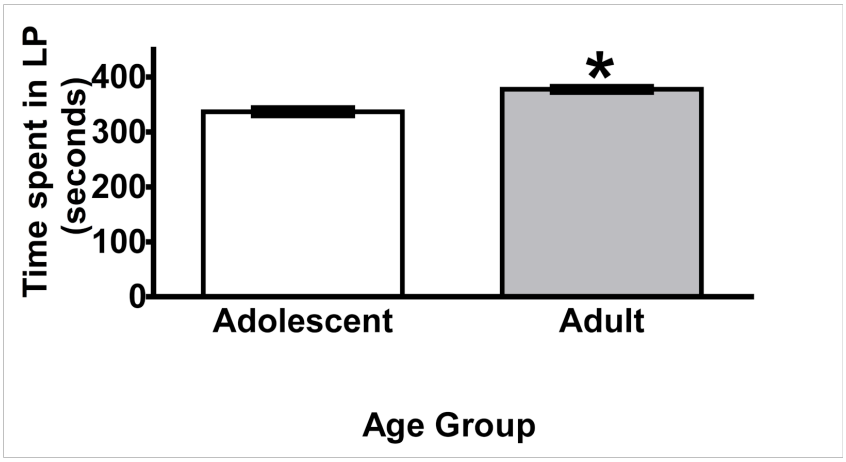

Figure 2. Initial Differences Prior to Drug Conditioning. Data are presented as group means for the amount of time spent in the LP compartment. Adolescent (PND 30; N = 72) rats spent less time in the LP, as compared to their adult (PND 60; N = 79) counterparts [$F(1, 152) = 18.773$, P = 0.000] prior to experimentation. * Denotes statistical significance.

To control for Age-dependent differences in the magnitude of initial place preference bias, data for the individual drug-treatment groups were transformed to percent change at baseline (individual preference [(Tests score/ Baseline group mean)*100]. Group means for raw data is presented in Table 1. A 2(Dose: 0.5 g/kg; 1.75 g/kg) x 2(Age: adolescent, adult) x 4(Drug: saline-saline; ethanol-saline; saline-cocaine; ethanol-cocaine) ANOVA and appropriate post-hoc analysis [(i.e. Fisher's Least Significant Difference (LSD)] were conducted to assess statistical significance across Age, Dose and Drug treatment conditions during the post-conditioning test. To determine the interaction effects between Dose, Age and/or Drug treatment at Test, subsequent independent ANOVAs with Dose, Age and/or Drug as the between-subject factors were conducted. Fisher's LSD post hoc tests were calculated to detect significant effects of Drug on conditioned behavior within drug-treatment groups at the time of Test. Separate Univariate ANOVAs were computed to investigate the effects of 0.5 g/kg and 1.75 g/kg ethanol and 20.0 mg/kg cocaine in isolation and in combination on conditioned behavior at Test. These analyses were carried out separately for adolescent and adult groups. A drug-induced CPP

Table 1. Aim 1 Means Table. Data are presented as means for the amount of time adolescent (PND 30, N = 72) and adult (PND 60, N = 79) spent in the least preferred (drug-paired) compartment (seconds).

	SALINE-SALINE		ETHANOL-SALINE		SALINE-COCAINE		ETHANOL-COCAINE	
Adolescent (0.5 g/kg)	BL	T	BL	T	BL	T	BL	T
Mean	366	389	323.1	379.5	341.5	524	332.5	332.9
SD	53	149	81	161	48.5	103.9	82.8	136.8
N	9	9	7	7	10	10	8	8
Adult (0.5 g/kg)	BL	T	BL	T	BL	T	BL	T
Mean	358.4	357.6	357.6	343.7	343.7	434.6	368.4	409.4
STD	52.6	66.3	66.3	58.7	58.7	114.8	58.9	129.9
N	11	11	9	9	10	10	11	11
Adolescent (1.75 g/kg)	BL	T	BL	T	BL	T	BL	T
Mean	339.2	360.4	336.1	289.5	301.6	457.6	357.4	436.1
SD	51.5	104.8	70.7	116.1	85.7	126.2	48.9	21.1
N	12	12	10	10	9	9	7	7
Adult (1.75 g/kg)	BL	T	BL	T	BL	T	BL	T
Mean	412.7	410.1	390.2	283.4	410.8	524.3	389	162.4
SD	30.4	134.9	39.6	71.6	37	90.9	28.8	79
N	11	11	8	8	9	9	10	10

response was defined as a significant increase in the amount of time spent in the least preferred (drug-paired) compartment on Test relative to saline controls (Bardo et al., 1995). Previous studies have shown increasing doses of ethanol to be aversive (Philpot et al., 2003; Busse et al., 2004). Therefore, behavior was also assessed to determine a drug-induced conditioned place aversion (CPA) response, which was defined as a significant decrease in the amount of time spent in the least preferred (drug-paired) compartment at Test relative to saline controls. All data were analyzed and graphed by using SPSS Version 21 statistical software and Graph Pad Prism, respectively. An alpha level of $p < 0.05$ and a confidence interval (CI) of 95% was set to determine statistical significance and indicate the range of values within the estimate for all analyses.

Results

The aim of the experiment was to determine the impact of ethanol Dose (0.5 or 1.75 g/kg) on cocaine (20.0 mg/kg) reward in adolescent and adult male rats (PND 30 and 60, respectively). Results show escalating doses of ethanol attenuated cocaine reward in an age-dependent manner, as a significant 3-way ANOVA [Dose (2: 0.5 g/kg, 1.75 g/kg) x Age (2: Adolescent and Adult) x Drug (4: saline-saline; ethanol-saline; saline-cocaine; ethanol-cocaine] was observed on conditioned behavior at Test [$F(3, 150) = 4.651$, $P < 0.01$]. Significant main effects for the between-subject factors Age [$F(1, 150) = 20.002$, $P = 0.000$] and Drug [$F(3, 150) = 17.904$, $P = 0.000$] were observed. Fisher's LSD post hoc analyses showed significant effects of Drug treatment between groups, regardless of Age (see Figure 3). Adolescent and adult rats conditioned to cocaine alone, exhibited CPP (saline-saline v. saline-cocaine, $P = 0.000$). Rats conditioned to ethanol (collapsed across Dose) alone, exhibited a trend towards a significant

CPA (saline-saline v. ethanol-saline, P = 0.060). Regardless of Dose and Age, ethanol significantly blocked cocaine CPP preference, as a significant difference between saline controls and the ethanol-cocaine treatment group was not observed (P = 0.187). Such results demonstrate a complete blockade of cocaine reward when ethanol was co-administered. Significant Drug effects were observed in adolescent and adult rats within the 0.5 and 1.75 g/kg ethanol Dose conditions following Univariate ANOVAs within Age groups (see Figure 4). Within the 0.5 g/kg ethanol condition, adult rats demonstrated a significant cocaine CPP effect (P = 0.01), which was attenuated by ethanol [$F(3, 40) = 5.346$, $P < 0.01$] (saline-saline v. saline-cocaine: P = 0.158). The aversive properties of 1.75 g/kg ethanol blocked cocaine CPP and induced a CPA [$F(1, 29) = 7.112$, P = 0.01], as rats conditioned to ethanol/cocaine spent significantly less time in the drug-paired compartment when compared to rats conditioned with cocaine alone (P = 0.000) and to saline controls on Test day (P = 0.000). Main Drug effects were also observed in adolescent rats within the 0.5 g/kg [$F(3, 33) = 3.293$, $P < 0.05$] and 1.75 g/kg [$F(3, 37) = 6.919$, P = 0.001] ethanol Dose condition. Cocaine CPP was more susceptible to disruption in adolescent rats within the 0.5 g/kg ethanol dose condition, as this Dose blocked cocaine CPP ($P < 0.05$) and restored preference levels to saline controls (P = 0.788). Adolescent rats conditioned with 1.75 g/kg ethanol and cocaine showed neither a preference nor an aversion to the drug combination, when compared to saline controls (P = 0.310), as the 1.75 g/kg ethanol dose was sufficient to decrease but not block cocaine CPP (P = 0.08).

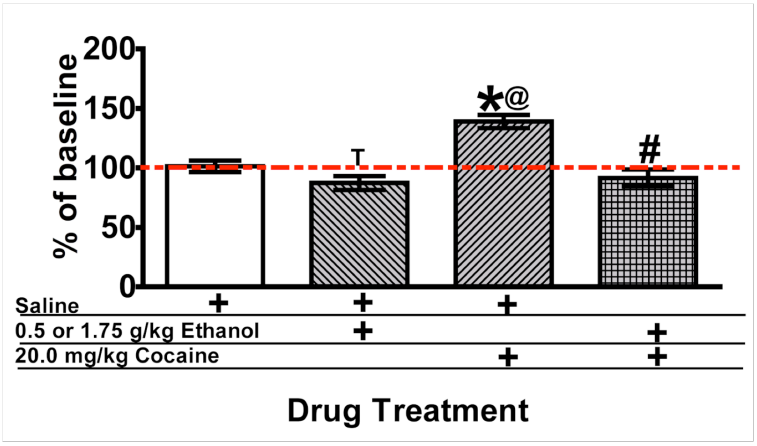

Figure 3. Ethanol Blocked Cocaine Reward in All Rats. Preference scores presented as percent change of Baseline at Test (mean +/- SEM). Overall, a significant 3-way interaction between Dose, Age and Drug was observed [$F(3, 150) = 4.651$, $P < 0.01$]. "T" denotes a trend from saline; * denotes a significant cocaine effect from saline controls @ denoting significant differences from ethanol only treated rats and # denoting significance from cocaine only treated rats. A cocaine CPP was observed (P = 0.000) and ethanol blocked this effect (P = 0.000).

Aim 2: The Role of NMDA Receptors in Ethanol and Cocaine Polysubstance Abuse

A biased CPP paradigm was utilized in the following two experiments to investigate the role of NMDA receptors in ethanol/cocaine reward, as observed in Aim 1 (see above). In Experiment 1, MK-801 (0.1 mg/kg or 0.2 mg/kg) and cocaine (20.0 mg/kg) were systemically administered in adolescent and adult rats in a fashion similar to Aim 1, with the exception that MK-801 pretreatment occurred 30 minutes prior to cocaine conditioning. This procedure has been shown to reliably attenuate cocaine CPP in adult male rats (Brown, Lee and Sorg, 2008). Detailed illustration for this experimental timeline is found in Figure 5. In Experiment 2, a separate group of rats underwent bilateral cannulation for intracerebral delivery of 1mM MK-801 into each side of the NAcc in adolescent and adult male rats. Detailed illustration for surgical procedures and drug conditioning are presented in Figure 10.

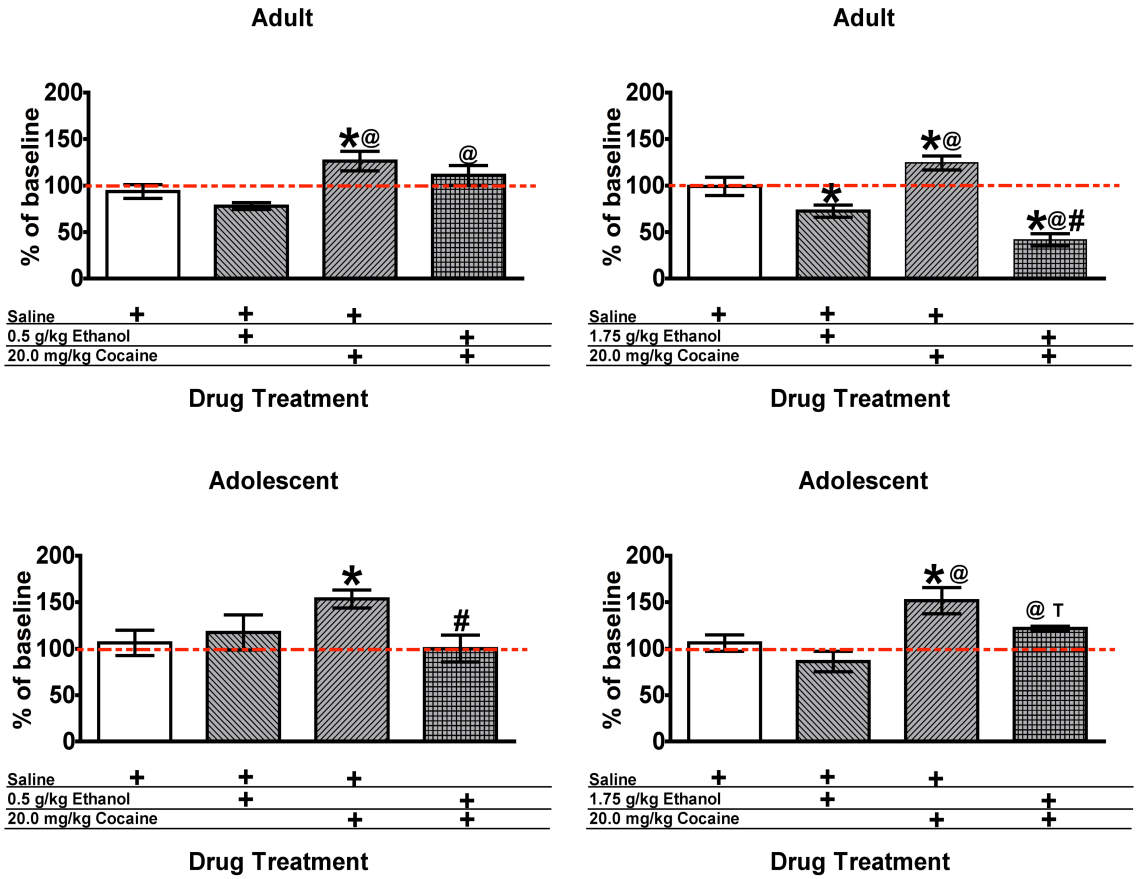

Figure 4. Escalating Doses of Ethanol Decreased Cocaine Reward in an Age-Dependent Fashion. Preference scores are presented as percent change of Baseline at Test (mean +/- SEM) for adult rats in the 0.5 and 1.75 g/kg Dose condition (Top left and Top right, respectively) and for adolescent rats in the 0.5 and 1.75 g/kg Dose condition (Bottom left and bottom right, respectively). * denotes significant differences from saline controls; @ denotes significant differences from ethanol-saline; # denotes significant differences from saline-cocaine; and "T" denotes a trend between saline-cocaine and 1.75 g/kg ethanol-cocaine adolescent drug-treatment group. Cocaine CPP was established across all conditions ($P < 0.05$). Adult rats displayed ethanol CPA following 1.75 g/kg ethanol conditioning ($P < 0.05$). Regardless of Dose, ethanol did not induce a CPA in adolescent rats (0.5 g/kg, $P = 0.603$ and 1.75 g/kg, $P = 0.158$). Adult rats were more sensitive to the opposing effects of 1.75 g/kg ethanol on cocaine reward (Figure 4B), while adolescent rats were more sensitive to the opposing effects of 0.5 g/kg ethanol on cocaine reward (Figure 4C).

Experiment 1: Systemic administration of MK-801 and cocaine

Aim. The purpose of the present experiment was to investigate the role of NMDA receptors as a potential mechanism mediating the opposing effects of ethanol on cocaine place preferences observed in Aim 1.

Methods.

Subjects. Fifty-three adolescent (PND 30) and sixty-five adult (PND 60) male rats, weighing between 100 and 370g, respectively, were used for experimentation. Breeding, selection and maintenance and treatment of rats were conducted identical to Aim 1 (see above).

Drugs and solutions. Cocaine hydrochloride (Sigma Aldrich Co., St. Louis, MO) was dissolved in pharmaceutical grade sterile saline (0.9%) to a concentration of 1.0 mg/ml and administered intraperitoneally (i.p.) at a dose of 20.0 mg/kg. The NMDA receptor antagonist MK-801 (Sigma Aldrich Co., St. Louis, MO) was dissolved in pharmaceutical grade sterile saline (0.9%) to a concentration of 1.0 mg/ml and administered at a dose of 0.1 or 0.2 mg/kg for systemic administration. MK-801 (0.1 or 0.2 mg/kg, i.p.) was administered 30 minutes prior to cocaine (20.0 mg/kg, i.p.) administration. Regardless of MK-801 dose, adolescent (PND 30) and adult (PND 60) rats were co-administered MK-801 (or saline equivalent) followed by 20.0 mg/kg cocaine (or saline equivalent; i.p.) within 30 minutes from each other, in this order. Control groups were administered two saline vehicle injections, a volume equivalent to appropriate drug dose. The doses and timing of drug injections and perfusions were chosen based on previous research demonstrating reliable cocaine place preference expression across development (Badanich and et al., 2006; Balda et al., 2006; Spyraki et al., 1982; Brown et al., 2008; for reviews see Bardo et al., 1995; Tzschentke, 1998; Tirelli et al., 2003), effectiveness of

systemic (Brown, Lee and Sorg, 2008) and intracerebral administration (Kawasaki et al., 2011) of MK-801 in blocking drug reward in adult male rats.

Place preference testing apparatus. The same 2-way place preference testing apparatus used in Aim 1 was used in the present experiment. Identical to previous procedures, initial pre-conditioning (Baseline) and post-conditioning preference (Test) assessments occurred in the testing apparatus, while drug conditioning was conducted in the compartment-specific conditioning apparatus.

Place preference conditioning apparatus. All rats underwent drug conditioning in the same place preference conditioning apparatus as previously described in Aim 1. Post-conditioning place preferences were conducted in the place preference testing apparatus.

Conditioning and testing procedures. The procedure for the present experiment was similar to those conducted in Aim 1 with the exception that MK-801 (0.1 or 0.2 mg/kg) was administered (i.p.) 30 minutes prior to cocaine conditioning (see Figure 5).

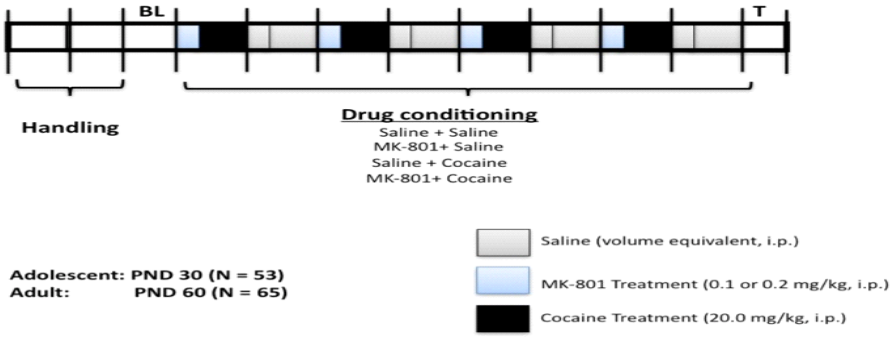

Figure 5. Aim 2 Experiment 1 Timeline. Schematic representation of the experimental timeline. Adolescent (PND 30; N = 53) and adult (PND 60; N = 65) underwent a two-day handling procedure, followed by a pre-conditioning assessment Baseline (BL), drug conditioning phase and a post-conditioning assessment Test (T).

In brief, fifty-three adolescent (PND 30) and sixty-five adult (PND 60) rats completed the 4 experimental phases: handling, Baseline, drug conditioning and Test. With the exception of handling (5 minutes each day), all other experimental phases were conducted in 15 minutes. Following Baseline, adolescent and adult, respectively, male rats were separated into 6 drug treatment groups: saline-saline (N = 8; N = 12); 0.1 MK-801-saline (N = 9; N = 12); 0.2 MK-801-cocaine (N = 8; N = 10); saline-cocaine (N = 8; N = 9); 0.1 MK-801-cocaine (N = 9; N = 12); and 0.2 MK-801-cocaine (N = 11; N = 10). A 20.0 mg/kg cocaine dose was used during cocaine conditioning, regardless of MK-801 pretreatment and Age condition. Rats were administered either dose of MK-801 (0.1 or 0.2 mg/kg) or a saline control equivalent and were placed into the respective homecage for 30 minutes. Following, rats were administered cocaine (20.0 mg/kg, i.p) or saline and placed into the least preferred (drug-paired) compartment for a 15-minute conditioning trial. MK-801 and cocaine control rats (saline-saline) were administered 2 saline injections in the same fashion. MK-801 pretreatment and cocaine conditioning occurred on Days 2, 4, 6, and 8 and on alternating days, all rats received 2 injections of saline (with the 30 minute homecage interval) followed by placement into the preferred (vehicle-paired) compartment for 15 minutes. Therefore, a total of 4 drug and 4 saline pairings in the drug-paired and vehicle-paired compartments, respectively, occurred throughout the 8-day conditioning phase.

On Day 10, all rats were placed into the testing apparatus in a drug-free state to examine post-conditioning place preference expression. Using the Noldus Ethovision® behavior tracking software, the amount of time (seconds) spent in each compartment during Baseline and Test was recorded and analyzed to determine cocaine CPP and MK-801 effectiveness in blocking cocaine place preferences within Age and Drug treatment groups. Identical analyses as those used in

Aim 1 were employed in the present experiment. An alpha level of $p < 0.05$ and a 95% CI were set to determine statistical significant effects of MK-801 (0.1 or 0.2 mg/kg, i.p.) on cocaine (20.0 mg/kg, i.p.) place preferences across development.

Data analysis . The amount of time (seconds) spent in each compartment on Baseline and Test were recorded and analyzed (See Table 2). A Univariate ANOVA examining the amount of time (seconds) spent in the least preferred (drug-paired) compartment was conducted to determine initial age-dependent differences in behavior prior to experimentation. All adolescent (N = 53) and adult (N = 65) rats in both Dose conditions were included in the analysis. Adolescent rats spent less time in the least preferred (drug-paired) compartment during the 15 minute Baseline assessment, as compared to adult rats [$F(1, 117) = 5.462$, $P < 0.05$] (See Figure 6).

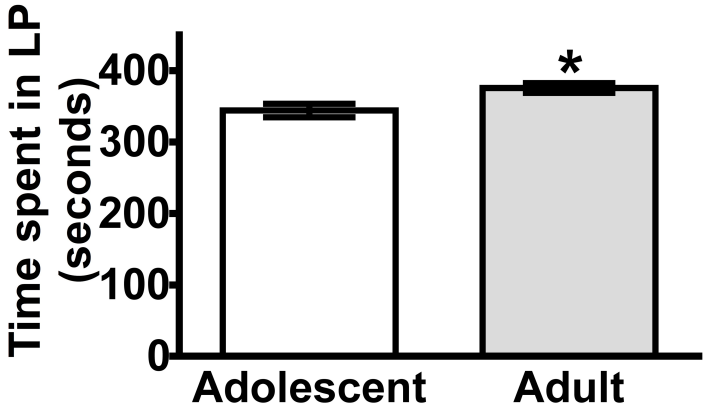

Figure 6. Initial Differences Prior to Drug Conditioning. Data are presented as group means for the amount of time spent in the LP compartment. Adolescent (PND 30; N = 53) rats spent less time in the least preferred (drug-paired), as compared to their adult (PND 60; N = 65) counterparts prior to experimentation [$F(1, 117) = 5.462$, $P < 0.05$]. * Denotes statistical significance.

Table 2. Means Table for Aim 2 Experiment 1. Data are presented as group means for the amount of time adolescent (N = 53) and adult (N = 65) spent in the least preferred (drug-paired) compartment (seconds).

	SALINE-SALINE		0.1 MK-801-SALINE		0.2 MK-801-SALINE		SALINE-COCAINE		0.1 MK-801-COCAINE		0.2 MK-801-COCAINE	
Adolescent	BL	T	BL	T	BL	T	BL	T	BL	T	BL	T
Mean	374.5	445.9	333	295.3	362	262.9	337.6	608.6	350.2	298	318.5	385
SD	64.6	35.7	79.3	95.2	43.5	68.4	74.2	96.2	54	117.6	84.5	46.2
N	8	8	9	9	8	8	8	8	9	9	11	11
Adult	BL	T	BL	T	BL	T	BL	T	BL	T	BL	T
Mean	409.3	422.9	364.3	394	372.2	425.5	353.9	518.9	357.1	456.4	398.4	421.7
STD	33.8	89.7	49.9	93.3	41.7	91.9	72.2	51.7	74.6	108.4	31.7	74.9
N	12	12	12	12	10	10	9	9	12	12	10	10

To control for age-dependent differences in the magnitude of initial place preference bias, data for the individual drug-treatment groups were transformed to percent change at Baseline (individual preference [(Tests score/ Baseline group mean)*100]. A 2(Age: adolescent, adult) x 6(Drug: saline-saline; 0.1 MK-801-saline; 0.2 MK-801-saline; saline-cocaine;

0.1 MK-801-cocaine; 0.2 MK-801-cocaine) ANOVA and appropriate post-hoc analysis (i.e. Fisher's LSD) were conducted to assess statistical significance across Age and Drug treatment conditions during the post-conditioning test. To determine the interaction effects between Age and Drug treatment at Test, subsequent independent ANOVAs with Age and Drug as the between-subject factors were conducted. Fisher's LSD post hoc tests were calculated to detect significant effects of Drug on conditioned behavior within drug-treatment groups at the time of Test. Separate Univariate ANOVAs were computed to investigate the effects of MK-801 and

20.0 mg/kg cocaine in isolation and in combination on conditioned behavior at Test. These analyses were carried out separately for adolescent and adult groups. A drug-induced CPP response was defined as a significant increase in the amount of time spent in the least preferred (drug-paired) compartment on Test relative to saline controls (Bardo et al., 1995). Behavior was also assessed to determine a MK-801-induced CPA response, which was defined as a significant decrease in the amount of time spent in the least preferred (drug-paired) compartment at Test relative to saline controls. All data were analyzed and graphed by using SPSS Version 21 statistical software and Graph Pad Prism, respectively. An alpha level of $p < 0.05$ and a confidence interval (CI) of 95% was set to determine statistical significance and indicate the range of values within the estimate for all analyses.

Results. Given that ethanol is an NMDA receptor antagonist (Lovinger, 1998) and NMDA receptors are implicated in the expression of cocaine place preferences in adult rats (Brown, Lee and Sorg, 2008), the purpose of the present experiment was to investigate the role of NMDA receptors as a potential mechanism mediating the opposing effects of ethanol on cocaine place preferences observed in Aim 1 (see Figure 4). A 2-way ANOVA with Age(2: adolescent and adult) and Drug(6: saline-saline; 0.1 MK-801-saline; 0.2 MK-801-saline; saline-cocaine; 0.1 MK-801-cocaine; 0.2 MK-801-cocaine) as the between subject factors was employed to analyze significant interactions and main effects between Age and Drug treatment following drug conditioning at the time of Test. Results demonstrated a significant 2-way interaction between Age and Drug on conditioned behavior at Test [$F(5, 117) = 8.965$, $P = 0.000$]. Significant main effects for the between-subject factor Drug [$F(3, 117) = 20.108$, $P = 0.000$] was observed. Fisher's LSD post hoc analyses showed significant effects of Drug

treatment between groups, regardless of Age (see Figure 7). Adolescent and adult rats conditioned to cocaine alone, exhibited CPP (saline-saline v. saline-cocaine, P = 0.000). A subsequent Univariate ANOVA, examining Age effects within Drug treatment, demonstrated a significant difference in the magnitude of cocaine CPP, with adolescent rats displaying a heightened cocaine CPP response, as compared to adult counterparts [$F(1, 16) = 10.406$, $P < 0.01$]. Rats conditioned with 0.2 mg/kg MK-801 alone demonstrated a trend towards a significant CPA (saline-saline v. 0.2 MK-801-saline, P = 0.070), with adolescent rats displaying greater sensitivity to the aversive properties of MK-801, as compared to the adult counterparts [$F(1, 17) = 15.521$, $P < 0.001$]. Repeated pretreatment of 0.1 mg/kg MK-801 neither induced an aversion nor a place preference, following conditioning (P = 0.171). Regardless of Age, 0.1 and 0.2 mg/kg MK-801 significantly blocked cocaine CPP preference, as a significant difference between saline-cocaine and both MK-801-cocaine treatment groups was observed ($P < 0.000$ and $P < 0.000$, respectively). In addition, 0.1 and 0.2 MK-801-cocaine treatment groups were not statistically significant from saline controls (P = 0.951 and P = 0.610, respectively), further supporting MK-801 effectiveness on blocking cocaine reward. Significant differences were not observed between 0.1 MK-801-cocaine and 0.2-MK-801-cocaine (P = 0.563). However, a Univariate ANOVA demonstrated differences between Age groups within both MK-801-cocaine Drug treatment groups, showing adolescent rats were more sensitive to the effects of 0.1 mg/kg MK-801 [$F(1, 20) = 9.319$, $P < 0.01$] and to 0.2 mg/kg MK-801 [$F(1, 20) = 4.267$, $P = 0.05$] on cocaine reward, as compared to adult counterparts. Results demonstrate a significant effect of MK-801 on cocaine reward, with adolescent rats responding with greater sensitivity, as compared to adult counterparts (see Figure 8).

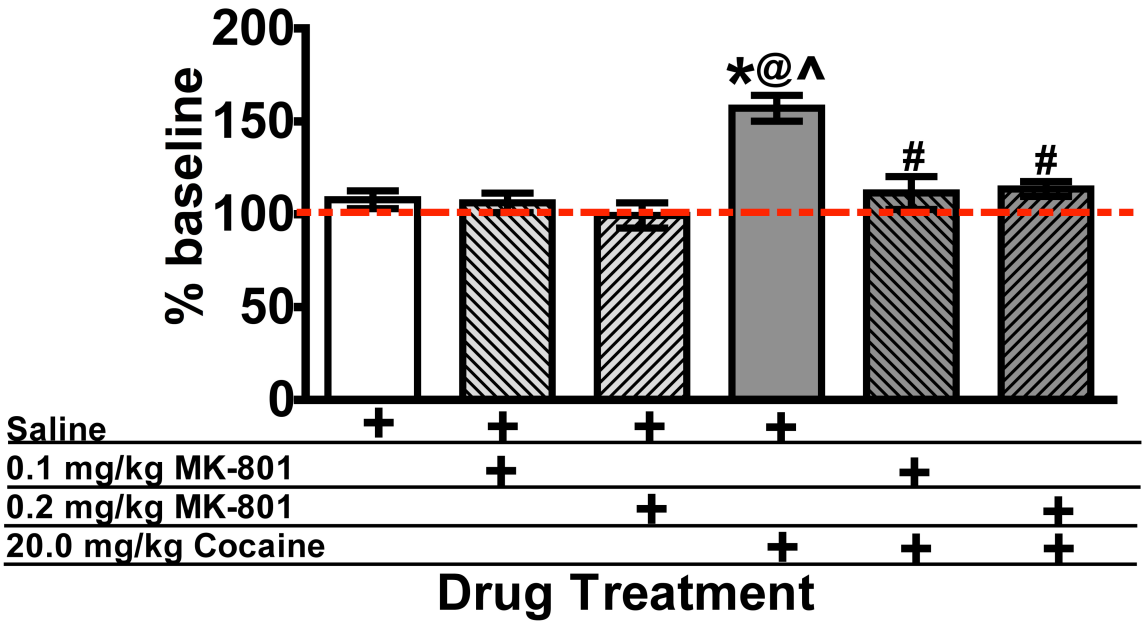

Figure 7. MK-801 Blocks Cocaine CPP. Preference scores are presented as percent change of Baseline at Test (mean +/- SEM) for rats in all Drug-treatment condition. * denotes significant differences from saline controls; @ denotes significant differences from 0.1 MK-801-saline; ^ denotes significant difference from 0.2 MK-801-saline; and # denotes significant differences from saline-cocaine. Overall, a main effect of Drug was observed on conditioned behavior at Test [$F(5, 117) = 20.108$, $P = 0.000$]. Cocaine CPP was established across all conditions ($P < 0.05$). Regardless of Dose, 0.1 and 0.2 MK-801 did not induce a CPA ($P = 1.603$ and $P = 0.07$, respectively), however both doses significantly blocked cocaine CPP ($P = 0.000$ and $P = 0.000$, respectively).

Individual Univariate ANOVAs were conducted to determine significant effects of Drug treatment [Drug (6: saline-saline; 0.1 MK-801-saline; 0.2 MK-801-saline; saline-cocaine; 0.1 MK-801-cocaine; 0.2 MK-801-cocaine)] within adolescent and adult Age groups at Test (see Figure 8).

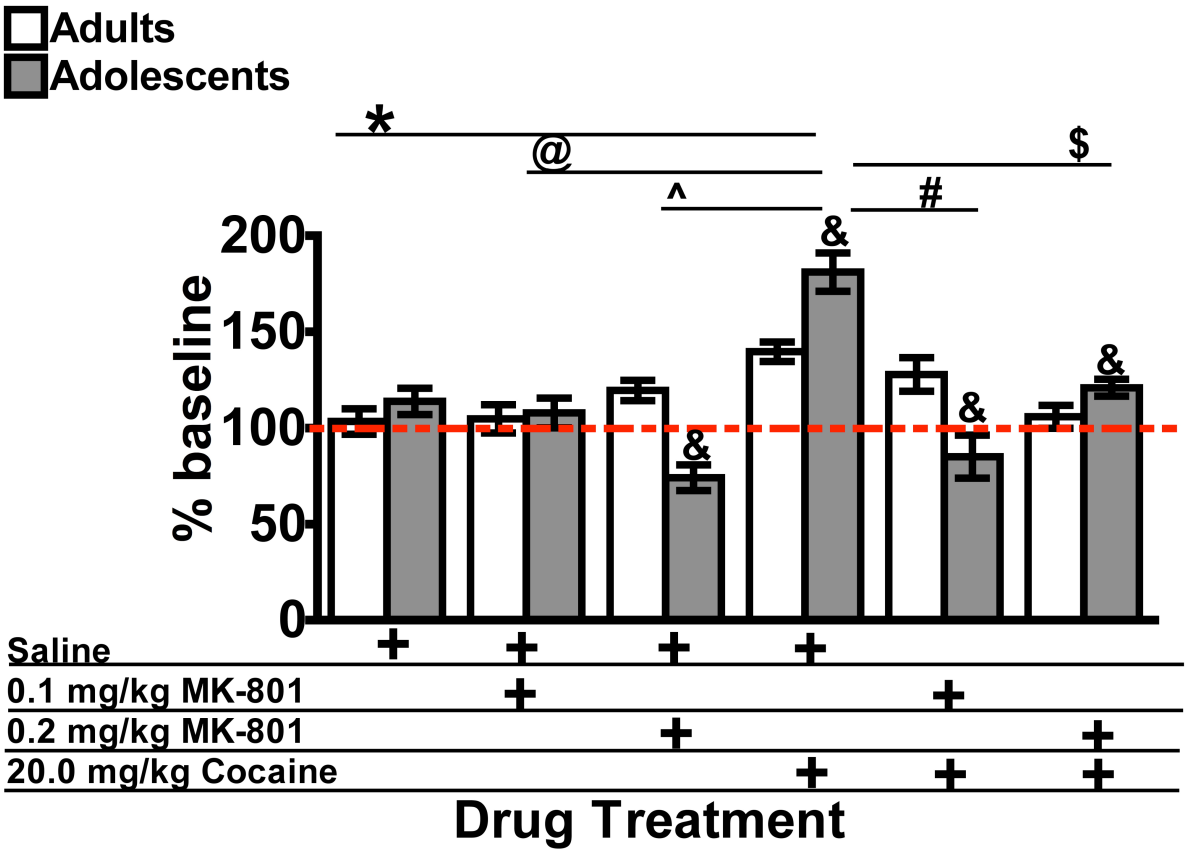

Figure 8. Age-Dependent Effects of MK-801 on Cocaine CPP. Preference scores are presented as percent change of Baseline at Test (mean +/- SEM) for rats in all Drug-treatment condition. * denotes significant differences from saline controls; @ denotes significant differences from 0.1 MK-801-saline; ^ denotes significant difference from 0.2 MK-801-saline; and # denotes significant differences from saline-cocaine at 0.1 MK-801-cocaine; $ denotes significant difference from saline-cocaine at 0.2 MK-801-cocaine; & denotes significant age-dependent differences. A significant 2-way interaction between Age and Drug treatment was observed on conditioned behavior at Test [$F(5, 117) = 8.965$, $P = 0.000$]. Cocaine CPP was established across all conditions ($P = 0.000$), with adolescent rats demonstrating greater sensitivity to the conditioned effects. Regardless of Dose, 0.1 and 0.2 MK-801 did not induce a CPA ($P = 1.603$ and $P = 0.07$, respectively), however both doses significantly blocked cocaine CPP ($P = 0.000$ and $P = 0.000$, respectively), with adolescent rats demonstrating greater sensitivity to MK-801 pretreatment on cocaine CPP (0.1 MK-801-cocaine, $P < 0.01$; 0.2 MK-801-cocaine, $P = 0.05$).

A significant effect of Drug treatment on conditioned behavior was observed in adolescent rats [$F(5, 52) = 22.556$, $P = 0.000$] (see Figure 9). LSD post hoc analyses demonstrated significant Drug effects between treatment groups. Specifically, 0.1 and 0.2 mg/kg MK-801 induced CPAs, as adolescent rats within the 0.1 MK-801-saline and the 0.2 MK-801-saline treatment groups spent significantly less time in the drug-paired compartment, as compared to saline controls ($P < 0.01$ and $P = 0.000$, respectively). Adolescent rats conditioned to cocaine alone demonstrated cocaine CPP, as the saline-cocaine treatment group spent more time in the drug-paired compartment on Test day, relative to saline controls ($P = 0.000$). The aversive properties of 0.1 mg/kg MK-801 blocked the rewarding properties of cocaine in adolescent rats pretreated with 0.1 mg/kg of MK-801 and conditioned to cocaine, as significant differences between saline-cocaine and 0.1 MK-801-cocaine ($P = 0.000$) and saline controls were observed ($P < 0.01$). The aversive properties of 0.2 mg/kg MK-801 also blocked cocaine CPP and eliminated place preference bias as significant differences were observed between saline-cocaine and 0.2 mg/kg MK-801 ($P = 0.000$) but not saline controls ($P = 0.868$). The magnitude of MK-801 effectiveness on blocking cocaine reward was statistically significant between the two MK-801 doses. Specifically, cocaine CPP was more sensitive to disruption following repeated 0.1 MK-801 pretreatment, as compared to the higher dose ($P < 0.01$). Disruption of cocaine CPP with 0.1 mg/kg MK-801 decreased the rewarding properties of cocaine to levels below those observed in adolescent rats pretreated with 0.2 mg/kg MK-801 and conditioned with cocaine ($P < 0.01$) and to saline controls ($P < 0.01$), thereby demonstrating drug-induced CPA within this treatment condition.

Significant Drug effects were observed in adult rats following drug conditioning [$F(5, 64) = 4.934$, $P = 0.001$] (see Figure 9). Systemic MK-801 pretreatment blocked cocaine CPP in

adult rats pretreated with 0.2 mg/kg MK-801 ($P = 0.000$) but not following repeated 0.1 mg/kg MK-801 pretreatment ($P < 0.08$). Dose-dependent differences in MK-801 effectiveness on cocaine CPP is observed when comparing 0.1 mg/kg MK-801-cocaine and 0.2 mg/kg MK-801-cocaine ($P < 0.05$). Furthermore, rats pretreated with the higher dose of MK-801 and conditioned with cocaine were not statistically significant from saline controls ($P = 0.846$), unlike rats pretreated with the lower dose of MK-801 and conditioned with cocaine ($P < 0.05$). Regardless of Dose, MK-801 administered alone or in combination did not induce CPA in adult rats, when compared to saline controls (0.1 or 0.2 mg/kg MK-801-saline: $P = 0.658$ and $P = 0.306$, respectively; 0.1 or 0.2 mg/kg MK-801-cocaine: $P < 0.05$ and $P = 0.846$, respectively).

Overall, age-dependent differences were observed in cocaine CPP sensitivity to MK-801 pretreatment. Adolescent rats displayed greater sensitivity to MK-801 pretreatment on cocaine reward, as compared to the adult counterparts.

Experiment 2: MK-801 Microinfusions into the NAcc

Aim. The aim of the current experiment was to assess the involvement of NMDA receptors within the NAcc in the age-dependent modulating effects of ethanol on cocaine reward.

Methods.

Subjects. Fifty-six adolescent and sixty adult at PND 30 and PND 60, weighing between 100 and 370g at the start of the experiment, respectively, were used for experimentation. Breeding, husbandry, selection, maintenance and treatment of rats were conducted identical to Aim 1 and Aim 2 Expriment 1 (see above), with the exception of bilateral intracranial cannulation into the NAcc (see Surgical Procedures).*Drugs and solutions.*

Drugs and solutions. Cocaine hydrochloride (Sigma Aldrich Co., St. Louis, MO) was dissolved in pharmaceutical grade sterile saline (0.9%) to a concentration of 1.0 mg/ml and administered intraperitoneally (i.p.) at a dose of 20.0 mg/kg. For Experiment 2, the NMDA receptor antagonist MK-801 was prepared in pharmaceutical grade saline to a concentration of 1mM and bilaterally perfused into the NAcc at a flow rate of 0.5µl/min for 1 min/side. Control groups for perfusion (Sham) and surgical, drug-treatment procedures (non-manipulated) were not administered saline or cocaine throughout 8 day conditioning phase. The doses and timing of drug injections and perfusions were chosen based on previous research demonstrating reliable cocaine place preference expression across development (Badanich and et al., 2006; Balda et al., 2006; Spyraki et al., 1982; Brown et al., 2008; for reviews see Bardo et al., 1995; Tzschentke, 1998; Tirelli et al., 2003), effectiveness of intracerebral administration of MK-801 in blocking drug reward in adult male rats (Kawasaki et al., 2011).

Surgical procedures. Adolescent (PND 30) and adult (PND 55) male rats were transported to the laboratory, separated from their litter mate, weighed and administered a weight-appropriate dose of ketamine (1.0 mg/kg, i.p) and xylazine (0.15 mg/kg, i.p) to induce anesthesia. For each surgery, the head of the rat was shaved and excess fur was removed. The rat was mounted onto the stereotaxic apparatus and an aseptic surgical area was prepared with the application of a germicidal scrub followed by an ethanol rinse.

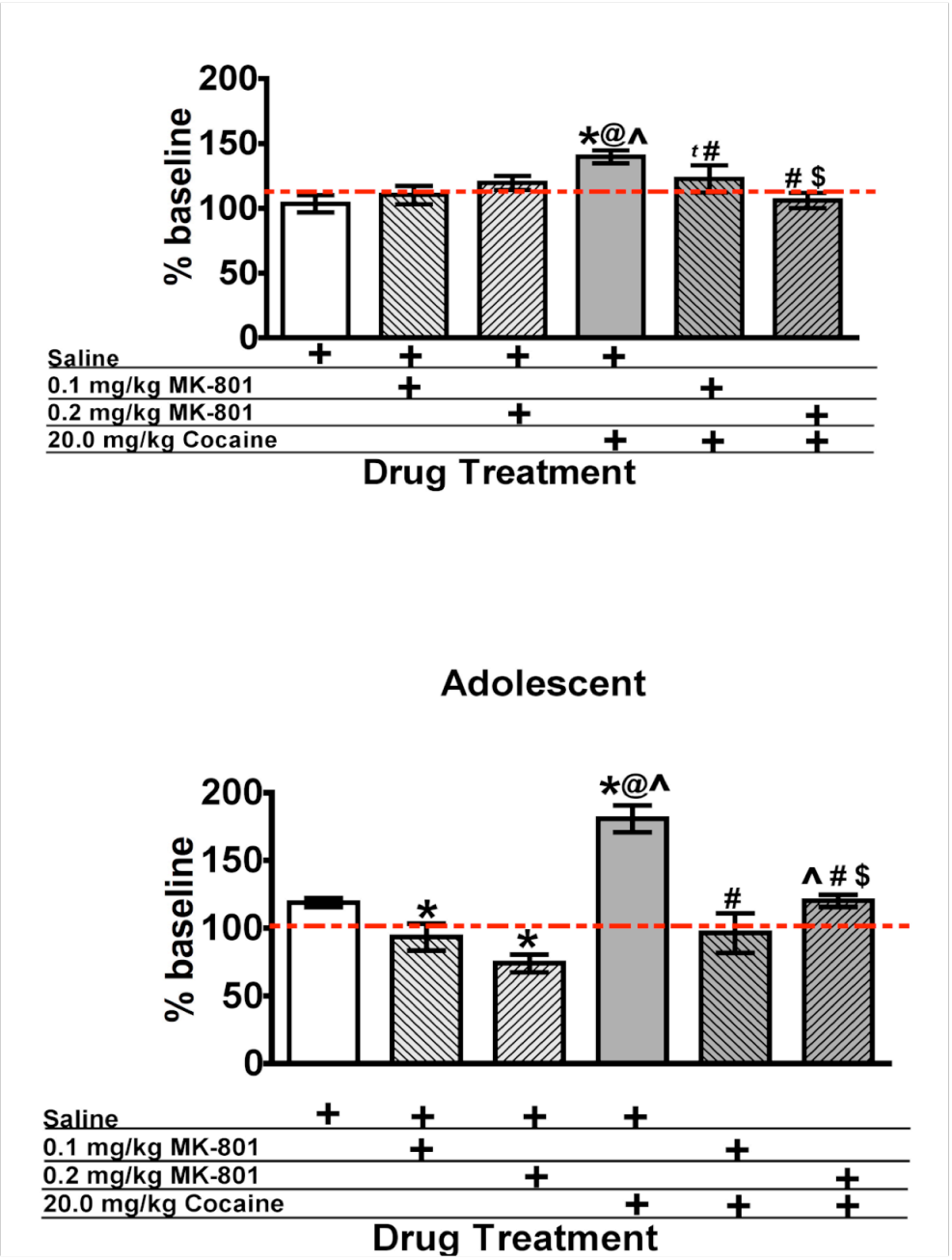

Figure 9. Adolescent Rats Were More Sensitive to MK-801 Disruption of Cocaine CPP. Preference scores are presented as percent change at Test (mean +/- SEM) for adult (top) and adolescent rats (bottom). An interaction between Age (2) and Drug (6) [$F(5, 117) = 8.965$, $P = 0.000$] and Drug treatment [$F(5, 117) = 20.108$, $P = 0.000$] were observed. * significant differences from saline controls; @ significant differences from 0.1 MK-801-saline; ^ significant differences from 0.2 MK-801-saline; # significant differences from saline-cocaine; $ significant difference from 0.1 MK-801-cocaine; and "T" denotes a trend between saline-cocaine and 0.1 MK-801-cocaine adult drug-treatment group. Cocaine CPP was established across all conditions ($P < 0.000$). 0.2 but not 0.1 mg/kg MK-801 significantly blocked cocaine CPP in adult rats. 0.1 and 0.2 mg/kg MK-801 blocked cocaine CPP in a dose-dependent manner in adolescent rats.

The surgical area was painted with an iodine solution and the cranial vault was draped with a sterile, disposable, surgical cloth. A sagittal incision was made over the skull and all tissue was pushed as far to the side as possible to expose bregma and lambda. Tissue was infiltrated with 2.5 mg/ml of bupivacaine hydrochloride. Skin was clamped away from the top of the skull to isolate the surgical area. Once accurate skull placement was ensured, the position of the guide cannula over bregma was zero-ed. Two holes were drilled into the left posterior and right posterior quadrants of the skull, lateral to the midline, for a total of 4 skull screw placements. Two holes were drilled for cannula implantation. Stereotaxic placement of the guide cannula, targeting the NAcc was based on individual weights (Philpot et al., 2001). The dura matter was gently retracted, exposing the outer layer of cortex. A guide cannula and accompanied dummy cannula, manufactured for CMA 11 (outer diameter 0.6 mm) probe insertion, was lowered at a rate of 1 mm per 5 minutes into the brain, targeting the NAcc on the left and right hemispheres. Slowly introducing the cannula into the brain was conducted to prevent rupture of blood vessels and unnecessary inflammatory processes, which may negatively impact results. Since the adolescent rat undergoes rapid, physical development and surgical procedures took place 16 days prior to termination of all testing procedures (including additional testing conducted for Aim 3), extensive brain growth was expected. Therefore, to optimize the probability of maintaining cannula placement within the NAcc throughout the experimental timeline, weight-based measurements and adjustments determined by Philpot and colleagues (2001; A:P +2.11, M:L + 0.46, D:V -6.60), targeting the NAcc were used in surgical procedures. In addition, further adjustments were made to the M:L weight-based coordinates to permit implantation of cannula at an 8 degrees angle. Entry at an angle ensured both cannula were successfully implanted without touching each other. A small amount of gel foam was applied to

the surface of cortex, prior to securing the guide cannulae with cyanoacrylate adhesive. Due to the longetivity of surgical procedures, a booster of ketamine (i.e. 1/3 of the original dose previously injected to induce anesthesia) was administered as necessary. At the termination of surgical procedures, the rat was gently removed from the stereotaxic apparatus and monitored until purposeful movement was observed for approximately 30 minutes. The typical recovery rate from anesthesia was approximately 60 +/- 10 minutes and 45 +/- 10 minutes for adolescent and adult rats, respectively. Following the experiment, rats were individually housed in a new homecage and a 4-day recovery period was in place (see Figure 9 for timeline).

Recovery. Adolescent and adult animals underwent a recovery period of 4 days (PND 31-34 or PND 56-59, respectively) to acclimate to the affixed guide cannula and to ensure adequate recovery for experimental procedures. All animals were handled for 20 minutes total during the recovery phase to re-establish a non-stressful state during experimenter handling (Marinelli and Piazza, 2002; Maldonado and Kirstein, 2005).

Place preference testing apparatus. Identical to previous experiments, a 2-way place preference testing apparatus was used during initial pre-conditioning (Baseline) and post-conditioning preference (Test) assessments. Drug conditioning was conducted in the place preference conditioning apparatus.

Place preference conditioning apparatus. All rats underwent drug conditioning in the same place preference conditioning apparatus, as previously described. Post-conditioning place preferences were conducted in the place preference testing apparatus.

Neutral habituation apparatus. All rats, regardless of treatment or surgical condition were placed into the neutral habituation apparatus, which was a clear Plexiglas ® bowl [40 cm (diameter) x 50 cm (height)], mounted onto the Raturn® table (Bioanalystical Systems, Inc.,

Indianapolis, IN), permitting unrestricted movement of the rat. The bowl consisted of bedding distinct from the homecage. The neutral habituation apparatus served as a holding area during microinfusion pretreatment procedures.

MK-801 microinfusion pretreatment procedures. In lieu of systemic MK-801 administration, rats were transported to the laboratory, weighed and placed into the neutral habituation apparatus for MK-801 microinfusion pretreatment procedures. CMA 11 probes without membrane were continuously perfused for 5 minutes with 1mM MK-801 or 0.9% saline at a flow rate set at 0.5µl/minute. Adequate flow was confirmed by collecting a small sample of the perfusate and measuring the output volume following a 10-minute time period. If the total output volume did not equate to 5µl, following the 10-minute collection time period, appropriate testing of PE tubing and probe function was assessed. Failed probes or PE-50 tubing were replaced and adequate flow was determined once again. The probes were connected PE-50 tubing, which was affixed to an automatic pump-activated, gastight syringe, motorized to permit steady perfusion of 1mM MK-801 or saline during microinfusion pretreatment procedures. Following, both stylets were removed and the functional CMA 11 probe was inserted. 1mM MK-801 or saline was perfused directly into the NAcc of the right hemisphere at a flow rate of 0.5µl/minute. A 1-minute diffusion period occurred and then identical procedures were repeated for the left side. After microinfusion procedures were completed for both sides of the NAcc, the probes were removed and stylets were replaced. Rats then immediately returned to their homecage for a 30-minute time period, prior to drug conditioning procedures. Rats scheduled for 1mM MK-801 microinfusion pretreatment were treated on drug-conditioning days (days 2, 4, 6,and 8). On alternating days, 0.9% saline was perfused into the NAcc. Rats within the MK-801 control condition, were administered 0.9% saline on days 1-9.

An appropriate control group was in place to control for microinfusion procedures. Adolescent (N = 9) and adult (N = 11) rats within the Sham condition underwent bilateral cannulation and identical procedures for microinfusion. However probes inserted into the NAcc of Sham rats were not perfused with 1mM MK-801 or saline. To control for surgical and drug administration procedures, a final Non-manipulated group of adolescent (N = 10) and adult (N = 10) rats was run. Non-manipulated rats did not undergo surgical procedures and drug administration, however all other procedures remained the same.

Conditioning and testing procedures. The procedures for the present experiment were similar to those conducted in Aim 2 Experiment 1 in which all rats in Aim 2 Experiment 2 completed the pre-conditioning (Baseline) assessment, the 8 day drug conditioning phase and post-conditioning (Test) assessment (see Figure 10 for timeline). The 15-minute Baseline assessment determined the least preferred, drug-paired, compartment during the conditioning phase. During the conditioning phase, adolescent and adult cannulated rats that underwent 1.0 mM MK-801 (or saline) pretreatment, were conditioned with either cocaine (20.0 mg/kg, i.p.) or saline with the least preferred (drug-paired) compartment on days 2, 4, 6, and 8 for 15 minutes. On alternating days, drug-treated rats were pretreated with saline (microinfusion), administered saline systemically (i.p.) and placed in the preferred (vehicle-paired) compartment. Sham rats underwent identical procedures, however neither cocaine nor saline were administered. Instead, a syringe with needle tip removed was gently placed in the intraperitoneal area, mimicking systemic administration. These procedures were carried out through the entire conditioning phase. Rats within the Non-manipulated group were simply placed in the least preferred compartment on days 2, 4, 6 and 8 and then in the preferred compartment on alternating days. Therefore based on all conditions (surgery, perfusion, drug treatment), adolescent (N = 56) and

adult (N = 60) rats, respectively, were scheduled into one of the following 6 conditions: Non-manipulated (N = 10; N = 10); Sham (N = 9; N = 9); saline-saline (N = 10; N = 11), MK-801-saline (N = 9; N = 9), saline-cocaine (N = 8; N = 9), and MK-801-cocaine (N = 10; N = 10).

Data analysis. The amount of time (seconds) spent in each compartment on Baseline and Test were recorded and analyzed for all Age groups and Drug treatment conditions (See Table 3). Initial Age differences were not observed at Baseline, following an Univariate ANOVA analysis, examining the amount of time (seconds) spent in the least preferred (drug-paired) compartment on Baseline day [$F(1, 115) = 0.050$, $P = 0.824$]. However, significant differences secondary to surgical procedures were observed [$F(1, 115) = 3.114$, $P = 0.01$], as LSD post hoc analyses demonstrated a significant difference between Non-manipulated controls and Sham rats ($P < 0.05$; see Figure 11).

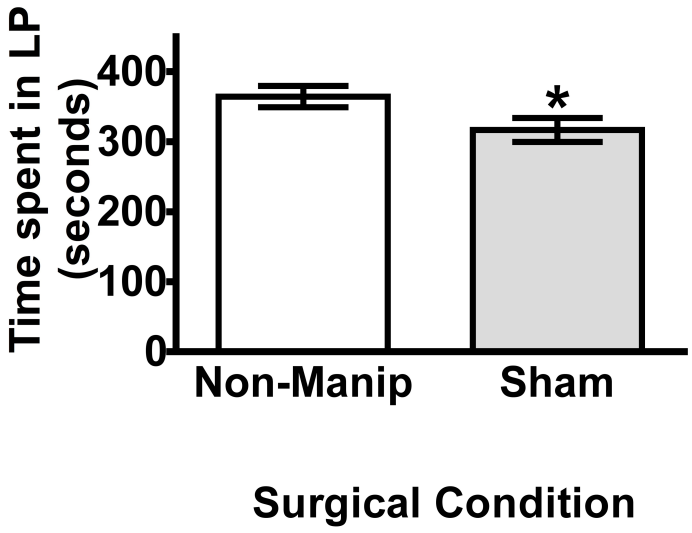

Figure 11. Initial Differences Prior to Experimentation Are Attributed to Surgical Procedures. Data are presented as means +/- SEM for the amount of time (seconds) spent in the least preferred (drug-paired) compartment on Baseline day for the non-manipulated and Sham surgical conditions. Regardless of Age, surgical procedures impacted initial preference bias to the preferred compartment at Baseline [$F(1, 115) = 3.114$, $P = 0.01$]

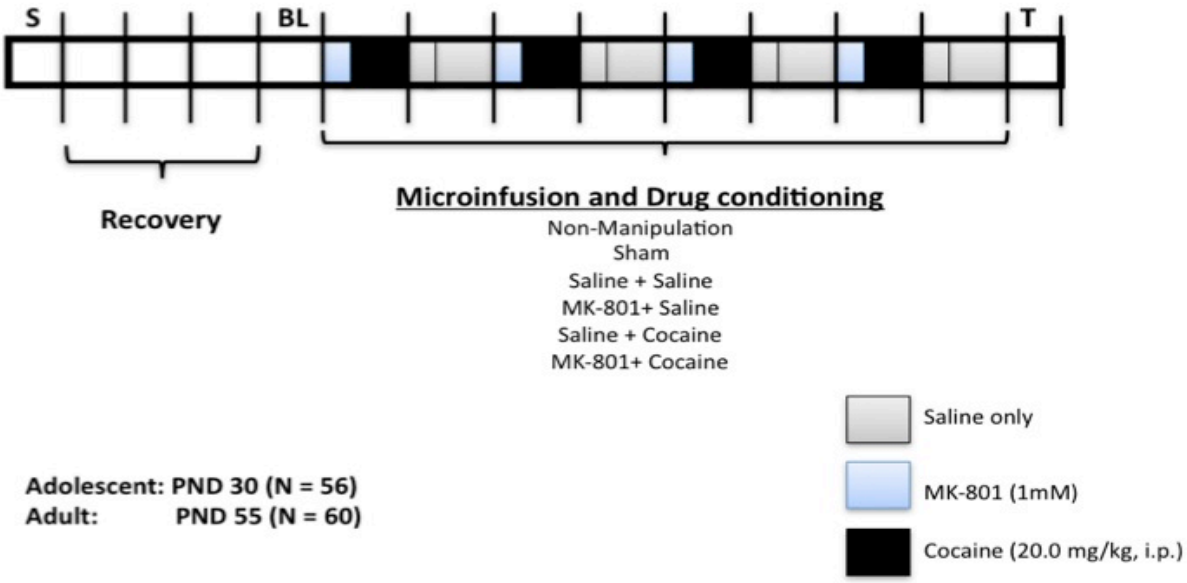

Figure 10. Aim 2 Experiment 2 Timeline. Schematic representation of the experimental timeline. Adolescent (PND 30; N = 72) and adult (PND 60; N = 79) underwent surgical procedures for bilateral cannula implantation into the NAcc (S), a 4-day recovery period (including handling) followed by a pre-conditioning assessment Baseline (BL), drug conditioning phase and a post-conditioning assessment test (T).

Table 3. Means Table for Aim 2 Experiment 2. Data are presented as group means for the amount of time adolescent (N = 56) and adult (N = 60) spent in the LP compartment (seconds).

	NON-MANIP		SHAM		SALINE-SALINE		MK-801-SALINE		SALINE-COCAINE		MK-801-COCAINE	
Adolescent	BL	T	BL	T	BL	T	BL	T	BL	T	BL	T
Mean	330.7	412.5	319	427.7	385.8	458.4	320.7	494	321	564	351.8	500.3
SD	78.7	34.7	77	115.5	52.8	116.3	93	167	70.6	86	62.7	162.1
N	10	10	9	9	10	10	9	9	8	8	10	10
Adult	BL	T	BL	T	BL	T	BL	T	BL	T	BL	T
Mean	398.4	461.5	315	419	381.1	493.8	324.4	482.7	310.6	562.3	319.7	474.5
STD	61	55.4	79.8	112	61	55.4	82.7	93.1	81.3	98.4	63.4	101.9
N	10	10	11	11	11	11	9	9	9	9	10	10

To control for differences secondary to surgical procedures, data for all groups were transformed to percent of Non-manipulated at Test for the respective Age condition [(Individual Test score/ group mean of Non-manipulated condition at Test)*100]. Therefore, the group means for the non-manipulated groups within the adolescent and adult Age condition at Test were normalized to 0. The amount of time spent in the least preferred (drug-paired) compartment at Test, was expressed as percent of non-manipulated at T and were compared between Sham and Drug-treatment groups (saline-saline; MK-801-saline, saline-cocaine, MK-801-cocaine) to the Age-respective Non-manipulated group. A 2 (Age: adolescent, adult) x 6 (Drug: non-manipulated; Sham; saline-saline; MK-801-saline; saline-cocaine; MK-801-cocaine) ANOVA and appropriate post-hoc analysis (i.e. Fisher's LSD) were conducted to assess statistical significance within and across Age and Drug treatment conditions during the post-conditioning test. Fisher's LSD post hoc tests were calculated to detect significant effects of Drug treatment on conditioned behavior within drug-treatment groups at the time of Test. Separate Univariate ANOVAs were computed to investigate the effects of intra-accumbal MK-801 and 20.0 mg/kg cocaine in isolation and in combination on conditioned behavior at Test within Age groups. Cocaine CPP was defined as a significant increase in the amount of time spent in the least preferred (drug-paired) compartment on Test relative to Non-manipulated controls. CPA defined as a significant decrease in the amount of time spent in the least preferred (drug-paired) compartment at Test relative to Non-manipulated controls was also assessed among all Drug treatment groups. Data were analyzed and graphed by using SPSS Version 21 statistical software and Graph Pad Prism, respectively. An alpha level of $p < 0.05$ and a confidence interval (CI) of 95% was set to determine statistical significance and indicate the range of values within the estimate for all analyses.

Results. The aim of the current experiment was to expand findings from Aim 1 and Aim 2 Experiment 1 in the investigation of NMDA receptors within the NAcc, as a potential mechanism contributing to the conditioned motivational effects of ethanol/cocaine polysubstance abuse across development. It was hypothesized that microinfusion of MK-801 into the NAcc would block cocaine CPP expression in an Age-dependent manner similar to that observed with ethanol-cocaine co-administration and MK-801-cocaine co-administration. Results demonstrated a significant effect of inra-accumbal MK-801 pretreatment on cocaine reward, regardless of Age (see Figure 12).

A 2-way ANOVA with Age (2: adolescent and adult) and Drug (6: non-manipulated; sham; saline-saline; MK-801-saline; saline-cocaine; MK-801-cocaine) as the between subject factors demonstrated a significant main effect of Drug treatment on conditioned behavior at Test, regardless of Age [$F(5, 115) = 4.006$, $P < 0.01$]. Microinfusion of MK-801 directly into the NAcc of rats effectively blocked cocaine CPP, as a significant decrease in the amount of time spent in the drug-paired compartment was observed in MK-801-cocaine rats, as compared to rats within the saline-cocaine condition ($P < 0.05$). A significant disruption rather than a mere attenuation of cocaine CPP following MK-801 pretreatment was noted, as the amount of time spent in the drug-paired compartment was not significantly different from saline controls ($P = 0.707$). A trend towards significance was observed in MK-801-cocaine rats, as compared to sham ($P = 0.06$) and non-manipulated controls ($P = 0.08$).

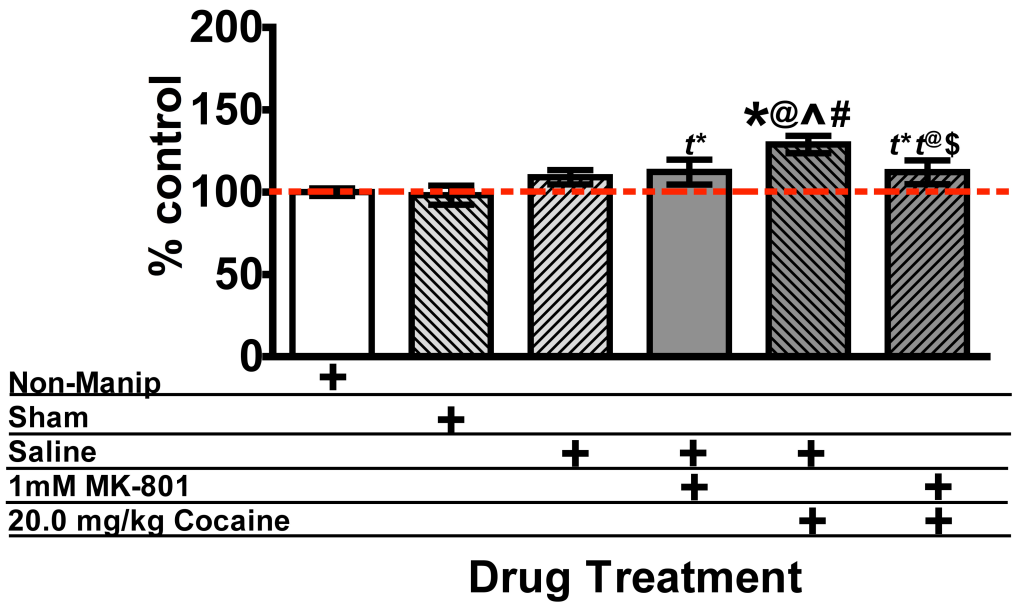

Figure 12. MK-801 Microinfusions into the NAcc Blocked Cocaine CPP. Repeated MK-801 microinfusions into the NAcc of rats disrupted cocaine CPP, regardless of Age [$F(5, 115) = 4.006$, $P < 0.01$]. * denotes significant difference from Non-manipulatated controls; @ denotes significant difference from Sham controls; ^ denotes significant difference from saline controls; # denotes significant difference from MK-801-saline controls; $ denotes significant difference from saline-cocaine; t* denotes trend towards significance from Non-manipulated controls; and $t^{@}$ denotes trend towards significance from Sham controls.

Aim 3: Identifying the Role of NMDA Receptors in Acute and Repeated Ethanol and Cocaine Polysubstance Abuse

Aim

Results from Aim 1 and 2 suggest ethanol decreases cocaine reward at the NMDA receptor site, as systemic treatment and intra-accumbens treatment with the NMDA antagonist MK-801 effectively blocked cocaine CPP. Drug effects were expressed as an interaction between Dose and Age. Findings depict a role for NMDA receptors following repeated polysubstance abuse, rather than acute exposure. Therefore, the aim of the below experiment was to examine the impact of NMDA receptor antagonism on cocaine reward, following acute MK-801 (i.p. and microinfusion) pretreatment. A second aim was to investigate long-term

effects of repeated NMDA antagonism with MK-801 pretreatment in blocking cocaine reward, following a cocaine challenge. A third aim was to explore potential Age and Dose-dependent effects in the involvement of NMDA receptors during acquisition and expression in a reactivation-dependent manner (Brown, Lee and Sorg, 2008; Sorg, 2012). In order to address these aims, rats from Aim 2 Experiments 1 and 2 remained in the experiment following the post-conditioning Test for additional testing. The extended experimental timeline for the current experiment is illustrated in Figure 13.

Methods

Additional testing was conducted on adolescent and adult rats from Aim 2 Experiments 1 and 2 (N = 53 and 65; N = 54 and 54, respectively) to examine the role of NMDA receptors in the acquisition and expression of ethanol/cocaine polysubstance reward across developmental time periods. Drug preparation, doses, treatment regimen and testing conditions were identical to those previous described in Aim 2. Briefly, rats were either pretreated with MK-801 systemically (0.1 or 0.2 mg/kg, i.p.; Experiment 1) or directly into the NAcc (1mM MK-801/0.5 µl/minute/side; Experiment 2) prior to cocaine conditioning in the least preferred (drug-paired) compartment. Following drug conditioning, all rats were assessed for drug preferences. Twenty-fours hours following the post-conditioning assessment (Test), all rats regardless of previous drug treatment (with the exception of the Non-manipulated rats in Experiment 2) were administered 20.0 mg/kg cocaine (i.p.) and placed into the place preference

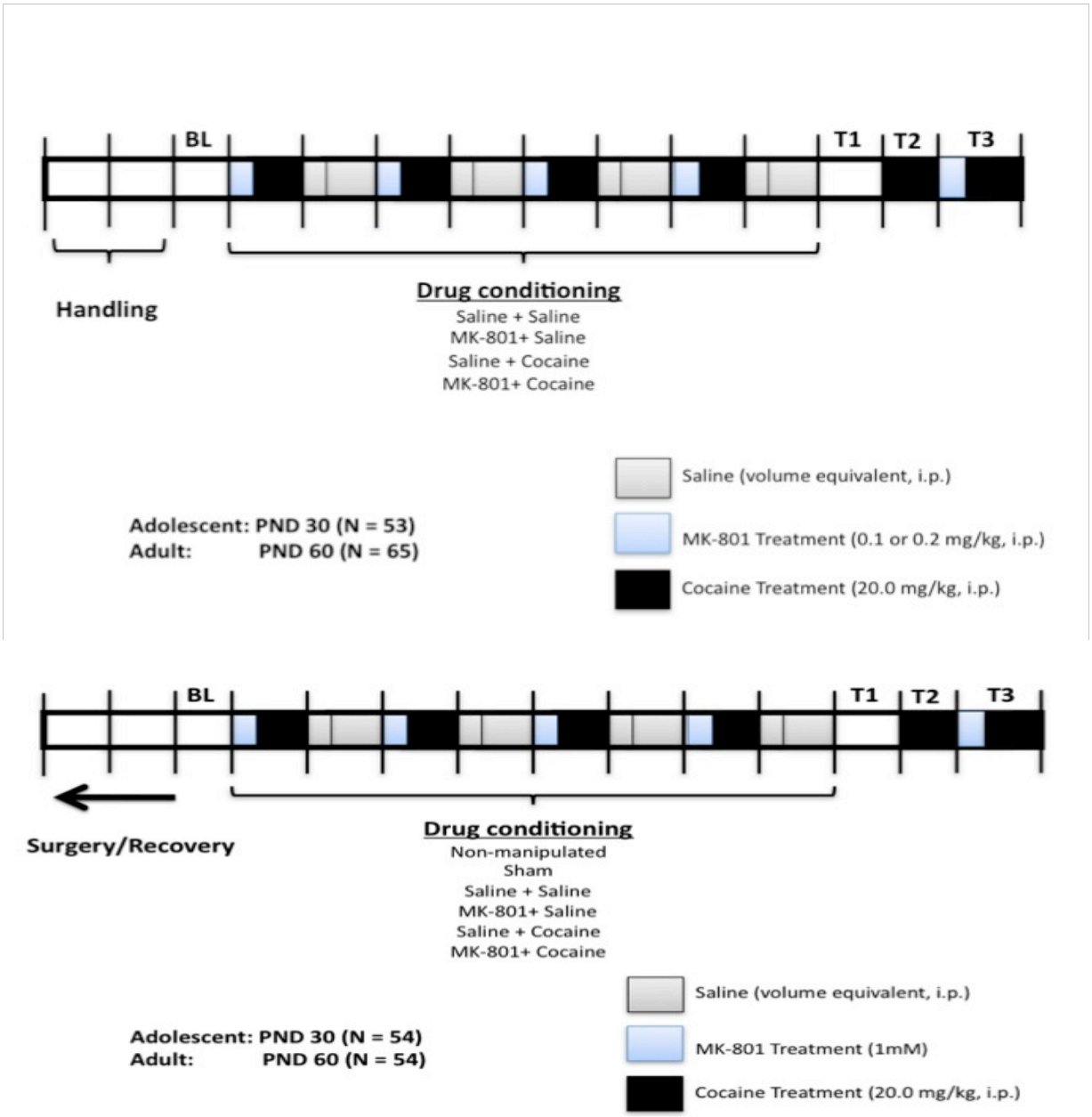

Figure 13. Aim 3 Experimental Timelines. Schematic representation of the experimental timelines. Following drug conditioning, adolescent and adult rats underwent the post-conditioning assessment (T1) followed by 2 additional assessments (T2 and T3). Rats were tested in a drug-free state on Test 1 (T1). On Test 2 (T2) all rats (except for non-manipulated controls in Panel B) were administered cocaine (20.0 mg/kg, i.p.) prior to testing. All rats, except for non-manipulated controls, were administered MK-801 and cocaine prior to Test 3 (T3).

testing apparatus for 15 minutes (Test 2). Non-manipulated rats were placed into the testing apparatus, however cocaine was not administered. On the next day, all rats were administered MK-801 (0.1 or 0.2 mg/kg, i.p. or 1mM MK-801) depending on previous treatment condition, followed by systemic administration of cocaine (20.0 mg/kg, i.p.) and placed in the testing apparatus for the 15 minutes Test 3 session. For Sham rats, perfusion of 1mM Mk-801, followed by cocaine administration (i.p.) occurred prior to Test 3. Non-manipulated rats neither received MK-801 nor cocaine treatment prior to Test 3. The amount of time (seconds) spent in the drug-paired compartment was recorded and assessed.

Histological Procedures

Following the termination of Test 3 procedures, all rats were euthanized with carbon dioxide and brains were removed, flash frozen and stored in a -80 freezer until sectioning. Brain tissue was sectioned at 40 microns, using a cryostat, and then stained with cresyl violet to confirm cannula placement within the NAcc (see Figure 16). Data collected from rats with correct placement were used in all analyses.

Data Analysis

The amount of time spent in the drug-paired compartment on Test 1, Test 2 and Test 3 were assessed separately for rats from Aim 2 Experiment 1 and Experiment 2.

Experiment 1. The amount of time spent in the drug-paired compartment was expressed as percent of Baseline. A 3-way repeated measures ANOVA with Age(2: adolescent and adult) and Drug(6: saline-saline; 0.1 MK-801-saline; 0.2 MK-801-saline; saline-cocaine; 0.1 MK-801-cocaine; 0.2 MK-801-cocaine) as the between subjects factors and Session(3: Test 1,

Test 2, Test 3) as the repeated measures, within subject factor was used to determine drug-induced changes in behavior. Fisher's LSD post hoc analyses were used when appropriate to determine significant effects of Drug treatment within Session conditions.

Experiment 2. Time (percent of Non-manipulated at Test) across Drug treatment groups was analyzed with a 3-way repeated measures ANOVA [Age(2: adolescent and adult) x Drug(6: non-manipulated, sham; saline-saline; MK-801-saline; saline-cocaine; MK-801-cocaine) x Session(3: Test 1, Test 2, Test 3)] to assess significant differences in behavior from non-manipulated. Drug effects within Test sessions were determined with Fisher's LSD post hoc analyses. For both experiments, alpha was set < 0.05 for determining significant effects.

Results

Experiment 1. A 3-way repeated measures ANOVA demonstrated a significant interaction effect between Age and Drug treatment across Sessions [$F(10, 198) = 2.482$, $P < 0.01$] (see Figure 14).

Test 2 results. Prior MK-801 treatment was effective in blocking cocaine CPP expression following a cocaine challenge [$F(10, 198) = 2.482$, $P < 0.01$], as significant differences were not observed in MK-801-cocaine treated rats when compared to saline controls (0.1 MK-801-cocaine v. saline-saline: $P = 0.268$; 0.2 MK-801-cocaine v. saline-saline: $P = 0.5487$). Dose and Age-related differences in the magnitude of the effect was observed. Specifically, repeated 0.2 but not 0.1 MK-801 pretreatment prior to cocaine conditioning was effective in blocking the expression of cocaine CPP, following a cocaine challenge in adult rats. The amount of time spent in the drug-paired compartment on Test 2 was significantly less in 0.2 MK-801-cocaine rats, as compared to rats conditioned to cocaine alone (0.2 MK-801-cocaine v. saline-cocaine:

[$F(5, 60) = 10.830$, $P = 0.000$] and compared to saline controls (saline-saline v. 0.2 MK-801-cocaine ($P < 0.05$). Significant changes in CPP expression were not observed between rats conditioned to cocaine alone and those pretreated with MK-801 prior to cocaine conditioning ($P = 0.524$).

In adolescent rats, repeated 0.1 and 0.2 MK-801 pretreatment prior to cocaine conditioning effectively blocked cocaine CPP following a cocaine challenge [$F(5, 49) = 4.225$, $P < 0.01$]. Regardless of Dose, adolescent rats conditioned with cocaine following MK-801 pretreatment spent less time in the drug-paired compartment relative to rats conditioned to cocaine alone (saline-cocaine v. 0.1 MK-801-cocaine, $P < 0.01$; saline-cocaine v. 0.2 MK-801-cocaine, $P < 0.01$). Moreover, adolescent rats pretreated with 0.1 and 0.2 mg/kg of MK-801 and conditioned with cocaine were not significantly different from saline controls (saline-saline v. 0.1 MK-801-cocaine, $P = 0.901$; saline-saline v. 0.2 MK-801-cocaine, $P = 0.992$).

Test 3 results. Adolescent and adult rats were administered 0.2 mg/kg of MK-801 prior to cocaine administration and the Test 3 session. Overall, A One-way repeated measures ANOVA within the factor of Age demonstrated that acute MK-801 pretreatment blocked established cocaine CPP in adolescent [$F(2, 23) = 4.785$, $P < 0.05$; T v. T3: $P < 0.01$] but not in adult rats [$F(2, 26) = 0.821$, $P = 0.458$; T v. T3: $P > 0.05$]. In adolescent rats, conditioned behavior decreased to control levels [$F(5, 51) = 1.647$, $P = 0.167$; saline-saline v. saline-cocaine: $P = 0.239$]. Relative lack of differences was not observed in adult rats, as saline controls exhibited an overall increase in behavior following MK-801 and cocaine pretreatment. This effect may have been secondary to the locomotor activating effects of the drug combination. Nonetheless, acute MK-801 pretreatment did not decrease conditioned reward in this Age group. In all, results suggest that adolescent rats were more sensitive to acute MK-801 as compared to

adults, while adult rats were more sensitive to the long-term effects of repeated MK-801 pretreatment on cocaine reward.

Experiment 2. The procedures in the following experiment were identical to those conducted in Experiment 1. All rats were administered 20.0 mg/kg cocaine (i.p.) prior to the 15 minute Test 2 session. All rats were administered 1mM MK-801 bilaterally into the NAcc prior to cocaine (20.0 mg/kg, i.p.) and the 15 minutes Test 3 session. The exception was the non-manipulated rats, in which this treatment group was not administered MK-801 or cocaine, before placement in the place preference testing apparatus. A 2(Age: adolescent and adult) x 6(Drug: Non-manipulated, Sham, saline-saline, MK-801-cocaine, saline-cocaine, MK-801-cocaine) x 3(Session: Test 1, Test 2, Test 3) repeated measures ANOVA with Age and Drug as the between subject factors and Session as the within-subject repeated factor did not show significant interaction effects within subject factors [$F(10, 192) = 0.192$, $P = 0.452$] (see Figure 15). Between-subject tests revealed a main effect of Drug [$F(5, 96) = 2.875$, $P < 0.05$] and Age [$F(1, 96) = 9.884$, $P < 0.01$], however a significant interaction effect between these two factors was not observed [$F(5, 96) = 0.916$, $P = 0.474$]. Independent analyses within Session, showed cocaine CPP expression [$F(5, 59) = 2.742$, $P < 0.05$; Non-manipulated, Sham and saline-saline v. saline-cocaine, $P < 0.05$) on Test 1 was neither impacted by the cocaine challenge on Test 2 [$F(5, 113) = 1.232$, $P = 0.229$] nor acute MK-801 administration on Test 3 [$F(5, 108) = 1.257$, $P < 0.288$].

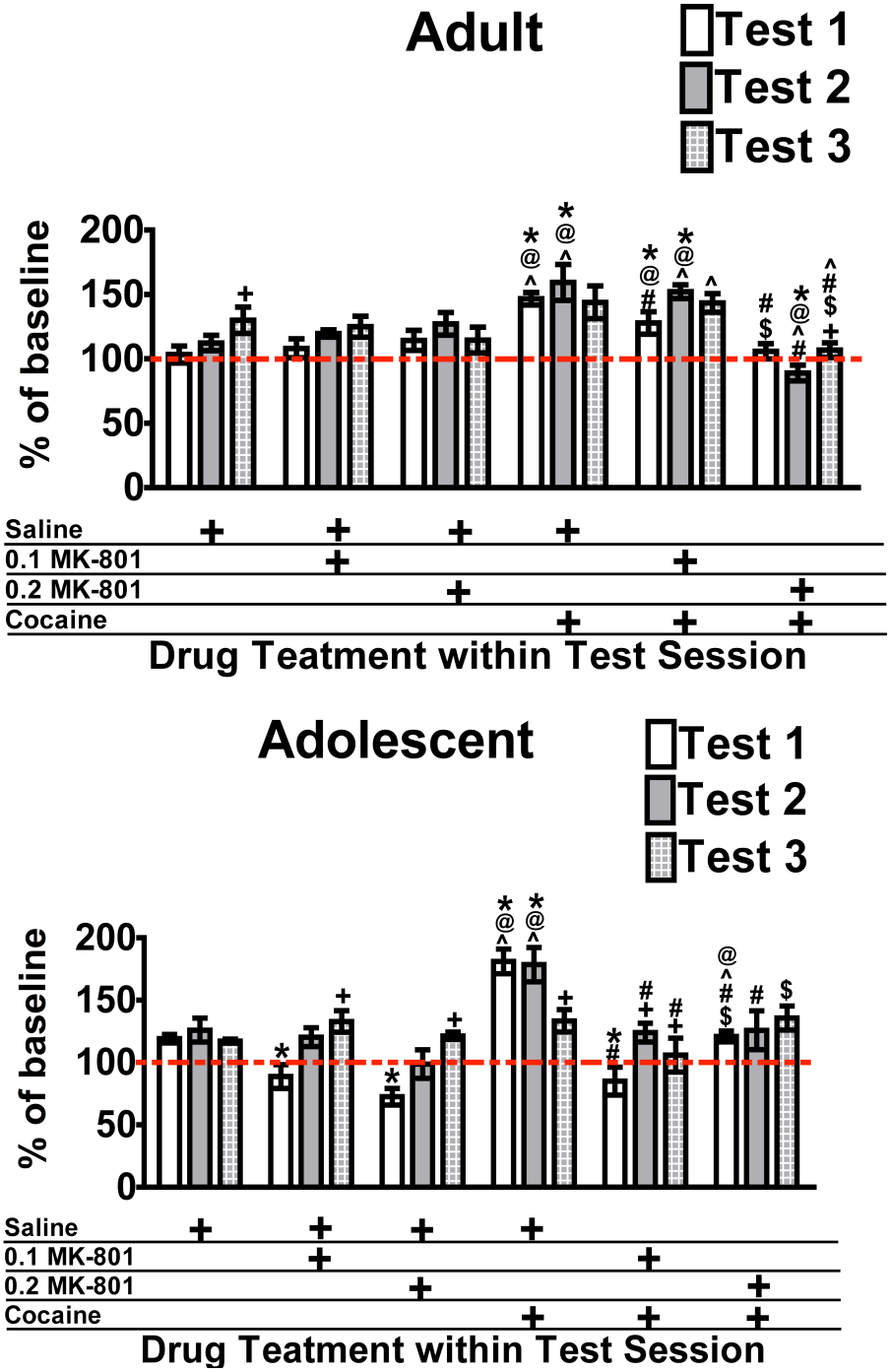

Figure 14. Acute and Repeated MK-801 Pretreatment Blocked Cocaine CPP in an Age-Dependent Manner. Overall, A 3-way interaction between Age and Drug treatment on conditioned behavior across test Sessions was observed [$F(10, 198) = 2.482$, $P < 0.01$]. Specific effects in adult and adolescent rats are presented in 14 (top) and 14 (bottom) graphs, respectively. Acute MK-801 pretreatment blocked cocaine CPP in adolescent but not adult rats. Long-term effects of repeated MK-801 pretreatment on cocaine CPP were observed in adult but not in adolescent rats. + denotes a significant difference from prior test session; * denotes significant difference from saline controls; @ denotes significant difference from 0.1 MK-801-saline; ^ denotes significant difference from 0.2 MK-801 cocaine; # denotes significant difference from saline-cocaine; $ denotes significant difference from 0.1 MK-801-cocaine.

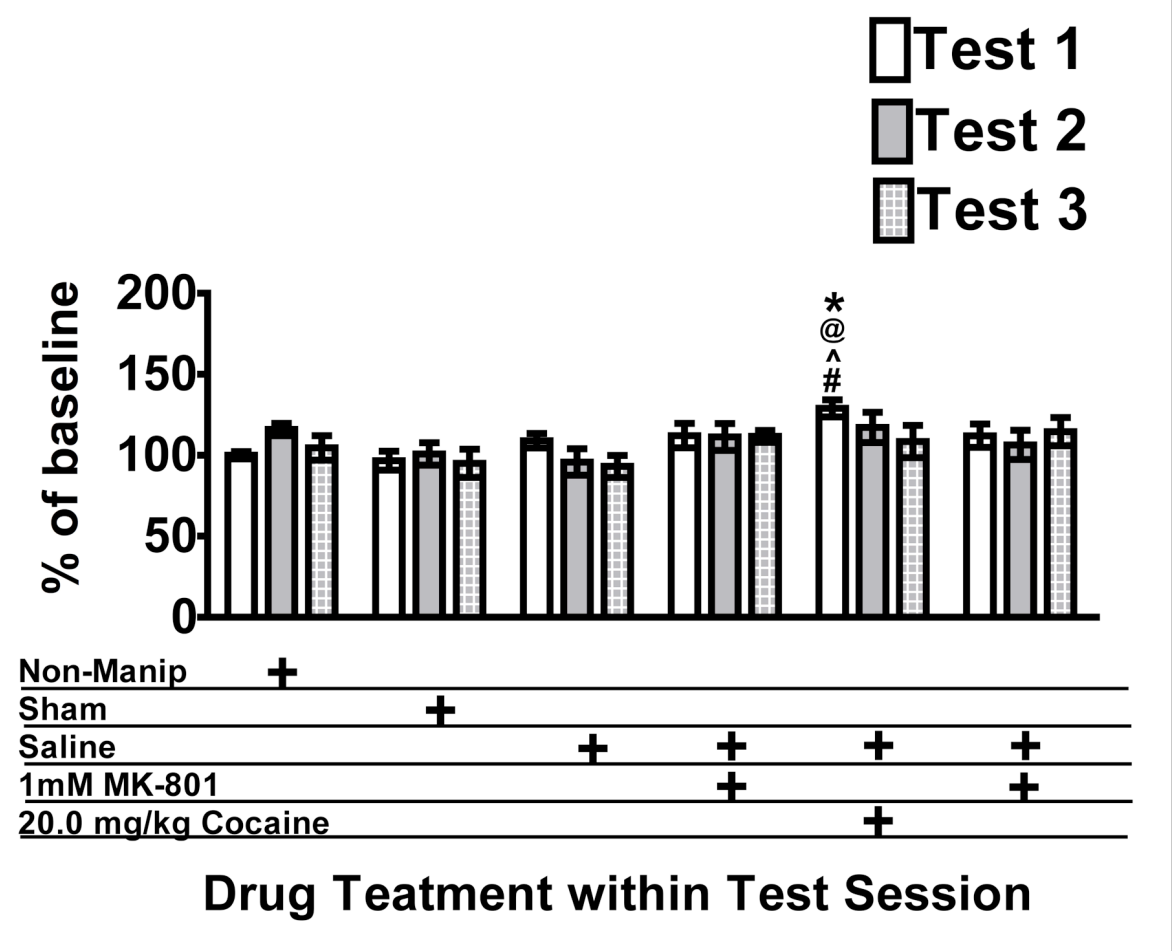

Figure 15. Acute MK-801 Microinfusions into the NAcc Did Not Block Cocaine CPP Across Development. An interaction between Age and Drug treatment on conditioned behavior across Sessions was not observed [$F(10, 192) = 0.192$, $P = 0.452$]. Analyses demonstrate significant effects at Test 1, but not at Test 2 and Test 3. * denotes significant difference from saline controls; @ denotes significant difference from 0.1 MK-801-saline; ^ denotes significant difference from 0.2 MK-801 cocaine; # denotes significant difference from saline-cocaine.

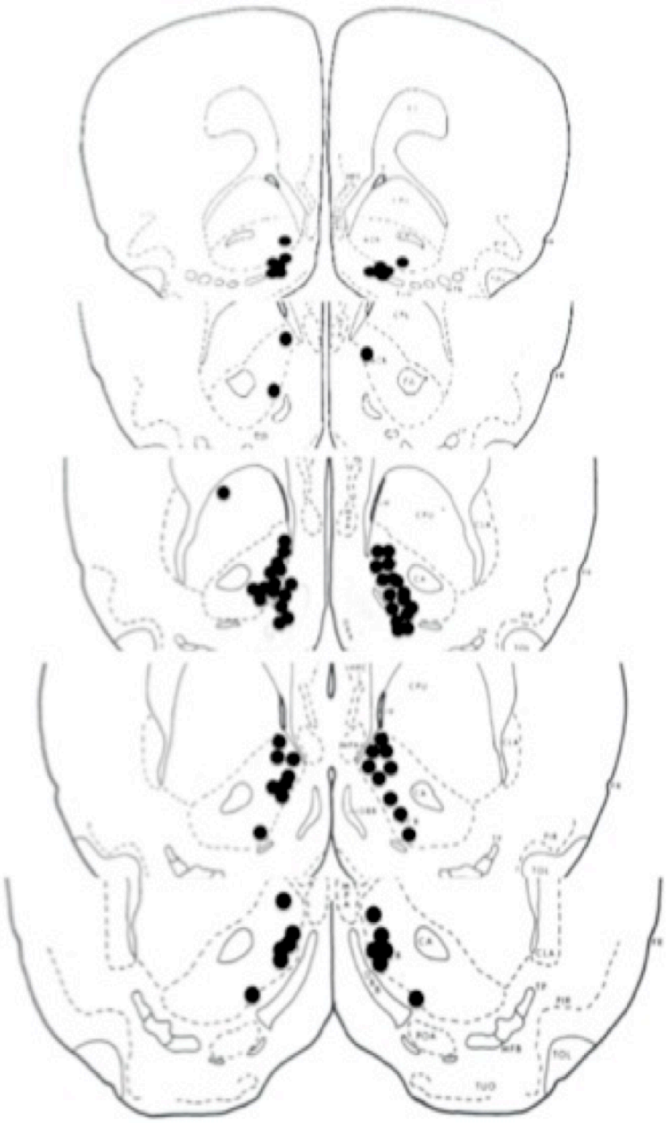

Figure 16. Schematic Representation of Cannula Placement within the NAcc.

CHAPTER THREE:

DEVELOPING AN ANIMAL MODEL OF POLYSUBSTANCE ABUSE

Discussion

The current series of experiments utilized the biased CPP to develop an animal model of polysubstance abuse, sensitive enough to determine age-dependent differences in ethanol/cocaine reward. Nucleus accumbens NMDA receptors were identified as a contributing mechanism mediating ethanol/cocaine polysubstance reward. The main findings of the current experiments demonstrated are as follows: 1) adolescent rats were less sensitive to the modulating effect of ethanol on cocaine reward 2) NMDA receptors contribute to ethanol and cocaine polysubstance reward 3) the NAcc is a site of action for ethanol and cocaine polysubstance reward.

Findings showed adult and adolescent rats responded similarly to co-administration of ethanol/cocaine with both ages showing a decrease in the rewarding properties of cocaine. What differed between the age groups was the aversive properties of ethanol with adolescents being less sensitive to the aversive properties of ethanol and its modulating effects on cocaine reward. Adolescent insensitivity to the aversive properties of the ethanol/cocaine combination suggests an increased vulnerability for this age group to continue engaging in polysubstance abuse. Evidence suggests that NMDA receptors within the NAcc contributed to the ethanol modulation of cocaine reward effect, however further investigation is needed to elucidate the mechanisms associated with conditioned aversion in adult but not in adolescent age groups. Such

mechanisms may provide useful information to the reasons why adolescents are less sensitive and therefore more vulnerable to engaging in continual polysubstance abuse.

Adolescents are Less Sensitive to the Modulating Effects of Ethanol on Cocaine Reward

All rats conditioned with 20.0 mg/kg of cocaine expressed a significant cocaine CPP, with adolescent rats demonstrating greater cocaine CPP as compared to their adult counterparts. These effects are consistent with previous findings demonstrating cocaine is rewarding across developmental time periods (Badanich et al., 2006; for reviews see Tzschentke, 1998; Bardo and Bevins, 2000; Tirelli et al., 2003; Sorg, 2012), with particular sensitivity during adolescence (Badanich et al., 2006). Adult rats conditioned with ethanol alone did not express place preferences but rather a conditioned avoidance to the ethanol-paired compartment, an effect strengthened with escalating doses. Adolescent rats did not express an ethanol-induced CPA, regardless of conditioned dose. Such findings were anticipated and are in accordance with previous research demonstrating age-dependent differences in ethanol sensitivity across conditioning doses (Philpot et al., 2006). Differences in ethanol sensitivity were also observed when ethanol and cocaine were co-administered. Adult rats conditioned with ethanol and cocaine actively avoided the ethanol/cocaine-paired compartment, an effect that significantly increased in a dose-dependent fashion. However, adolescent rats showed neither a preference nor an aversion to the ethanol/cocaine-paired compartment following conditioning procedures. Therefore in adolescent rats, ethanol decreased the rewarding properties of cocaine without inducing an aversive state, suggesting adolescent rats are less sensitive to the modulating effects of ethanol on cocaine reward.

The rewarding properties of most drugs are enhanced, while the aversive secondary properties remain insensitive during adolescence (Spear and Varlinskaya, 2010). Adolescent insensitivity to repeated ethanol treatment might have served as a protective mechanism against the profound effect of ethanol on cocaine reward. Indeed, adolescent rats demonstrated insensitivity to the dose-dependent effects of ethanol on cocaine reward. Adolescent insensitivity to ethanol and ethanol/cocaine reward may increase the likelihood of continued polysubstance abuse (Bailey, 1992), as negative consequences are not experienced. Such implications are important as human ethanol consumption increases following simultaneous cocaine use, which increases the risk of cardiotoxicity, hepatotoxicity and lethality and hospital admissions (Gossop et al., 2006; Barrett et al., 2006; Boyer and Petersen, 1992; Jatlow et al., 1995; McCance-Katz et al., 1998).

Polysubstance abuse during adolescence may also have long-term effects on neurochemical mechanisms involved in reward (Badanich et al., 2007; Maldonado-Devincci et al., 2010) and learning and memory (White and Swartzwelder, 2008). Such drug-induced changes further increase vulnerability for persistent polysubstance use. Even though neurobiological development may initially serve as a protective mechanism, it may induce vulnerability for persistent use later in life.

NMDA Receptors Contribute to Ethanol and Cocaine Polysubstance Reward

The modulating effects of ethanol on cocaine reward were consistent with previous investigations (Busse and Riley, 2002; Busse et al., 2004) and expand the current literature to include adolescent rats. However the mechanism(s) contributing to such effects are yet to be determined. Therefore, NMDA receptor involvement in ethanol/cocaine reward across development was investigated.

The NMDA receptor antagonist, MK-801, has been shown to block the expression of cocaine CPP in adult rats in a reactivation-dependent manner (administered prior to re-exposure to the cocaine-paired compartment; Brown, Lee and Sorg, 2012). In addition, post-conditioning treatment with MK-801 blocked cocaine CPP expression in adult rats (Alaghband and Marshall, 2013). However, the purpose of the current experiment was to assess the role of NMDA receptors in the ethanol/cocaine polysubstance conditioned behavior. Therefore MK-801 pretreatment always occurred prior to cocaine conditioning: procedures mimicking ethanol/cocaine co-administration in Aim 1.

The age and dose-dependent modulating effects of ethanol on cocaine reward were also observed when MK-801 (0.1 or 0.2 mg/kg, i.p.), was administered prior to cocaine conditioning. However, findings demonstrated an inverse relationship between age and drug sensitivity. Overall, systemic MK-801 pretreatment blocked cocaine reward, regardless of age or pretreatment dose administered. Previous reports using similar MK-801 doses (0.05 and 0.20 mg/kg, i.p.) have demonstrated MK-801 effectiveness in blocking the expression of cocaine CPP in adult rats without inducing place aversions (Brown, Lee and Sorg, 2008). Consistent with such reports, repeated MK-801 pretreatment prior to saline conditioning (Aim2: MK-801-saline treatment groups) procedures neither induced a place preference nor aversion in adult rats.

Repeated MK-801 pretreatment, prior to cocaine conditioning blocked cocaine CPP in an age and dose-dependent manner. While 0.1 and 0.2 mg/kg MK-801 effectively blocked cocaine CPP in adolescent rats, only the 0.2 mg/kg dose was effective in adult rats. Adolescent sensitivity to MK-801 pretreatment was also observed in the expression of MK-801/cocaine place aversions. Previous reports investigating the effects of MK-801 on adolescent locomotor

behavior and drug-induced pathological changes within the developing brain have been investigated (Wozniak et al., 1996; Haberny et al., 2002).

Therefore, in the present experiment, the aversive properties of MK-801 decreased cocaine reward and the conditioned motivational properties of the cocaine-paired context. These results replicated previous experiments demonstrating the effectiveness of MK-801 in blocking cocaine CPP in adult rats (Brown, Lee and Sorg, 2008) and expanded results to include the adolescent age population. Despite the inverse relationship between the age-dependent sensitivity to ethanol and MK-801, combined findings from the two experiments suggest EtOH may decrease cocaine reward at the NMDA receptor site. However, since repeated MK-801/cocaine treatment did not induce a CPA in either age group, additional unknown mechanisms are likely associated with the aversive properties of the ethanol/cocaine combination observed in adult rats. In addition, the complete blockade of cocaine CPP in adolescent rats pretreated with MK-801 do not support NMDA receptors as a protective mechanism against adolescent insensitivity to the aversive properties of ethanol and cocaine co-administration.

Repeated drug exposure induces long-term neuroadaptations in glutamate and DA terminals in the NAcc, which are known to mediate maladaptive learning and stimulate drug craving following re-exposure to drug-associated stimuli (Vanderschuren and Kalivas, 2000; Robinson and Berridge, 2008; Wolf and Ferrario, 2010; Nestler, 2011). The involvement of NMDA receptors in the acquisition and expression of ethanol/cocaine conditioned behavior, suggests similar dopamine and glutamate interactions within the mesocorticolimbic circuit mediate the conditioned motivational properties of the ethanol/cocaine-paired compartment, across developmental time periods (McFarland et al., 2003; Berridge, 2007; Brenhouse et al., 2008; Sonntag et al., 2014). Therefore, further experimentation was conducted to elucidate the

role of NMDA receptors within the NAcc in associative learning across developmental time periods.

The Nucleus Accumbens is a Site of Action for Ethanol and Cocaine Polysubstance Reward

Systemic ethanol and MK-801 administration showed a decrease in cocaine reward. Since systemic administration of any drug impacts the central nervous system as a whole, the conditioned behavior expressed may have been indirectly influenced by additional, unknown mechanisms. Intracranial microinfusions of MK-801, directly into the NAcc of adolescent and adult rats were conducted to localize the NAcc as a target structure and NMDA receptors as the implicated mechanism involved in ethanol/cocaine conditioned behavior. MK-801 administration into the NAcc attenuated cocaine reward, regardless of age. These findings are in accordance with previous research demonstrating the reinforcing effects of cocaine are mediated by glutamate neurotransmission into the NAcc. Cocaine increased glutamatergic signaling and NMDA receptor activation within the mesocorticolimbic pathway (Schillstrom et al., 20006). Regional administration of MK-801 into the NAcc decreased the rate of cocaine self-administration (Cornish, Duffy and Kalivas, 1999) and presently cocaine place preference expression. Glutamatergic signaling pathways originating from the basal nucleus of the amygdala, hippocampus and the prefrontal cortex and terminating onto the NAcc regulated conditioned behavior following the presentation of a drug-associated cue (Ciccocioppo, Sanna and Weiss, 2001; Ambroggi et al., 2011). Therefore, ethanol likely decreased the conditioned motivational properties of the ethanol/cocaine-paired environment by decreasing cocaine reward via inactivation of glutamatergic sensitivity within the NAcc. The ethanol response on cocaine

reward was not attributed to peripheral effects following systemic administration of the drug combination, but rather localized NMDA antagonism within the NAcc.

Despite the relationship between ethanol and MK-801 on cocaine reward, additional mechanisms are likely involved in the conditioned expression of the polysubstance combination. Intracranial administration of MK-801 attenuated but did not completely block cocaine CPP. In addition, adult rats conditioned with 1.75 g/kg v and cocaine exhibited significant CPA behavior following conditioning procedures. Conditioned aversion was not observed following systemic administration with either doses of MK-801 or when administered directly into the NAcc. Similarly, the aversive properties of MK-801 blocked cocaine reward in adolescent rats, however adolescent rats pretreated with MK-801 and conditioned with cocaine did not demonstrate a MK-801/cocaine CPA. Taken together, the modulating effects of ethanol on cocaine reward are expressed as a function age and dose and are associated with NMDA antagonism. However additional mechanisms in combination with NMDA receptors are likely implicated in adult sensitivity and adolescent insensitivity to the ethanol/cocaine conditioned response.

Additional Mechanisms Contribute to Ethanol and Cocaine Polysubstance Aversion

The mesocorticolimbic circuit is comprised of the VTA, mPFC and NAcc respectively and is implicated in ethanol and cocaine reward (Wise, 1987). Within the circuit, the NAcc has been referred to as a critical interface of dopamine and glutamate interactions (Deniau et al., 1994; Rebec and Sun, 2005; Wolf, 2002) as it receives dense dopamine and glutamate innervation from the VTA and mPFC, respectively. *In vivo* and *in vitro* electrophysiological recordings have shown ethanol and cocaine independently increased VTA-dopamine release via disinhibition of VTA-GABA interneurons (Steffenson et al., 2008; Tateno and Robinson, 2011; Bunney et al., 2000). Consequentially, an increase in NAcc-dopamine was observed following

ethanol and cocaine administration, as measured by microdialysis and neurochemical procedures (Di Chiara and Imperato, 1988). When ethanol and cocaine were co-administered, VTA-GABA disinhibition and augmented dopamine release was potentiated (Bunney et al., 2000). These observations have been associated with a decrease inhibitory control, augmented conditioned reward and reinforcement (Wolf et al., 2002). However, in the present series of experiments, the drug combination expressed an opposite, aversive response, which was enhanced with escalating doses of ethanol. These results suggest that an interaction between VTA-DA and NMDA receptors (i.e. antagonism) within the NAcc mediated the observed ethanol/cocaine conditioned behavior. Ethanol and cocaine in combination augments mesocorticolimbic dopamine release and availability increasing, thereby increasing the likelihood of ethanol/cocaine CPP. However, ethanol simultaneously blocks NMDA receptor function in the NAcc. NMDA receptor activation within the NAcc is essential for glutamatergic-induced stimulation of NAcc quiescent GABA neurons. Therefore, it is proposed that the absence of ethanol/cocaine CPP was NMDA dependent and secondary to decreased NAcc sensitivity to VTA-dopamine signaling.

Adult sensitivity to the aversive properties of 1.75 g/kg ethanol alone, leading to the dose-dependent expression of ethanol/cocaine conditioned avoidance suggests that NMDA-dependent processes regulate ethanol modulation of cocaine reward, however additional mechanisms mediate the behaviors expressed in relation to the aversive properties of the drug combination. Results from Aim2 support this theory, as conditioned aversion was expressed in adolescent rats following repeated MK-801 treatment but not following MK-801 pretreatment combined with cocaine conditioning.

AMPA receptor function may regulate ethanol/cocaine CPA expression. NMDA and AMPA receptors are implicated in the long-term associative memory between drug reward and

environmental stimuli. However AMPA receptors specifically, have shown to promote the expression of conditioned aversion following pharmacological treatment with the AMPA antagonist, DNQX into the NAcc (Wolf, 2002; Hyman, Malenka and Nestler, 2006; Malenka and Bear, 2004; Reynolds and Berridge, 2003). Repeated ethanol/cocaine exposure may have impacted NMDA/AMPA function, contributing to the absence of ethanol/cocaine CPP (NMDA antagonism) while promoting the acquisition and expression of ethanol/cocaine CPA (AMPA antagonism). Administration of the AMPA/kainate antagonist DNQX into the NAcc induces conditioned avoidance (Reynolds and Berridge, 2003) and EtOH is an AMPA receptor antagonist (Weiner et al., 1999). Therefore, the expression of ethanol/cocaine conditioned behavior may have occurred along a gradient, with successful expression of cocaine CPP in the absence of ethanol, NMDA-receptor induced attenuation/blockade of cocaine reward with lower doses of ethanol, and finally AMPA-receptor mediated ethanol/cocaine aversion, with high doses of ethanol. Similar dose-response effects of ethanol modulation on cocaine reward have been described (Busse and Riley, 2002; Busse et al., 2004). However, findings within the present experiments are the first to investigate the role of NMDA receptors in the expression of ethanol anol/cocaine conditioned behavior and expand findings to include the adolescent population. Considering adolescents were less sensitive to the ethanol/cocaine combination, it is possible that ethanol modulates cocaine reward in adolescent rats primarily at NMDA, and to a lesser degree at AMPA receptors.

An alternative explanation to the apparent age-sensitivity to ethanol/cocaine conditioned behavior maybe attributed to enhanced glutamate neurotransmission following repeated ethanol administration. Despite initial NMDA antagonism (Lovinger et al., 1989) chronic ethanol exposure is associated with increased glutamate neurotransmission and NMDA receptor

activation, particularly during withdrawal (Hoffman and Tabakoff, 1996; Zhu et al., 2007). The conditioning procedures used in the present experiments may have been extensive and sufficient enough to initially block and then stimulate NMDA receptor activation. Enhanced excitatory post-synaptic potentials increases Glu-R1 surface expression, a measure of increased AMPA receptor trafficking on excitatory postsynaptic sites (i.e. glutamate terminals on NAcc neurons), which has been associated with synaptic plasticity and the reconsolidation of learned associations between the affective properties of a drug and the drug-paired environment (Bolshakov and Siegalbaum, 1994; Citri and Malenka, 2008; Ungless et al., 2001; Wolf and Ferrario, 2010; Sorg, 2012). Since adult but not adolescent rats demonstrated CPA to ethanol alone and to the ethanol /cocaine combination and adolescent rats expressed MK-801 CPA, it is possible that the ontogeny of ethanol/cocaine conditioned motivation is regulated by age-dependent differences in NMDA/AMPA sensitivity. Such differences may further contribute to adolescent vulnerability of continued EtOH /cocaine polysubstance use.

Findings from Aim 1 and Aim 2 provide a role for NAcc NMDA receptors in ethanol/cocaine polysubstance abuse. Protective mechanisms preventing adolescent rats from ethanol/cocaine conditioned aversion remain unknown. However, it is likely that age-dependent differences occur within glutamate and dopamine neural pathways within the NAcc, impacting variants in ethanol/cocaine conditioned behavior. Due to significant findings observed, the biased CPP served as a reliable and valid animal model of polysubstance abuse, capable detecting age-specific sensitivities to ethanol/cocaine polysubstance reward. Future investigations should rule out age-dependent differences in AMPA mediated effects, as well as ontogenetic differences in synaptic plasticity, following repeated ethanol/cocaine co-administration. In addition, in vivo microdialysis procedures can offer valuable information on

ethanol/cocaine reward, as measured by extracellular dopamine concentration. Such investigations would elucidate the role AMPA receptors, synaptic plasticity and combined dopamine and glutamate interactions, in adolescent vulnerability to continued polysubstance abuse.

CHAPTER FOUR:

EFFECTIVENESS OF MK-801 TREATMENT ON THE ACQUISITION AND EXPRESSION OF CONDITIONED REWARD ACROSS DEVELOPMENT

Discussion

The CPP paradigm has been regarded as a valid animal model of learning and memory (Calcagnetti and Schechter, 1993), as it operates under the basic principles of Classical Pavlovian conditioning. Aims 1 and 2 provided evidence in support for the utility of the CPP paradigm to model polysubstance abuse in rats. In addition to assessing the affective (i.e. rewarding and aversive) properties of a drug/drug combination, the place preference paradigm has been extensively used to elucidate the learning and memory mechanisms underlying conditioned motivation and reinforcement (Bardo et al., 1995; Bardo and Bevins, 2000; Tzschentke, 2007). The underlying assumption is as follows: repeated drug-context pairings results in learned associations between the affective properties of the drug and the drug-paired environment, driving conditioned behavior (i.e. CPP and CPA) upon re-exposure. The following discussion utilizes learning theories to explain the prevalence of ethanol and cocaine polysubstance abuse.

Utilizing Learning Theories in Ethanol and Cocaine Polysubstance Conditioned Reward

NMDA receptor activation and AMPA receptor trafficking is necessary for the induction of long-term potentiation: signaling events critical for synaptic plasticity and conditioned behavior (Malenka and Bear, 2004; Wolf and Ferrario, 2010). Findings from Aims 2 and Aim 3 and previous investigations further support the role of the NMDA receptor in learning and memory processes associated with the expression of conditioned behavior (Brown, Lee and Sorg, 2008; Sorg, 2012). Given that NMDA antagonism occurs following acute ethanol administration and ethanol was consistently paired with cocaine since the first day of conditioning, it is possible that ethanol-mediated NMDA receptor antagonism suspended the development of associative memory for the drug-paired compartment. Learning and memory deficits attributed to human ethanol consumption in isolation and in combination with other drugs have been documented (Liljequist et al., 1975; Mattila et al., 1998; Ridley et al., 2013). A caveat to this interpretation is that learned associations did occur following repeated ethanol/cocaine pairings, as observed by conditioned avoidance. As previously mentioned, repeated ethanol treatment may have initially suspended and then facilitated the acquisition and expression of ethanol/cocaine conditioned aversion (Lovinger et al., 1989; Hoffman and Tabakoff, 1996; Zhu et al., 2007). Ethanol's impact on molecular mechanisms associated with learning and memory may provide a rationale for the apparent lack of drug reward potentiation observed here and previous investigations as well as the reports obtained from human polysubstance abusers (McCance-Katz et al., 1993 and 1998; Busse and Riley, 2002; Busse et al., 2004). However additional experiments investigating the direct effect of ethanol/cocaine on learning and memory is needed (i.e. Morris Water Maze, Radial Arm Water Maze, Novel Object Recognition, etc).

NMDA Receptors are Implicated in the Acquisition and Expression of Ethanol and Cocaine Polysubstance Conditioned Behavior

The involvement of NMDA receptors in the consolidation, drug-induced reactivation and reconsolidation of cocaine place preferences across developmental time periods was examined in Aim 3. The main findings are as follows: MK-801 pretreatment effectively blocked the acquisition of cocaine place preferences in both age groups, with particular sensitivity in the adolescent age group (Test 1); Repeated 0.2 mg/kg MK-801 pretreatment effectively blocked drug-induced reactivation of cocaine-associated memories, following a cocaine challenge (Test 2); adolescent rats were sensitive to the acute MK-801 treatment on disrupting the reconsolidation of cocaine place preferences in a drug-reactivation dependent manner (Test 3). These findings suggest that NMDA receptor function is an essential and common mechanism for the development and maintenance of cocaine-associated memories across developmental time periods. Overall, findings suggest that adolescent rats are more vulnerable to acquiring addictive-like behaviors, however they are more responsive to acute MK-801 treatment and are less likely to express addictive behaviors following a lapse in abstinence.

Additional testing was also conducted in cannulated rats to localize NMDA function specifically to the NAcc. However, findings from this experiment did not reveal significant effects of drug treatment or age. This is likely due to the longetivity of the experimental timeline (15 days) and the rapid growth adolescent rats undergo. For these reasons, discussion will focus on the results obtained from systemic MK-801 administration.

MK-801 effectiveness in disrupting the reconsolidation of cocaine CPP in a reactivation-dependent manner supported previous investigations in adult male rats (Kelley et al., 2007; Brown et al., 2008) and expanded previous findings to include the effectiveness of MK-801

treatment on the consolidation, drug-induced reactivation and reconsolidation of cocaine place preferences in adolescent male rats. Identifying the mechanisms contributing to cue- and context-induced drug craving and relapse during adolescence is critical, considering the increased likelihood of maintaining addictive behaviors secondary to adolescent drug use (Patton et al., 2004; Schramm-Saptya et al., 2009; Spear, 2010) and the risk for drug relapse following re-exposure to drug-associated cues (O'Brien et al., 1992; Childress et al., 1988; Childress and O'Brien, 2000; Volkow et al., 2008).

NMDA receptors are contributing factors in cocaine associative learning in adolescent and adult rats

NMDA receptors and the consolidation of cocaine-associated reward. The effectiveness of repeated MK-801 pretreatment on blocking the consolidation of cocaine place preferences across development was assessed in a drug-free state in the "Test 1" assessment. The impeding effects of MK-801 on the consolidation of cocaine place preferences in adolescent and adult rats suggest that NMDA receptors mediate the learning and memory processes of conditioned reward, facilitating conditioned behavior across developmental time periods. Cocaine-stimulated dopamine release within the mesocorticolimbic circuit is associated with the rewarding properties of the drug (Ritz et al., 1987). Stimulated dopamine release within the mesocortical pathway indirectly increases mPFC pyramidal neuron excitability, promoting corticolimbic glutamate neurotransmission (Lewis and O'Donnell, 2000) and ultimately NMDA receptor activation. Such dopamine/glutamate interactions activate downstream signaling events known to promote neuroplasticity and aberrant learning following repeated drug use (Pennartz et al., 1993; Kombian and Malenka, 1994; Everitt et al., 2001; Hyman et al., 2005; Sun et al., 2005; Gao and Wolf, 2007; Sun et al., 2008; Thomas et al., 2008; Wolf and Ferrario, 2010; Liu and

Steketee, 2011; Gao et al., 2006; Madhavan et al., 2013). The dopamine/glutamate interaction effects at the site of the NMDA receptor may serve as the initial steps of cocaine reward consolidation in adolescent and adult rats, as rats conditioned with cocaine and pretreated with 0.2 mg/kg MK-801 failed to acquire cocaine place preferences in the present study.

NMDA receptors and drug-induced reactivation of cocaine place preferences. Prior to Test 2, all rats were administered 20.0 mg/kg cocaine (i.p.) to examine the effectiveness of repeated MK-801 pretreatment in the consolidation and reconsolidation of cocaine place preferences in a drug-induced reactivation manner across developmental time periods. Results showed that regardless of age, repeated 0.2 mg/kg MK-801 pretreatment blocked the consolidation of learned associations between cocaine and the drug-paired compartment. Adolescent rats were more sensitive to MK-801 effects, as both doses of MK-801 effectively blocked the expression of cocaine CPP, despite the cocaine challenge. These findings suggest adolescent and adult rats possess similar mechanisms that promote the consolidation of learned drug-environment associations, however the adolescent age group is more responsive to treatment. In addition, obtained data support previous reports demonstrating MK-801 effectiveness in blocking cocaine-primed reinstatement of CPP in a reactivation dependent manner (Brown et al., 2008) and further implicate NMDA receptors in the consolidation and reconsolidation of conditioned reward and behavior in developing and matured rats.

NMDA receptor activation is essential for the consolidation of cocaine-associated cues, which are retrieved, expressed as conditioned behavior and reconsolidated following re-exposure to the drug-paired compartment or following a drug challenge. The involvement of NMDA receptors in the reconsolidation of cocaine place preferences in a reactivation dependent manner suggests NMDA-mediated processes stimulate synaptic plasticity permitting the acquisition and

expression of the cocaine conditioned effects across developmental time periods. Cocaine-stimulated dopamine release and enhanced glutamate neurotransmission was augmented following repeated cocaine exposure (Pierce et al., 1994; Ito et al., 2002). In addition, increased dopamine and glutamate neurotransmission stimulated synaptic plasticity within the mesocorticolimbic circuit by promoting NMDA/AMPA receptor trafficking via D1 receptor activation (Sun et al., 2005; Gao and Wolf, 2007; Sun et al., 2008; Liu and Steketee, 2011; Gao et al., 2006; Madhavan et al., 2013). The reconsolidation of cocaine-associated cues following drug-induced reactivation within the present study was NMDA dependent. These results further support the role for glutamate-dependent neuroplasticity in the reconsolidation of drug-associated cues (Valjent et al., 2006), an event associated with the increased likelihood of engaging in relapse behavior (O'Brien et al., 1992; Childress et al., 1988; Childress and O'Brien, 2000; Volkow et al., 2008).

NMDA receptors and memory reconsolidation. The impact of acute MK-801 treatment on the reconsolidation of cocaine place preferences in adolescent and adult rats was examined in a reconsolidation assessment (Test 3), in which all rats were administered 0.1 or 0.2 mg/kg MK-801 followed by 20.0 mg/kg cocaine (i.p.). Results showed acute MK-801 treatment effectively blocked the expression of cocaine CPP in adolescent but not adult rats. Such results suggest learned cocaine-context associations are more vulnerable to disruption in adolescent populations. It was hypothesized that reconsolidation would be disrupted with acute MK-801 pretreatment, regardless of age (Brown et al., 2008). One explanation for the apparent discrepancy may be that reconsolidation of cocaine conditioned reward and behavior following the cocaine challenge at "Test 2," may have strengthened and therefore became resistant to a single MK-801 treatment. Previous investigations have indicated a need for multiple

reactivation trials to sufficiently disrupt memory reconsolidation for drug-associated memories (Brown et al., 2007; Brown et al., 2008). In addition, previous work demonstrated complete disruption of cocaine place preferences with MK-801 following an extinction period (Brown et al., 2008). Therefore, application of extinction procedures, coupled with repeated MK-801 treatment may be necessary for the complete disruption of cocaine place preferences in a reactivation manner. Furthermore, successful disruption of cocaine place preferences with a single administration of MK-801 must be conducted in a context-dependent reactivation manner in the absence of the conditioning drug (Brown et al., 2007; Brown et al., 2008). This is likely the case as a cocaine challenge following repeated treatment has been shown to augment dopamine-modulated glutamate neurotransmission (Pierce et al., 1994; Lewis and O'Donnell, 2000; Ito et al., 2002). Therefore it is likely that the acute MK-801 dose, prior drug treatment history and/or the cocaine administered prior to testing increased reconsolidation resistance to disruption in adult rats.

A fourth alternative to cocaine CPP resistance may be that additional mechanisms are recruited and therefore serve as primary mediators of memory reconsolidation in the presence of cocaine. For example, LTP and LTD are associated with AMPA/NMDA receptor trafficking and increased surface expression of AMPA receptors within the NAcc (Wolf and Ferrario, 2010), facilitating cue-induced reinstatement of cocaine seeking behavior (Gipson et al., 2013). mPFC-stimulated GLU release and activation of AMPA receptors has shown to mediate cocaine-seeking behavior, as AMPA receptor antagonists disrupt reinstatement of cocaine self-administration and induce place aversion (Cornish and Kalivas, 2000; Renolds and Berridge, 2003). In addition, metabotropic glutamate receptors, glutamate transporters and several molecular targets associated with synaptic plasticity have also been implicated in the

reconsolidation of cocaine conditioned reward and behavior (for review see Sorg, 2012). Furthermore, VTA stimulated co-release of dopamine and glutamate to the NAcc may have induced excitatory postsynaptic currents, promoting synaptic plasticity and conditioned behavior (Tecuapetla et al., 2010; Stuber et al., 2010). Results from Aim 3 together with Aims 1 and 2 suggest a critical role for NMDA receptors in the blockade of cocaine reward, a potential mechanism involved in ethanol/cocaine polysubstance abuse.

Experimental Considerations

The series of experiments conducted were specifically designed with the intention of developing an animal model of polysubstance abuse. Simultaneous systemic administration of ethanol and cocaine modeled simultaneous ethanol/cocaine human use and abuse. The animal model also provided insight into the rewarding, reinforcing properties of the polysubstance combination, which is recognized as a societal issue with potential threat to the physical and psychological wellbeing. Experimental procedures were influenced from previous investigations within the drug abuse and learning and memory fields. Despite advancements from current experiments, additional research is needed to refine the polysubstance adolescent animal model, elucidate the role of additional mechanisms driving reward, and assess epigenetic factors so that the model can further develop into a translational animal model providing utility to the clinical field.

The application of the biased CPP paradigm in the current animal model of ethanol/cocaine polysubstance use had its advantages. First, adolescent rats were able to complete the entire experiment during the time period of adolescence (PND 30-45) and therefore direct age comparisons were able to be made. Second, the role for NMDA receptor involvement

was conducted by simply substituting MK-801 for ethanol in the CPP design. Special consideration for treatment doses and drug administration schedule were taken to include the adolescent age group. Third, the relatively short experimental timeline permitted sufficient time to conduct surgical procedures and permit recovery of all animals prior to behavioral experimentation. Fourth, the application of microinfusions, which substituted for systemic MK-801 and ethanol administration, controlled for peripheral drug effects, while maintaining consistent drug treatment procedures used in the prior two experiments. Lastly, the CPP paradigm has been recognized as a valid animal model of learning and memory and the current series of experiments replicated previous findings within the literature. Therefore, present results are informative, reliable and valid.

Few limitations are also recognized. In the clinic, polysubstance use is not passive and the routes of ethanol and cocaine administration typically involve oral consumption of alcohol and intranasal or inhalation of cocaine. Passive v. active drug administration and differences in routes of administration pose concern regarding generalizability. The apparent cognitive and motivational component in drug seeking is absent with passive drug administration and the pharmacokinetic profile of ethanol and cocaine co-administration varies according to route of administration. The application of voluntary ethanol consumption and cocaine self-administration can be employed, however the acquisition phase for voluntary ethanol consumption and the training phase for operant conditioning are long. Since adolescence occurs within a narrow time frame it would not possible to make direct age comparisons due to extended experimental timelines to include acquisition of consumption and operant behavior. Second, behavioral deficits and extensive adolescent brain growth following cannulation may have impacted conditioned behavior. Appropriate controls were included in the experiment to

account for drug treatment (saline-saline; MK-801-saline; saline-cocaine), perfusion (Sham) and surgical procedures (non-manipulated) and all behavior was compared to the non-manipulated controls. Therefore, appropriate controls and data transformations were employed limit the influence of external variables on conditioned behavior. Lastly, surgical procedures did occur prior to the behavioral experiment. In order to minimize extensive tissue damage and to maximize the probability of accurately implanting within the NAcc, cannula were lowered at a very slow pace and placement was adjusted according to weight and angle entry. Once implanted, cannula remained in the brain for 15 days. During this length of time, stylets were removed from the cannula and probes were inserted for microinfusion procedures. Following microinfusion, probes were removed and stylets were re-inserted. Repeated removal and replacement of cannula and stylets may have induced stress, discomfort or pain which could negatively impact results. However, appropriate controls were in place and behavior across all test sessions were compared to the non-manipulated controls.

In summary, despite the discussed methodological and application considerations, the biased CPP paradigm presents as a useful developmental animal model of polysubstance abuse.

CHAPTER FIVE:

ADOLESCENT VULNERABILITY IN ETHANOL AND COCAINE POLYSUBSTANCE REWARD

Mechanisms of Adolescent Vulnerability

Age-dependent differences observed across all experiments are attributed the extensive neurological changes that occur between adolescence to adulthood (For review see Spear, 2000 and 2002). Region specific time courses of overproduction followed by pruning of DA receptors are detected during maturation, with observations depicting an earlier time course within the striatum as compared to the PFC (Andersen et al., 2000), but sparing the NAcc (Teicher et al., 1995). In addition, basal dopamine levels increase during adolescence and then decrease to reach levels of maturation at PND 60 (Badanich et al., 2006). Differences in basal dopamine are likely due to age-dependent differences in DAT expression and dopamine reuptake during this time period (Coulter et al., 1996; Badanich et al., 2006). Mylenation within the PFC persists throughout development, despite the reduction in cortical synaptic density and overall cortical volume (Huttenlocher, 1984; Huttenlocher and Dabholkar, 1987; van Eden et al., 1990; Jernigan and Gamst, 2005). NMDA synaptic receptor expression and desensitization facilitates neuronal activity and synaptic plasticity, while protecting it from excitotoxicity (Tong et al., 1995; Dingledine et al., 1999; Li et al, 2003). NMDA function is dependent on NR1 subunit expression as assembly with NR3 subunits are shown to regulate NMDA receptor expression

(Monyer et al., 1992; Matsuda et al., 2003). NR1 expression progresses linearly throughout development, with peak levels observed at mature ages (Hansen et al., 2008).

The ontogenetic profile of dopamine and glutamate neural systems support current observations of age-dependent sensitivities to drug treatment, however adolescent increased sensitivity to MK-801, as the mesocorticolimbic circuit during this developmental time frame is mainly influenced by VTA-dopamine neural systems and dopamine receptor expression. Supporting evidence for this hypothesis is that adolescent rats are more sensitive to ethanol NMDA receptor antagonism, as compared to their adult counterparts (Swartzwelder et al., 1995). However, Teicher et al., (2003) also reports insensitivities to MK-801 treatment on D1 and D2 receptor expression across developmental time periods. Present findings suggest that consolidation and reconsolidation of cocaine conditioned reward and behavior involves the NDMA receptor across developmental time periods, with increased periods of sensitivity during adolescence.

The NMDA Receptor Mediates Psychological Processes of Addictive Behavior

Learning theories were used to interpret results from Aims 1, 2 and 3 experiments, however the application of these theories in the elucidation of the mechanisms underlying the psychological processes governing relapse behavior is needed. The effectiveness of acute and repeated MK-801 treatment on suppressing and blocking cocaine place preferences, despite re-exposure to the drug itself, suggests that conditioned reinforcement and motivation to approach the drug-paired environment may have been disrupted. The failure to express cocaine place preferences may also or instead be attributed to a decrease or lack of reinforcing properties within the drug-paired compartment. Following repeated pairings of neutral stimuli with cocaine (as in the case of CPP experiments), conditioned stimuli assume secondary reinforcing properties

and are thought to stimulate cocaine craving and relapse behavior, as an increase in extracellular dopamine is observed following re-exposure to drug-associated cues (Ito et al., 2002; Volkow et al., 2006). Repeated MK-801 pretreatment may have prevented the drug-paired compartment from acquiring secondary reinforcing properties, as acute and repeated MK-801 treatment blocked acquisition and full expression of cocaine place preferences. The failure to express conditioned reinforcement is not secondary to conditioned aversion, as repeated MK-801 treatment in isolation and in combination with cocaine did not induce a conditioned place avoidance response. In addition, it is likely that MK-801 did not extinguish cocaine-conditioned reward, as repeated MK-801 treatment was administered prior to cocaine conditioning and previous investigations show that NMDA receptor antagonism blocks the acquisition of extinction (Kelamangalath et al., 2007). This effect is most likely due to extinction being considered as a form of learning, requiring neuroplasticity, rather than forgetting. These results suggest that regardless of age, NMDA-dependent neuroplasticity may be essential in the development of psychopathology associated with drug abuse and relapse.

The persistency of addictive behavior and vulnerability to drug relapse is associated with conditioned reward, reinforcement and motivation (Everitt et al., 2001; Milton and Everitt, 2010) and results suggest that NMDA receptors may serve as a potential therapeutic agent in preventing the reconsolidation of drug-associated memories and drug relapse. However, clinical investigation of NMDA receptor antagonists, such as memantine, dextromethorphan, acomprosate, MK-801, and ketamine show limited clinical efficacy as these agents typically induce an aversive psychotomimetic state and have been shown to induce relapse behavior (for review see Tzschentke and Schmidt, 2003). As suggested by Tzschentke and Schmidt (2003), previously stated above and reviewed by Nestler (2013) advancement in the treatment of drug

abuse, addiction and relapse may involve targeting the drug-induced neuroadaptations and mechanisms underlying drug-induced synaptic plasticity, including epigenetic gene regulation and expression as well as protein transcription.

Conclusion

The present series of experiments portray a contributing role of NAcc NMDA receptors in ethanol/cocaine polysubstance abuse as well as in the consolidation, reconsolidation and expression of cocaine place preferences across development. Dopamine/glutamate interactions at the site of the NAcc NMDA receptors drive conditioned behavior associated with persistent ethanol/cocaine polysubstance exposure. NMDA-dependent processes contributing to the consolidation and reconsolidation of cocaine-associated memories are preserved throughout development. In addition, future directions in the investigation of additional mechanisms contributing to adolescent vulnerability of continued ethanol/cocaine polysubstance are discussed. Targeting NMDA receptors in the treatment and prevention of drug abuse and drug relapse is appealing, however the relative risks of utilizing pharmacological agents that present a high affinity for NMDA receptors poses risk. Therefore, continued research is needed to elucidate additional mechanisms underlying conditioned reward and behavior, promoting context-dependent drug relapse that may serve as potential therapeutic agents in the drug abuse field.

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