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Chronic Ethanol Drinking by Alcohol-preferring Rats Increases the Sensitivity of the Mesolimbic Dopamine System to the Reinforcing and Stimulating Effects of Cocaine

For the degree of Doctor of Philosophy

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CHRONIC ETHANOL DRINKING BY ALCOHOL-PREFERRING RATS  
INCREASES THE SENSITIVITY OF THE MESOLIMBIC DOPAMINE SYSTEM  
TO THE REINFORCING AND STIMULATING EFFECTS OF COCAINE

A Dissertation

Submitted to the Faculty

of

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by

Scott M. Oster

In Partial Fulfillment of the  
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of

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Indianapolis, Indiana

For Ty, Xaven, and Daxen.

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## LIST OF ABBREVIATIONS

5-HT <sub>1B</sub>	serotonin receptor type 1B
5-HT <sub>2A</sub>	serotonin receptor type 2A
5-HT <sub>3</sub>	serotonin receptor type 3
Ab	ethanol-abstinent pretreatment condition
AC	adenylyl cyclase
Acb	nucleus accumbens
AcbC	nucleus accumbens, core subregion
AcbSh	nucleus accumbens, shell subregion
ACh	acetylcholine
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
AP	anterior-posterior axis coordinate
aVTA	ventral tegmental area, anterior subregion
AUC	area under the curve
BNST	bed nucleus of the stria terminalis
C	Celsius
cAMP	cyclic adenosine monophosphate
CE	continuous ethanol-drinking pretreatment condition
CNS	central nervous system
cm	centimeter
CPP	conditioned place preference
CREB	cAMP response element binding protein
CRF	corticotrophin releasing factor
CRF <sub>1</sub>	corticotrophin releasing factor receptor type 1

CRF <sub>2</sub>	corticotrophin releasing factor receptor type 2
d	day
D <sub>1</sub>	dopamine receptor type 1
D <sub>2</sub>	dopamine receptor type 2
D <sub>3</sub>	dopamine receptor type 3
D <sub>4</sub>	dopamine receptor type 4
D <sub>5</sub>	dopamine receptor type 5
DA	dopamine
DAT	dopamine transporter
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
DV	dorsal-ventral axis coordinate
EDTA	ethylenediaminetetraacetic acid
EMIT	electrolytic microinfusion transducer
FR	fixed ratio
g	gram
GABA	gamma-aminobutyric acid
GABA <sub>A</sub>	GABA receptor type A
GluR <sub>1</sub>	glutamate receptor subunit 1
HPLC	high performance liquid chromatography
HR	high responder to novelty
h	hour
ICSA	intracranial self-administration
IVSA	intravenous self-administration
kg	kilogram
LR	low responder to novelty
LTP	long-term potentiation
MCL	mesocorticolimbic
mg%	milligram percent
min	minute

ML	medial-lateral axis coordinate
mm	millimeter
mM	millimolar
MSN	medium spiny neuron
mV	millivolt
MVL	meso-ventrolateral
MVM	meso-ventromedial
N	ethanol-naïve pretreatment condition
NET	norepinephrine transporter
nl	nanoliter
nM	nanomolar
nmol	nanomole
NMDA	<i>N</i> -methyl-D-aspartate
NMDAR <sub>1</sub>	NMDA receptor subunit 1
NP	alcohol-nonpreferring
ORX	orexin
OSA	1-octanesulfonic acid
OT	olfactory tubercle
P	alcohol-preferring
pA	picoampere
PFC	prefrontal cortex
PKA	protein kinase A
pmol	picomole
PR	progressive ratio
pVTA	ventral tegmental area, posterior subregion
s	second
SEM	standard error of the mean
SERT	serotonin transporter
SN	substantia nigra
THC	delta-9 tetrahydrocannabinol

V	volt
v	volume
VP	ventral pallidum
VTA	ventral tegmental area
wk	week
$\mu$ A	microampere
$\mu$ l	microliter
$\mu$ m	micrometer
$\mu$ M	micromolar

## ABSTRACT

Oster, Scott M. Ph.D., Purdue University, May, 2012. Chronic Ethanol Drinking by Alcohol-Preferring Rats Increases the Sensitivity of the Mesolimbic Dopamine System to the Reinforcing and Stimulating Effects of Cocaine. Major Professor: James M. Murphy.

Alcohol and cocaine are commonly co-abused drugs, and those meeting criteria for both cocaine and alcohol use disorders experience more severe behavioral and health consequences than those with a single disorder. Chronic alcohol (ethanol) drinking increased the reinforcing and dopamine (DA) neuronal stimulating effects of ethanol within mesolimbic regions of the central nervous system (CNS) of alcohol-preferring (P) rats. The objectives of the current study were to determine if chronic continuous ethanol drinking produced: (1) alterations in the sensitivity of the nucleus accumbens shell (AcbSh) to the reinforcing effects of cocaine, (2) changes in the magnitude and time course of the local stimulating effects of cocaine on posterior ventral tegmental area (pVTA) DA neurons, and (3) a persistence of alterations in the stimulating effects of cocaine after a period of protracted abstinence.

Female P rats received continuous, free-choice access to water and 15% v/v ethanol for at least 10 wk (continuous ethanol-drinking; CE) or access to water alone (ethanol-naïve; N). A third group of rats received the same period of ethanol access followed by 30 d of protracted abstinence from ethanol (ethanol-abstinent; Ab). CE and Ab rats consumed, on average, 6-7 g/kg/d of ethanol. Animals with a single cannula aimed at the AcbSh responded for injections of cocaine into the AcbSh during four initial operant sessions. Cocaine was not present in the self-infused solution for the subsequent three sessions, and

cocaine access was restored during one final session. Animals with dual ipsilateral cannulae aimed at the AcbSh and the pVTA were injected with pulsed microinfusions of cocaine into the pVTA while DA content was collected for analysis through a microdialysis probe inserted into the AcbSh.

During the initial four sessions, neither CE nor N rats self-infused artificial cerebrospinal fluid (aCSF) or 0.1 mM cocaine into the AcbSh. CE, but not N, rats self-administered 0.5 mM cocaine into the AcbSh, whereas both groups self-infused concentrations of 1.0, 2.0, 4.0, or 8.0 mM cocaine. When cocaine access was restored in Session 8, CE rats responded more on the active lever and obtained more infusions of 0.5, 1.0, 2.0, or 4.0 mM cocaine compared to N rats. Microinjection of aCSF into the pVTA did not alter AcbSh DA levels in N, CE, or Ab rats. Microinjections of 0.25 mM cocaine into the pVTA did not significantly alter AcbSh DA levels in N animals, moderately increased DA levels in CE rats, and greatly increased DA levels in Ab rats. Microinjections of 0.5 mM cocaine into the pVTA modestly increased AcbSh DA levels in N animals, robustly increased DA levels in CE rats, and did not significantly alter DA levels in Ab rats. Microinjections of 1.0 or 2.0 mM cocaine into the pVTA modestly increased AcbSh DA levels in N animals but decreased DA levels in CE and Ab rats.

Overall, long-term continuous ethanol drinking by P rats enhanced both the reinforcing effects of cocaine within the AcbSh and the stimulatory and inhibitory effects of cocaine on pVTA DA neurons. Alterations in the stimulatory and inhibitory effects of cocaine on pVTA DA neurons were not only enduring, but also enhanced, following a period of protracted abstinence from ethanol exposure. Translationally, prevention of chronic and excessive alcohol intake in populations with a genetic risk for substance abuse may reduce the likelihood of subsequent cocaine use.

## 1. INTRODUCTION

### Addiction and Polydrug Use

A definitive feature of substance abuse is an individual's transition from a controlled use of drugs or alcohol to a state where compulsive substance use involves a 'loss of control' (DSM-IV, American Psychiatric Association, 1994). Along these lines, addiction is generally expressed as maladaptive changes in spontaneous behavior and the behavioral response to a drug after continued use, resulting from drug-induced adaptations in the central nervous system (CNS; Kalivas, 2009). More than 50% of Americans over the age of 12 (120 million) are current alcohol users, and nearly 8% of those (18 million) met criteria for substance abuse or dependence (Substance Abuse and Mental Health Services Administration, 2008). Moreover, nearly 16% of adults in the United States (29 million) will participate in non-medical or illicit drug use in their lifetime, with 3% (5.4 million) meeting criteria for drug dependence at some point (Grant and Dawson, 1998; Grant et al., 2004). Of this illicit drug use, cocaine continues to be a significant problem. In 2005, 2.4 million and 1.5 million Americans were classified as users and abusers, respectively (Substance Abuse and Mental Health Services Administration, 2005). Cocaine use accounted for 29% of all drug-related emergency department visits in 2000, more than any other illicit drug. Substance abuse and dependence were associated with an increased risk of physical and mental illness, disability, lost work productivity, financial problems, committing and suffering violence, accidents, and death (Rehm et al., 2006). Estimates predict that 23% of alcohol users and 21% of cocaine users will become dependent at some time in their life (Lopez-Quintero et al., 2011b).

Those with a diagnosis of an alcohol use disorder (abuse or dependence) were seven times more likely to develop another drug use disorder as those without a diagnosis of alcohol abuse or dependence (Compton et al., 2007). Subjects with the diagnosis of alcohol dependence were more likely to become cocaine misusers and experience more adverse consequences of cocaine use, and alcohol abuse was a common problem among cocaine dependent patients (Heil et al., 2001; Staines et al., 2001). Those with a previous diagnosis of cocaine dependence were less likely to remit from alcohol dependence (Lopez-Quintero et al., 2011a). Moreover, a significant proportion of those seeking treatment for either alcohol or cocaine abuse concurrently used both drugs. For example, the majority of cocaine users reported co-administering alcohol during cocaine binges (Brookoff et al., 1996; Carroll et al., 1993; Grant and Harford, 1990; Magura and Rosenblum, 2000). Estimates suggest that 20-63% of alcohol-dependent patients qualified as cocaine-dependent (Miller and Giannini, 1991), while 62-85% of cocaine-dependent individuals were dependent on alcohol (Regier et al., 1990; Rounsaville et al., 1991). Individuals with comorbid drug and alcohol use disorders met a higher number of criteria for each disorder, and thus displayed more severe dependence-related problems than those with a single disorder. Those who co-abused alcohol and other drugs were also more likely to be diagnosed with psychiatric disorders, attempt suicide, and suffer health problems (Arnaout and Petrakis, 2008). Patients who were both cocaine- and alcohol-dependent displayed more psychosocial problems compared to patients with only alcohol dependence or cocaine dependence (Brady et al., 1995; Carroll et al., 1993; Walsh et al., 1991). Furthermore, chronic abuse of either cocaine or alcohol left multiple metabolic and structural brain defects after protracted abstinence, and concomitant dependence on both substances aggravated white-matter structural defects in frontal cortical brain regions (O'Neill et al., 2001).

In studies of drug use initiation order, alcohol and tobacco are most commonly used first, followed by marijuana, and finally other illicit drugs (Ellickson et al., 1992; Fergusson et al., 2006). Kandel and Faust (1975) initially

proposed this sequence as the 'gateway' hypothesis, suggesting use at each stage increases the number of users progressing to the next stage. While longitudinal studies support a causal effect of alcohol and tobacco use on subsequent illicit drug use (Van Gundy and Rebellon, 2006), one must account for the potential existence of one or more unmeasured common causes (Morrall et al., 2002). Accordingly, background prevalence at least partially affected substance use progression patterns when patterns from multiple countries around the world were analyzed (Degenhardt et al., 2010). Interestingly, the initiation of alcohol and tobacco use was more significantly associated with the subsequent onset of illicit drug use in countries with higher rates of alcohol/tobacco use (e.g. the United States) compared to countries with lower use rates (e.g. Nigeria and South Africa). While early onset drug use often predicted an increased risk of transition to dependence (Behrendt et al., 2009; Dawson et al., 2008; Grant et al., 2001), the risk of transitioning to dependence may depend as much upon the existence of a concurrent psychiatric or other substance use disorder (Behrendt et al., 2009; Degenhardt et al., 2009; Lopez-Quintero et al., 2011b). Associative learning processes, rapid neuroadaptations, and drug interactions potentially mediated the enhanced speed of transition from cocaine use to dependence resulting from a previous history of a distinct substance use disorder, via enhanced drug effects and/or decreased adverse effects (Bradberry et al., 1999; Kapusta et al., 2007; Leri et al., 2003; Schlaepfer et al., 2008).

Genetic factors played a significant role in the pattern of comorbidity of common psychiatric and substance use disorders when examined in family, twin, and genome-wide studies (Burmeister et al., 2008; Kendler et al., 2003; Li and Burmeister, 2009). Dependence on multiple substances had a high genetic liability, and polysubstance abuse was associated with a number of alleles on several genes (Agrawal et al., 2008; Schlaepfer et al., 2008). Moreover, while both genetic and environmental factors played a role in twin concordance of cocaine use, genetic factors more significantly impacted the concordance of

abuse and dependence (Kendler and Prescott, 1998; Kendler et al., 2000). For example, 59% of those diagnosed with an alcohol use disorder and 77% of those diagnosed with a cocaine use disorder had family histories with substance use disorders (Lopez-Quintero et al., 2011b). Furthermore, the rate of cocaine-dependence was nearly three times higher in individuals with a genetic predisposition for alcoholism compared to the general population (Nurnberger et al., 2004). The initiation of alcohol use predicts the subsequent use of illicit drugs, but questions regarding causality remain when examining epidemiological data. Experiments using animal models of drug use can be used to examine the sequence of drug initiation and elucidate putative neurobiological substrates for adaptive processes contributing to the observed changes in behavior.

### The Role of Mesocorticolimbic Dopamine

Compulsive and maladaptive drug-taking and drug-seeking behaviors are associated with neuroplastic changes within the mesocorticolimbic dopamine (DA) pathway and its afferent and efferent connections (Everitt and Robbins, 2005; Koob and Volkow, 2010; Nestler, 2005; Wise, 2004). The mesocorticolimbic (MCL) system consists of DA cell bodies in the ventral tegmental area (VTA), sending efferent projections to a number of cortical and basal 'limbic' forebrain structures, including the nucleus accumbens (Acb), ventral pallidum (VP), amygdala, olfactory tubercle (OT), septum, bed nucleus of the stria terminalis (BNST), hypothalamus, thalamus, habenula, hippocampus, prefrontal cortex (PFC), orbitofrontal cortex, and anterior cingulate (Hasue and Shammah-Lagnado, 2002; Nauta et al., 1978; Oades and Halliday, 1987; Phillipson, 1979a; Sesack and Grace; 2010; Swanson, 1982). VTA DA neurons were implicated in a number of functions, including: the facilitation of approach behaviors; the mediation of positive reinforcement; error prediction of reinforcement; and the mediation of associative learning processes, such as selection, initiation, and invigoration of learned behaviors (Baldo and Kelley,

2007; Fields et al., 2007; Grace et al., 2007; Horvitz, 2000; Ikemoto, 2007; Kelley and Berridge, 2002; Redgrave et al., 2008; Robbins and Everitt, 1996; Salamone et al., 2003; Schultz, 2007; Wise, 2004). While these functions are conceptually distinct, they are difficult to separate experimentally (Fields et al., 2007; Ikemoto and Panksepp, 1999). Moreover, firing patterns of VTA DA neurons and both increases and decreases in terminal DA levels were associated with stress, novelty, negative reinforcement, and aversive/noxious stimuli (Brischoux et al., 2009; Carlezon and Thomas, 2009; Liu et al., 2008; Ungless et al., 2010).

The MCL system facilitates complex hierarchical control of adaptive ingestive behavior by integrating temporally and spatially coincident events into associations, allowing for adaptive and goal-directed behaviors and ultimately, survival (Kelley, 2004). Briefly, the constructs of 'reward' and 'reinforcement' are related, but distinct. Reward can be conceptualized as an unconditioned stimulus eliciting an appetitive approach response from an organism (Fields et al., 2007; Ikemoto and Panksepp, 1999; Young, 1959), distinct from the concept of reward as a positive hedonic experience (i.e. euphoria or liking). Reinforcement refers to the strengthening of associations between an action, a stimulus contingent to an outcome, and an outcome contingent to an action (Skinner, 1938). A number of novel stimuli initially unpaired with behavioral outcomes but possessing salience due to high intensity and abrupt onset affected the activity of VTA DA neurons (Schultz et al., 1998). Unexpected natural rewards and conditioned cues predicting reinforcement, both motivationally relevant events, induced firing of VTA DA neurons (McClure et al., 2003; Phillips et al., 2003). Thus, DA release in terminal areas, such as the Acb, was potentially involved with the response to the delivery of a reinforcer and enabled motivated actions toward future reinforcement (Wise, 2004). As such, MCL DA potentially functions primarily to guide instrumental behavior and is less associated with the consummatory act (Robinson and Berridge, 1993; Salamone et al., 2003). Theoretically, DA released in terminal areas acts: to alert the organism to the appearance of a novel salient stimuli and promote learning through neuroplasticity, and to alert the

organism of a future presentation of a familiar motivationally relevant event based on learned associations with stimuli predicting the event (Keitz et al., 2003; McClure et al., 2003). Interestingly, different subsets of neurons within the Acb may respond preferentially to different motivationally relevant stimuli, such as water versus cocaine (Carelli and Wondolowski, 2003).

On the other hand, a number of similarities between drug and non-drug (e.g. food, sex, exercise, gambling) reinforcers exist with regards to both behavioral and neurobiological responses (Corwin and Hajnal, 2005; Levine et al., 2003; Pelchat et al., 2004; Potenza, 2008; Volkow and Wise, 2005). For example, acute exposure either drugs of abuse (e.g. ethanol, cocaine, nicotine) or sucrose resulted in increased DA levels within terminal regions of the MCL system (Carr, 2002; Di Chiara and Imperato, 1988; Hajnal et al., 2004; Rada et al., 2005). Furthermore, hunger was not required to produce food or sucrose craving (Grimm et al., 2007; Pelchat et al., 2004), just as withdrawal is not necessary for drug craving (Shaham et al., 2003). Interactive effects between drug and non-drug reinforcement processes potentially resulted from common neural substrates. For example, the process of oral ethanol consumption activated reinforcement circuitry through both direct mechanisms and indirectly via the stimulation of neural substrates associated with the taste of sugar (Lemon et al., 2004). In addition, excessive seeking and consumption of food and sucrose followed a similar progression of compulsive and maladaptive behaviors associated with drug addiction (Ahmed, 2005; Avena et al., 2008; Carr, 2007; Koob and Volkow, 2010; Wise, 2004). Sucrose and drug reinforcers displayed both cross-sensitization (Avena and Hoebel, 2003a,b; Colantuoni et al., 2001; Gosnell, 2005) and cross-tolerance (D'Anci et al., 1996). Moreover, sequential exposure to drug and non-drug reinforcers produced interactive effects with regard to intake escalation (Avena et al., 2008; Colantuoni et al., 2002), withdrawal effects (Colantuoni et al., 2002), and relapse (Shalev et al., 2006).

Drugs of abuse produced a functional reorganization of the MCL system in the form of robust and persistent neuronal plasticity, leading to the formation of

maladaptive behaviors and ultimately compulsive drug seeking (Kalivas and Volkow, 2005; Self and Nestler, 1998; Steketee and Kalivas, 2011; Wolf et al., 2004; Zweifel et al., 2008). While drugs of abuse induced functional alterations throughout the MCL and its afferent and efferent connections, the mesoaccumbens division of the MCL is of particular interest in the initiation and early formation of maladaptive neuroplasticity. The mesoaccumbens (or meso-ventrostriatal) circuit can be divided into two functionally distinct, but interrelated systems (Ikemoto, 2007). These systems are: the meso-ventromedial (MVM) circuit from the caudomedial (posterior) VTA (pVTA) to the medial Acb shell (AcbSh) and medial OT, and the meso-ventrolateral (MVL) circuit from the rostromedial (anterior) VTA (aVTA) to the lateral AcbSh, Acb core (AcbC), and lateral OT. For future reference, AcbSh will refer to the medial AcbSh, as nearly all experiments evaluating the function of the AcbSh have specifically targeted medial regions. Research into the mechanisms of drugs of abuse within the MVM is of particular interest, as both the pVTA and AcbSh are critical substrates for the acute reinforcing effects of drugs and for the initiation of associative learning (Gonzales et al., 2004; Ikemoto, 2007; Ikemoto and Panksepp, 1999; Kelley, 2004; Koob and Volkow, 2010; Koob et al., 1998; McBride et al., 1999; Nestler, 2005; Wise, 2004). The dynamic role of MVM dopaminergic functioning in reinforcement and learning processes has frequently been assessed using a number of well established behavioral and neurochemical assays.

### Behavioral and Neurobiological Assays

*In vivo* microdialysis, voltammetry, and electrophysiological measurement (for review, see Marinelli et al., 2006; Salamone et al., 1996, Torregrossa and Kalivas, 2008), as well as intracranial self-administration (ICSA) and intracranial place conditioning (for review, see Ikemoto, 2007, 2010; McBride et al., 1999; Sanchis-Segura and Spanagel, 2006; Tzschentke, 1998, 2007; Wise and

Hoffman, 1992), are examples of assays implemented in the characterization of specific brain sites involved in reinforcement and learning processes. The investigation of the neurocircuitry and mechanisms associated with drug reinforcement using both neurochemical (e.g. *in vivo* microdialysis) and behavioral (e.g. ICSA) techniques provide convergent evidence associating functional alterations in neurotransmitter output with changes in behavioral output (Torregrossa and Kalivas, 2008).

The ICSA technique is a valuable method for the identification and classification of discrete brain regions responsible for the initiation and maintenance of response-contingent delivery of a chemical reinforcer (Goeders and Smith, 1987). ICSA procedures have reliably identified brain sites involved in mediating the reinforcing effects of drugs of abuse and have examined brain sites, neuronal pathways, and receptor systems involved in mediating operant reinforcement behavior (for review, see Goeders and Smith, 1987; Ikemoto, 2007, 2010; Wise, 2002; McBride et al., 1999). In some cases, a chemical agent's neural 'trigger-zone' contains cell bodies, whereas in other cases it is within a terminal region. Drugs of abuse can act within the brain in a reinforcing manner by mimicking, enhancing, or blocking the actions of endogenous transmitters in the brain within particular anatomical substrates of the MCL DA system (Wise and Hoffman, 1992) and sites sending afferent innervation to MCL sites (Ikemoto, 2010). Using the ICSA paradigm, animals receive the delivery of nanoliter volumes of a drug solution into a discrete brain region a response-contingent manner. The ICSA procedure has been used to elucidate specific central nervous system sites, neuronal systems, and receptor types involved in reinforcement and the interactions between these systems (McBride et al., 1999). By using intracranial drug delivery, the ICSA technique presents fewer anatomical and pharmacological limitations compared to the intracranial electrical self-stimulation and intravenous self-administration (IVSA) methods.

The microinjection-microdialysis procedure has identified specific brain sites involved in the actions of drugs and other pharmacological agents and the

corresponding neurotransmitter responses in associated brain sites (Ding et al., 2009a,b; Guan and McBride, 1989; Ikemoto et al., 1997b). For example, microinjection-microdialysis techniques have explored the specific 'downstream response' of ICOSA-like pulsed drug microinfusions to the pVTA (which contain MVM DA neurons) on extracellular DA levels in terminal regions such as the AcbSh. Drug administration is contingent upon operant responding in the ICOSA paradigm, but not during microinjection-microdialysis. Along these lines, certain neurobiological assays captured the effects of drug contingency on the expression of functional alterations to the MCL system (Chen et al., 2008), while other assays did not find significant effects of contingency (Geisler et al., 2008).

The microinjection-microdialysis paradigm examines the initial (non-contingent) pharmacological effects presumably observed during the initiation of response-contingent behaviors in parallel ICOSA experiments. Nevertheless, changes in Acb DA levels are extremely similar after either operant oral ethanol consumption (Melendez et al., 2002; Weiss et al., 1996) or pVTA microinjection of ethanol (Ding et al., 2009a). Moreover, microinjections of the ethanol metabolites acetaldehyde or salsolinol, or cocaine into the pVTA, but not the aVTA, increased extracellular DA levels within the Acb (current results; Deehan et al., 2007; Rodd et al., 2006b). Accordingly, rats will self-administer these reinforcing compounds (cocaine, ethanol, acetaldehyde, salsolinol) into the pVTA, but not aVTA (Rodd et al., 2004a,b, 2005a,b,c,d, 2008; Rodd-Henricks et al., 2000a, 2002b, 2003). Of note, *in vivo* microdialysis is a measurement tool best utilized to assess 'tonic', opposed to 'phasic', dopaminergic functioning (Grace, 2000; Grace et al., 2007). In this regard, observed changes in extracellular DA were correlated with the number of spontaneously active VTA DA neurons, but not with the average firing frequency or burst activity of VTA DA neurons (Floresco et al., 2003; Marinelli et al., 2006).

## Mesoaccumbens Circuit: Connectivity and Heterogeneity

### Ventral Tegmental Area: Connectivity

The VTA consists of a number of adjacent heterogeneous groups of cells positioned medially and ventrally within the midbrain (Tsai, 1925a,b; Dahlstrom and Fuxe, 1964). The VTA contains the majority of what is historically known as the A10 DA cell bodies, although there is no clear boundary between A9 nigral and A10 tegmental neurons (Dahlstrom and Fuxe, 1964; Lindvall and Bjorklund, 1978; Oades and Halliday, 1987). The VTA contains mostly DA neurons (around 65%; Swanson, 1982; Nair-Roberts et al., 2008), with gamma-aminobutyric acid (GABA; Swanson, 1982; Steffensen et al., 1998) and glutamate (Nair-Roberts et al., 2008) neurons present to a lesser degree (around 30-35% and 3%, respectively). These DA neurons send significant projections to limbic and cortical areas, while less prominently innervating striatal, diencephalic, and rhombencephalic areas (Oades and Halliday, 1987).

The VTA receives abundant afferent projections, forming a continuous band of neurons not organized into discrete nuclei, from a number of regions (predominantly 'limbic' structures) including the: PFC, lateral septum, medial septum-diagonal band complex, Acb, VP, BNST, amygdala, medial and lateral preoptic area, lateral hypothalamus, lateral habenula, dorsal raphe, rostromedial tegmental nucleus, pedunculo-pontine tegmental nucleus, laterodorsal tegmentum, periaqueductal gray, and mesencephalic and pontine reticular formation (Carter and Fibiger, 1977; Dahlstrom and Fuxe, 1964; Geisler and Zahm, 2005; Lindvall and Bjorklund, 1978; Mena-Segovia et al., 2008; Oades and Halliday, 1987; Phillipson, 1979b). These afferent inputs provide excitatory, inhibitory, and modulatory innervation to the DA, GABA, and glutamate neurons within the VTA via the release of a number of neurotransmitters and neuromodulators, including: acetylcholine (ACh), GABA, glutamate, norepinephrine, serotonin, endogenous opioids, corticotrophin releasing factor

(CRF), and orexin (ORX). The VTA sends efferent projections from DA and GABA neurons, with minimal branching and considerable overlap, innervating cortical regions (e.g. prefrontal, insular, medial frontal, entorhinal, cingulate, anterior suprarhinal), Acb, lateral septum, BNST, amygdala, OT, dorsal hippocampus, lateral habenula, thalamus, reticular formation, central gray, locus coeruleus, median and dorsal raphe nuclei, and the anteromedial striatum (Beckstead et al., 1979; Carter and Fibiger, 1977; Fallon and Moore, 1978; Ikemoto, 2007; Nauta et al., 1978; Oades and Halliday, 1987; Swanson, 1982).

Anatomically, the VTA is a heterogeneous structure composed of five identified nuclei: paranigral, parabrachial, interfascicular, rostral linear, and caudal linear (for review, see Oades and Halliday, 1987). VTA cytoarchitecture includes morphologically distinct dopaminergic cells exhibiting specialized axonal projections (Halliday and Tork, 1984; Phillipson, 1979a) and topographic afferent and efferent projections (Brog et al., 1993; Fallon and Moore, 1978; Fallon, 1988; Hasue and Shammah-Lagnado, 2002; Kalen et al., 1988; Tan et al., 1995). For instance, the raphe nuclei send serotonin projections primarily innervating the interfascicular and paranigral of the VTA, with less innervation of the dorsorostral VTA (Herve et al., 1987). Moreover, reciprocal projections to the dorsal raphe nucleus originate almost exclusively from the caudal VTA (Kalen et al., 1988). Moreover, the paranigral region contains a greater density of serotonergic varicosities and sends projections primarily to the AcbSh, whereas the lateral PB region has less serotonergic innervation and projects predominantly to the AcbC (Van Bockstaele et al., 1994). While the majority of DA neurons originate from within the pVTA, most GABA and glutamate neurons are located in the aVTA (German and Manaye, 1993; Ikemoto, 2007; Nair-Roberts et al., 2008; Olson and Nestler, 2007; Swanson, 1982; Yamaguchi et al., 2007). ACh neurons preferentially innervate mesoaccumbens neurons in the VTA (Omelchenko and Sesack, 2006), and activation of nicotinic ACh receptors within the pVTA, but not aVTA, activated DA neurons (Zhao-Shea et al., 2011). ORX neurons synapse on DA neurons projecting to the PFC and AcbSh, but not the AcbC (Vittoz et al.,

2008). Medial portions of the parabrachial project to the AcbSh and medial OT, while the lateral parabrachial projects to the AcbC, lateral AcbSh, and lateral OT (Ikemoto, 2007).

### Nucleus Accumbens: Connectivity

The Acb is part of the ventral striatal complex (Zahm, 2000; Zahm and Brog, 1992) receiving extensive excitatory afferents from the cortex and thalamus (Sesack and Grace, 2010). The AcbSh and AcbC share striatal characteristics, as approximately 90% of the cells are medium spiny projection GABA neurons (MSN; Meredith, 1999). The remaining cells are primarily ACh interneurons, with an extensive network of axons and terminals innervating MSNs within both the AcbSh and AcbC (Meredith and Chang, 1994; Kawaguchi et al., 1995). The Acb receives afferent innervation from the VTA, VP, substantia nigra (SN) pars compacta, dorsal raphe, locus coeruleus, periaqueductal gray, pedunculopontine tegmental nucleus, lateral hypothalamus, cortex (PFC, limbic, orbital, and insular), subiculum of the hippocampus, and amygdala (Kelley et al., 1982; Reynolds and Zahm, 2005; Van Bockstaele and Pickel, 1995; Voorn et al., 1986). These afferents are excitatory, inhibitory, and modulatory through the release of GABA, glutamate, norepinephrine, serotonin, and ORX (Sesack and Grace, 2010). The Acb sends efferent projections to the hypothalamus, VP, VTA, SN, and other brainstem areas (Haber et al., 1990; Heimer et al., 1991; Zahm, 2000). The AcbSh and AcbC differ in their afferent and efferent projection patterns, neurochemistry, cellular morphology, and overall function (Heimer et al., 1991; Meredith et al., 1992, 1996; Brog et al., 1993; Zahm and Heimer, 1993).

Both dopaminergic and non-dopaminergic innervation of the Acb appears to display a similar medial-lateral topography (Ikemoto, 2007; Sesack and Grace, 2010). For example, ventral portions of the subiculum and limbic and orbital cortices innervate the AcbSh, whereas the dorsal aspects of these regions project to the AcbC (Brog et al., 1993). In addition, ORX neurons from the

hypothalamus innervate the AcbSh, but not the AcbC. DA afferents from the VTA to the Acb synapse onto GABA MSNs (Pickel and Chan, 1990; Pickel et al., 1988). These MSNs also receive excitatory synapses from cortical axon terminals often organized in a 'triad' arrangement, allowing DA to display inhibitory or modulatory synapses (Sesack and Pickel, 1990, 1992). DA axons in the Acb do not appear to selectively modulate particular glutamate afferents, but potentially act more commonly through volume transmission (Sesack and Grace, 2010). Supporting this idea are the findings that DA receptors in the Acb are primarily extrasynaptic (Sesack, 2009), DA communicates through both synaptic and volume transmission (Moss and Bolam, 2008) and modulates the general excitability of glutamate neurons (Nicola et al., 2000; Surmeier et al., 2007).

The AcbSh innervates the ventromedial VP, VTA, SN pars compacta, lateral hypothalamus, lateral preoptic area, periaqueductal gray, and pedunculopontine tegmental nucleus; while the AcbC innervates the dorsomedial VP, SN zona reticulata, and entopeduncular nucleus (Haber et al., 1990; Heimer et al., 1991). The dorsolateral VP then sends projections to the SN reticulata and subthalamic nucleus, while the ventromedial VP innervates the VTA and basal forebrain. This allows for not only an indirect and direct feedback loop from the forebrain to the midbrain, but also a spiral loop moving from ventromedial to dorsolateral striatal areas (Nauta et al., 1978; Zahm and Heimer, 1993). This spiral loop forms a pVTA-AcbSh-aVTA-AcbC-SNreticulata-striatum pattern of connectivity allowing 'limbic' structures to affect transmission in more motor-related structures of the basal forebrain (Sesack and Grace, 2010). This corresponds to Mogenson's conceptualization of the Acb as an interface where motivations from limbic regions interact with motor circuitry to drive motivated behavior (Mogenson et al., 1980).

Overall, firing of DA neurons in the VTA produces the release of DA from terminals in the Acb (Somers et al., 2009) and from the soma and dendrites within the VTA (Beart et al., 1979; Kalivas and Duffy, 1991) in an impulse-dependent manner (Beckstead et al., 2007; Kita et al., 2009). DA released within

the Acb activates DA-receptor-1-like ( $D_1$  and  $D_5$ ) and DA-receptor-2-like ( $D_2$ ,  $D_3$ , and  $D_4$ ) receptors. These receptors will be referred to from this point as simply  $D_1$  and  $D_2$  unless otherwise noted. Activation of  $D_1$  receptors increase adenylyl cyclase (AC) activity via stimulatory G-proteins to increase cyclic adenosine monophosphate (cAMP) levels, while the  $D_2$  receptor is negative coupled to AC and cAMP (Lachowicz and Sibley, 1997). While co-localization of presynaptic  $D_1$  and  $D_2$  receptors in the Acb is common (Wong et al., 1999), different populations of MSNs express either  $D_1$  or  $D_2$  receptors postsynaptically almost exclusively (Le Moine and Bloch, 1996; Lee et al., 2006). However, mixed physiological response-patterns to receptor manipulation are often observed due to the co-expression of the  $D_3$  receptors on  $D_1$ -expressing MSNs in the AcbSh (Le Moine and Bloch, 1996; Ridray et al., 1998). Activation of  $D_1$  receptors in the Acb enhances glutamatergic drive (Chergui and Lacey, 1999; West and Grace, 2002) via increased AC activity (Sibley et al., 1993), while activation of  $D_2$  receptors inhibits Acb neurons via decreased AC activity (White and Wang, 1986; O'Donnell and Grace, 1996). MSNs with  $D_2$  receptors project almost exclusively to the VP, whereas  $D_1$ -containing MSNs project to both the VP and VTA (Lu et al., 1997, 1998).

### Ventral Tegmental Area: Regional Heterogeneity

In addition to differences in morphology and connectivity, the VTA is also functionally heterogeneous with regards to drugs of abuse. Using viral-mediated gene transfer, over-expression of glutamate receptor subunit type one (Carlezon et al., 2000) or phospholipase C gamma-1 (Bolanos et al., 2003) in the aVTA increased morphine place preference, while over-expression in the pVTA resulted in morphine place avoidance. Moreover, over-expression of cAMP-response-element-binding protein (CREB) in the aVTA increased place preference for either cocaine or morphine, while over-expression in the pVTA resulted in place avoidance for these drugs (Olson et al., 2005). Furthermore, a

number of drugs of abuse and receptor ligands are self-administered directly into the pVTA, but not the aVTA, using the ICSA paradigm. These drugs include: cocaine (Rodd et al., 2005a), nicotine (Ikemoto et al., 2006), ethanol (Rodd et al., 2004a,b, 2005b,c,d; Rodd-Henricks et al., 2000a, 2003), the ethanol metabolites acetaldehyde and salsolinol (Rodd et al., 2005d, 2008; Rodd-Henricks et al., 2002b), delta-9 tetrahydrocannabinol (THC; Zangen et al., 2006), endomorphin-1 (Zangen et al., 2002), a serotonin receptor type 3 (5-HT<sub>3</sub>) agonist (Rodd et al., 2007), and a muscarinic ACh receptor agonist (Ikemoto and Wise, 2002). Moreover, THC, endomorphin-1 and the muscarinic agonist produced a place preference when injected into the pVTA, but not aVTA, using an intracranial place-conditioning paradigm (Ikemoto and Wise, 2002; Zangen et al., 2002, 2006).

#### Nucleus Accumbens: Regional Heterogeneity

A number of experiments observed a functional divide in the Acb in addition to the observed heterogeneity found in the VTA. Repeated injections of amphetamine into the AcbSh, but not AcbC, produced both a behavioral sensitization to locomotor stimulation and a neurochemical sensitization to increases in DA (Pierce and Kalivas, 1995). Moreover, amphetamine sensitization increased presynaptic proteins associated with synaptic plasticity and the regulation of DA release in the AcbSh, but reduced protein levels in the AcbC (Subramaniam et al., 2001). Similarly, chronic cocaine treatment produced a robust and persistent depression in MSN activity in the AcbSh, while MSNs in the AcbC were transiently activated (Kourrich and Thomas, 2009). Systemic administration of cocaine, morphine, and amphetamine preferentially increase extracellular DA in the AcbSh compared with the AcbC (Pontieri et al., 1995). The AcbSh was also more responsive than the AcbC to systemic administration of D<sub>1</sub> receptor agonists and antagonists when measuring ACh output (Consolo et al., 1999). Interestingly, injections of N-methyl-D-aspartic acid (NMDA)

antagonists into the AcbC, but not the AcbSh, reduced cocaine-induced locomotor activity (Pulvirenti et al., 1994).

Direct stimulation of D<sub>1</sub> or D<sub>2</sub> receptors within the AcbSh, but not the AcbC, produced a cocaine-primed 'reinstatement of responding' following cocaine IVSA and extinction (Bachtell et al., 2005; Schmidt et al. 2006) blocked by co-infusion of the respective receptor antagonist (Schmidt and Pierce, 2006). Conversely, D<sub>1</sub> or D<sub>2</sub> receptor antagonists injected into the AcbSh, but not the AcbC, blocked the 'reinstatement of responding' (Anderson et al., 2003, 2006; Bachtell et al., 2005). In addition, 6-hydroxydopamine lesions to the AcbSh, but not the AcbC, attenuated the conditioned place preference (CPP) for cocaine, while lesions to the AcbC, but not the AcbSh, reduced cocaine-induced increases in locomotor activity (Sellings et al., 2006). Both contingent (Hemby et al., 1997; Parsons et al., 1995) and non-contingent (Cadoni and Di Chiara, 1999; Hedou et al., 1999; Pontieri et al., 1995) administration of cocaine produced greater increases in DA within the AcbSh than the AcbC. Lastly, DA terminals in the AcbSh, but not the AcbC, can co-release glutamate (Stuber et al., 2010).

Utilizing the ICSA method, rats learned to self-administer the following agents into the AcbSh, but not the AcbC: cocaine (Katner et al., 2011, Rodd-Henricks et al., 2002a), ethanol (Engleman et al., 2009), the ethanol metabolite salsolinol (Rodd et al., 2003b), amphetamine (Ikemoto et al., 2005), THC (Zangen et al., 2002), a DA reuptake inhibitor (Carlezon et al., 1995), a combination of D<sub>1</sub> and D<sub>2</sub> agonists (but neither individually; Ikemoto et al., 1997a), a muscarinic ACh agonist (Ikemoto et al., 1998), and NMDA antagonists (Carlezon and Wise, 1996). Additionally, rats displayed a CPP for injections of THC into the AcbSh, but not into the AcbC (Zangen et al., 2002). Overall, connections from the pVTA to the AcbSh appear to be important for modulating motivational salience and establishing learned associations between motivational events and concurrent environmental stimuli, whereas the aVTA-AcbC circuit primarily mediates the expression of learned behaviors in response to stimuli

predicting motivational relevant events (Bassareo and Di Chiara, 1999; Di Ciano and Everitt, 2001; Kelley, 2004; Ikemoto, 2007).

### Drugs of Abuse Produce Alterations in Mesocorticolimbic Functioning

Compulsive and maladaptive behaviors associated with excessive drug taking and drug seeking result from persistent, and possibly, permanent alterations in the functioning of the MCL DA system and associated structures (Hyman et al., 2006; Kalivas, 2009; Koob and Volkow, 2010; Nestler, 2001; Wise, 2004). A key feature of addiction is the compromised ability to suppress compulsive drug seeking, even when faced with serious adverse consequences (Kalivas, 2009; Kalivas and Volkow, 2005). A susceptibility to relapse can remain for years after abstinence from drug use, and underscores the contributions of repeated drug exposure, genetic background, and associations between environmental stimuli and drug use (learning). During the initial experiences with drugs of abuse, the acute pharmacological drug effects and the relevant associative learning processes concomitantly activate the same neural circuitry (e.g. MVM DA: neuronal firing in the pVTA and increased DA release in the AcbSh). With continued drug use, the dopaminergic response within the AcbSh became sensitized to the acute drug effects (Hooks et al., 1994; Kalivas, 2009; Lecca et al., 2007; Madayag et al., 2010). Moreover, continued drug use produced maladaptive functional changes in the VTA, by increasing the strength of excitatory inputs along with a reduction in inhibitory input (Chen et al., 2010).

Research continues to elucidate specific mechanisms by which different drugs disrupt tonic and phasic DA signaling in MVM DA and other circuits, the functional significance of this disruption, and the cellular and molecular substrates by which drugs modify synapses and circuits. The initial stages of addiction likely involve the acquisition of excessive incentive–motivational properties by drug conditioned stimuli, as a result of drugs effectively ‘stamping-in’ maladaptive associations between the primary reinforce and drug cues via the

coincident activation of the MVM DA pathway (Di Chiara, 1999; Ikemoto and Panksepp, 1999; Robinson and Berridge, 1993). Furthermore, the development of addiction (e.g. progression from actions to habits to compulsive behavior) coincides with the progression of neuronal adaptations from limbic to cortical areas, from ventromedial to dorsolateral striatal systems, and from dopaminergic to glutamatergic control (Everitt and Robbins, 2005; Kalivas, 2009; Lane et al., 2010; Schmidt and Pierce, 2010; Sesack and Grace, 2010).

### Acute Reinforcing and Dopamine Neuronal Stimulating Effects of Cocaine within the Mesoaccumbens Circuit

Abused drugs (e.g. ethanol, cocaine, nicotine, amphetamine, morphine, THC) produced increased levels of DA in the Acb (Di Chiara and Imperato, 1988), albeit often through divergent substrates (Koob et al., 1998). For example, cocaine binds to norepinephrine, serotonin, and DA transporters (NET, SERT, and DAT, respectively) to inhibit the uptake of these neurotransmitters (Reith et al., 1986; Ritz et al., 1990). While the potency for cocaine reinforcement is strongly correlated with the affinity at the DAT (Ritz et al., 1987), knockout studies suggest SERT also plays a significant role in cocaine reinforcement (Filip et al., 2005; Hall et al., 2004; Sora et al., 2001). The net effect of cocaine to increase DA levels in the Acb activates local D<sub>1</sub> and D<sub>2</sub> receptors, and the concurrent stimulation of these receptors is required for cocaine sensitization (Henry et al., 1998). Injections of cocaine into the AcbSh, but not the AcbC, produced locomotor activation, CPP, and operant responding for self-infusion (Filip and Siwanowicz, 2001; Ikemoto, 2002; Katner et al., 2011; Liao et al., 2000; Rodd-Henricks et al., 2002a). The local effects of cocaine on DA output require neuronal impulse flow (Nomikos et al., 1990), both in the VTA (Chen and Reith, 1994a) and in the Acb (Westerink et al., 1987). Furthermore, VTA DA neuronal firing is required for cocaine to increase DA levels within the AcbSh (Aragona et al., 2008; Sombers et al., 2009).

In human imaging studies, VTA activation was associated with the subjective experience of euphoria following cocaine administration (Breiter et al., 1997; Breiter and Rosen, 1999). In animals, injections of cocaine into posterior portions of the VTA produced behavior sensitization and operant responding for self-infusion (Cornish and Kalivas, 2001; David et al., 2004; Rodd et al., 2005a). Similar to the Acb, cocaine inhibited monoamine uptake within the VTA, increasing local levels of DA, serotonin, and norepinephrine (Chen and Reith, 1994a,b). While the relative uptake potency has not been examined in the VTA, cocaine was more potent inhibiting serotonin versus DA uptake in the Acb (Matecka et al., 1996; Uchimura and North, 1990). Cocaine-induced increases in VTA serotonin levels activated local serotonin type 1B receptors (5-HT<sub>1B</sub>), serotonin type 2A receptors (5-HT<sub>2A</sub>), and 5-HT<sub>3</sub> receptors to putatively stimulate VTA DA neurons and increase DA levels in the Acb (McMahon and Cunningham, 2001; O'Dell and Parsons, 2004; Rodd et al., 2005a).

Furthermore, cocaine potentially acts indirectly through local glutamatergic functioning to increase VTA DA neuronal activity. Stimulation of glutamatergic tone to the VTA induced burst firing of DA neurons and increased the number of spontaneously active DA neurons (Kalivas, 1993; White, 1996). Cocaine-induced increases in VTA DA levels activated presynaptic D<sub>1</sub> receptors to increase local glutamate levels (Kalivas and Duffy, 1995), while D<sub>1</sub> receptor blockade in the VTA reduced the reinforcing effects of IVSA cocaine (Ranaldi and Wise, 2001). Moreover, impairment of VTA glutamatergic function attenuated: responding for cocaine conditioned stimuli (You et al., 2007), the acquisition of cocaine CPP (Harris and Aston-Jones, 2003), and behavioral sensitization to cocaine (Kalivas and Alesdatter, 1993). A single injection of cocaine, amphetamine, nicotine, morphine, or ethanol produced both a long-term potentiation (LTP) of excitatory glutamate synapses (Saal et al., 2003) and an impairment of LTP of inhibitory GABA synapses (Guan and Ye, 2010; Niehaus et al., 2010) on VTA DA neurons.

### Chronic Ethanol Exposure: Effects on Neurobiology and Behavior

Ethanol appears to produce acute reinforcement through direct activation of VTA DA neurons (Brodie et al., 1999; Gessa et al., 1985; Verbanck et al., 1990). Chronic ethanol exposure produces neuronal alterations in dopaminergic functioning in both the Acb and VTA (Fadda and Rossetti, 1998; Vengeliene et al., 2008). For example, chronic continuous voluntary ethanol drinking by alcohol-preferring rats produced an up-regulation in dopaminergic functioning within the Acb. Specifically, ethanol-drinking experience produced increased extracellular DA levels (Thielen et al., 2004), decreased D<sub>2</sub> autoreceptor function (Engleman et al., 2003), accelerated DA uptake (Carroll et al., 2006), and increased the effectiveness of a DAT-inhibitor to increase extracellular DA levels (Engleman et al., 2000). In the VTA, chronic ethanol drinking by alcohol-preferring rats increased the number of spontaneously active VTA DA neurons (Morzorati et al., 2010), while decreasing pVTA DA levels (Engleman et al., 2011). Furthermore, chronic voluntary ethanol drinking in outbred rats increased the number of DAT binding sites in the Acb and VTA (Jiao et al., 2006). Chronic ethanol drinking increased the sensitivity of VTA DA neurons to the DA-neuronal-stimulating and reinforcing effects of ethanol (Brodie, 2002; Rodd et al., 2005b,c). In addition, repeated local injections of ethanol sensitized pVTA DA neurons to the stimulating actions of ethanol (Ding et al., 2009a). Chronic ethanol drinking also influenced subsequent behavioral and neurobiological responses to cocaine.

Wistar rats with ethanol-drinking experience displayed a greater increase in Acb DA in response to the local application of cocaine compared to naïve controls (Yoshimoto et al., 2000), and mice with repeated ethanol exposure displayed a CPP for cocaine associated with enhanced synaptic plasticity of NMDA receptors on VTA DA neurons (Bernier et al., 2011). Furthermore, Wistar rats with high levels of ethanol drinking were more sensitive to cocaine CPP and locomotor activation than low ethanol-drinking rats (Stromberg and Mackler, 2005). Other studies also observed an enhanced locomotor response to cocaine

after chronic ethanol exposure (Hopf et al., 2007; Manley and Little, 1997) Rats with high levels of operant lever responding for oral ethanol subsequently displayed a more rapid acquisition of lever responding for IVSA cocaine compared to animals with lower levels of ethanol responding (Mierzejewski et al., 2003). Chronic ethanol exposure produced abolished the subsequent conditioned taste aversion to cocaine seen in ethanol-naïve animals (Kunin et al., 1999; Grakalic and Riley, 2002).

Other drugs of abuse produced neurobiological and behavioral alterations similar to those seen with chronic ethanol exposure. For example, chronic ethanol, cocaine, or morphine treatment increased levels of NMDA type 1 (NMDAR<sub>1</sub>) and glutamate type one (GluR<sub>1</sub>) ionotropic glutamate receptor subunits, while decreasing levels of the GABA receptor A (GABA<sub>A</sub>) alpha-1 subunit in the VTA (Fitzgerald et al., 1996; Ortiz et al., 1995). Repeated systemic ethanol, nicotine, morphine, or cocaine treatments produced an enhanced locomotor response to a subsequent injection of cocaine, associated with a long-term enhancement of evoked DA and ACh release in the Acb (Nestby et al., 1997). Repeated cocaine or ethanol injections produced an enhanced locomotor response to a subsequent injection with the opposite drug, an effect associated with an increased number of striatal DAT binding sites (Itzhak and Martin, 1999).

### Genetic Factors Associated with Drug and Alcohol Abuse

An increased risk for the development of a drug or alcohol use disorder is significantly greater for those with an identified genetic risk factor (Heath et al., 1997; Lopez-Quintero et al., 2011b; Nurnberger et al., 2004). These risks are often associated with differences in the expression of specific genes associated with neurotransmission (Edenberg and Kranzler, 2005; Kohnke, 2008). Moreover, certain genes are associated with multiple substance use disorders, such as the gene encoding the tachykinin 3 receptor, which is associated with both alcohol and cocaine dependence (Foroud et al., 2008).

Animal research has attempted to investigate the role of genetic background on drug and alcohol use and abuse through the characterization of individual differences in outbred rat strains or with the selective breeding of a specific phenotype from a heterogeneous stock of animals. Selective bi-directional breeding has established high- and low-alcohol drinking lines to serve as reliable models for assessing genetic predispositions associated with the contrasting extremes of alcohol use observed in human populations (Murphy et al., 2002). To date, bi-directional breeding for ethanol preference versus non-preference has produced five sets of alcohol-preferring and -nonpreferring lines. These include the University of Chile A and B lines (Mardones and Segovia-Riquelme, 1983), the Sardinian alcohol-preferring/nonpreferring lines (Colombo, 1997), the ALKO alcohol/nonalcohol lines (Eriksson, 1968), the Indiana University alcohol-preferring/nonpreferring (P/NP) lines (Lumeng et al., 1977), and the Indiana University high/low alcohol-drinking replicate lines (Li et al., 1993). The Indiana P rat line, derived by selective breeding from an outbred Wistar stock, has been thoroughly tested and found to fulfill the necessary criteria for an animal model of alcoholism originally proposed by Lester and Freed (1973) and Cicero (1979).

The P line of alcohol-preferring rats will orally self-administer significant levels of ethanol; reach pharmacologically relevant blood ethanol levels (200 mg%) during 24-h free-choice ethanol drinking; consume alcohol for pharmacological effects and not caloric value or taste; work for the operant oral, intracranial, and intragastric self-administration of ethanol to show positive reinforcement; show expression of functional and metabolic tolerance after chronic consumption of ethanol; and show withdrawal symptoms (a sign of dependence) after chronic ethanol consumption and termination of ethanol access (McBride and Li, 1998; Murphy et al., 2002). In addition, the P line also meets an additional criterion proposed by McBride and Li (1998) – the alcohol-deprivation effect. P rats show an alcohol-deprivation effect, represented by a transitory increase in the level of ethanol consumption after a single period or

multiple periods of forced abstinence. The alcohol-deprivation effect may be a useful model for studying the human phenomenon of relapse, 'loss of control', or 'binge' drinking (Murphy et al., 2002).

P and NP rats represent two divergent neurobiological phenotypes of alcohol preference. P rats, compared to NP rats, had significantly lower levels of serotonin and the primary metabolite 5-hydroxyindoleacetic acid in a number of cortical and limbic regions, including the Acb (Murphy et al., 1982, 1987). Appropriately, P rats had less dense serotonergic innervation of many of these regions, including the Acb, than NP rats (Zhou et al., 1991). P and NP rats also displayed differences in serotonin receptor densities in a number of limbic brain regions (Murphy et al., 2002). Moreover, P rats had lower levels of DA and the metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid in the Acb and fewer dopaminergic projections from the VTA to the Acb, compared to NP rats (Murphy et al., 1982, 1987; Zhou et al., 1995). Compared to NP rats, P rats possessed pVTA DA neurons with more action potentials in bursts and a greater number of bursts (Morzorati and Marunde, 2006). Lastly, P rats had relatively higher densities of mu-opioid receptors in the AcbSh and AcbC, but lower levels of CRF in the amygdala (Ehlers et al., 1992; McBride et al., 1998).

In addition to alcohol-preference and neurobiology, high and low alcohol-preferring lines were characterized by phenotypic differences in the response to other drugs of abuse. Alcohol-preferring rats were more sensitive to the effects of cocaine compared to alcohol-non-preferring and outbred rat strains when measured by: operant reinforcement (Katner et al., 2011; Marttila et al., 2007), locomotor activation (Honkanen et al., 1999; Mikkola et al., 2002), AcbSh dopaminergic response (Leggio et al., 2003), neurochemical sensitization of AcbSh DA (Mikkola et al., 2001), and drug-induced reinstatement of cocaine-seeking behavior (Le et al., 2006). In addition, peripheral injections of either cocaine or ethanol produced significantly greater Acb DA release in mice bred for sensitivity to the locomotor activating effects of ethanol compared to ethanol-insensitive mice (Meyer et al., 2009). Selective breeding for alcohol preference

potentially produced pleiotropic effects of the genes underlying the selected phenotype for a general preference for drugs of abuse.

Reciprocal genetic factors may affect both the intake of sweetened substances and drug self-administration (Carroll et al. 2008). Compared to rats selectively bred for saccharin non-preference (LoS), rats bred for saccharin preference (HiS) displayed enhanced acquisition, maintenance, escalation, extinction, and reinstatement of cocaine IVSA (Carroll et al., 2002, 2007, Dess et al., 1998; Perry et al., 2006). HiS animals also displayed a greater cocaine-induced locomotor activation and a potentiated locomotor sensitization compared to LoS animals (Carroll et al., 2007). In addition to selective breeding, studies also examined individual differences in drug-related behaviors. A model of spontaneous addiction liability characterizing rats by the locomotor activity to a novel environment was developed, resulting in a divergent response to psychostimulants between high-responder (HR) and low-responder (LR) to novelty rats. Compared to LR rats, HR rats: exhibited a greater locomotor and Acb dopaminergic response to cocaine or amphetamine (Hooks et al., 1991, 1992; Piazza et al., 1989), acquired cocaine or amphetamine IVSA more rapidly (Marinelli and White, 2000; Piazza et al., 1990), were quicker to escalate cocaine intake (Grimm and See, 1997), displayed increased maximal responding at peak doses of cocaine (Piazza et al., 2000), and more robust and persistence increases in VTA DA neuronal firing during acute withdrawal from cocaine (McCutcheon et al., 2009). The interactions between genetic background and drug exposure also produced unique behavioral effects. For example, repeated ethanol injections increased basal DA levels in P and Wistar rats, but not NP rats (Smith and Weiss, 1999). Ethanol exposure also increased basal serotonin levels in NP and Wistar rats, but decreased levels in P rats.

### Objectives and Experimental Rationale

The objectives of the present study are to determine if chronic continuous ethanol-drinking experience produces: (1) alterations in the sensitivity of the AcbSh to the reinforcing effects of cocaine, (2) changes in the magnitude and time course of the local stimulating effects of cocaine on pVTA DA neurons, (3) a persistence of alterations in the stimulating effects of cocaine after a period of protracted abstinence. The first hypothesis is that long-term continuous voluntary ethanol-drinking experience will produce an increased sensitivity to the reinforcing effects of cocaine within the AcbSh of P rats. This will be evidenced by the ability of a previously subthreshold dose of cocaine to reliably support self-administration on a fixed ratio (FR) schedule of reinforcement (i.e. leftward shift in the dose-response curve). The second hypothesis is that the protocol of ethanol drinking from Experiment 1 will produce an increase in the local stimulating effects of cocaine on pVTA DA neurons. This will be evidenced by the ability of a previously subthreshold dose of intra-pVTA cocaine to produce a significant increase in AcbSh DA release. The ability of suprathreshold doses to produce a greater maximal dopaminergic response (i.e. upward shift in the dose-response curve) would also support the second hypothesis. The third hypothesis is that the potential alterations in the local stimulating effects of cocaine on pVTA DA neurons observed in Experiment 2 will remain after a 30-d period of protracted abstinence from ethanol drinking. This will be evidenced by similar profiles of AcbSh dopaminergic response across the range of cocaine doses examined for ethanol-abstinent rats relative to animals with continuous ethanol-drinking experience. The current experiments will determine if chronic continuous voluntary ethanol drinking by animals selectively bred for ethanol preference and resulting in moderate to high levels of ethanol intake: (1) initiates alterations in the sensitivity to the reinforcing effects of cocaine within the AcbSh and (2) produces long-lasting functional changes in cocaine-evoked mesolimbic DA neurotransmission associated with changes in reinforcement.

The rationale for this study is supported by evidence suggesting polysubstance abuse: was more common than misuse of a single drug of abuse, was more likely to occur in those with a genetic predisposition to substance abuse, and generally occurred in a sequence with the initiation of 'gateway' drug (e.g. alcohol) use preceding the initiation of illicit drug (e.g. cocaine) use. Ethanol and cocaine use activates reinforcement circuitry in similar manner, albeit through distinct mechanisms of action. As a result, chronic ethanol drinking has the potential to produce functional alterations in cocaine's central effects via ethanol-induced plasticity to reinforcement substrates unique to cocaine, as well as through changes in shared targets. Animal models can examine the effects of so-called 'gateway' drugs on the initiation and escalation of illicit drug use at a number of levels, including molecular, neurotransmitter, electrophysiological, systems, and behavioral. These experimental studies control factors with significant influence in clinical studies, such as the relative drug availability. Moreover, animal models of drug and alcohol also examined genetic contributions to drug use and maladaptive behavioral profiles, providing an additional line of evidence to complement human genetic studies.

Results demonstrating chronic ethanol-drinking experience contributed to an increased sensitivity to the central reinforcing and neuronal activating effects of cocaine would support the concept of alcohol as a 'gateway' drug to increase the likelihood of future cocaine use. Translationally, these findings would suggest effective behavioral and/or pharmaceutical interventions would not only reduce the initiation and escalation of alcohol drinking, but could also reduce the risk of progression to illicit drug use. Future experimental studies should elucidate the genetic contributions of the observed effects, as well as the putative substrates of ethanol-induced adaptations. This, in turn, could identify human risk factors and develop targeted pharmaceutical treatments.

## 2. METHODS

### Animals

Selectively-bred adult female P rats from the 61<sup>st</sup> to 68<sup>th</sup> generations, weighing 220 to 280 g at the time of initial ethanol exposure, were obtained from the Indiana University breeding colony. Rats were pair-housed inside standard opaque plastic tubs upon arrival and were maintained on a 12-h reverse light/dark cycle (lights off at 1000 hr) in a temperature and humidity controlled environment. Food and water were freely available except in the test chambers. All experimental procedures, including surgery, ICSA, and microdialysis were conducted during the dark phase.

The current study used female P rats because females maintain body weight and head size better than males, producing more accurate stereotaxic placements (Rodd et al., 2004a,b, 2005a,b,c,d; Rodd-Henricks et al., 2000a, 2003). Although it is possible to delineate the anterior and posterior VTA in male P rats, small variations in skull thickness and shape (which appear to be greater in male than female rats) require alteration of coordinates from animal to animal, reducing placement reproducibility. Most of our previous ICSA studies used female rats (Gatto et al., 1994; Rodd et al., 2003b, 2004a, 2005 a,b,c,d; Rodd-Henricks et al., 2000a, 2002a,b, 2003). Although not systematically studied, the estrous cycle did not appear to significantly alter self-administration behavior, as indicated by stable responding across several sessions when the same concentration of ethanol (Rodd-Henricks et al., 2003) was given. Furthermore, a significant gender effect for the self-infusion of ethanol was not observed in a recent study using male rats (Rodd et al., 2004b). Lastly, peripheral

administration of ethanol produced the same brain ethanol pharmacokinetic profile across different stages of the estrous cycle (Crippens et al., 1999; Robinson et al., 2002), although the rate of ethanol clearance within the brain may be different between males and females (Robinson et al., 2002). Additionally, no consistent effects of estrous cycle in female P rats were observed in previous studies on DA efflux (Ding et al., 2009a,b; Engleman et al., 2003; Kohl et al., 1998)

Animals used in this experiment were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care. All research protocols were approved by the institutional animal care and use committee and are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, NIH, and the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). For ICOSA experiments, the number of animals indicated for each experiment represents approximately 90% of the total number undergoing surgery. The remaining 10% were not included in the analysis because their injection sites could not be verified due to the loss of the cannulae or because the animal failed to complete all experimental test sessions. For microdialysis experiments, the number of animals indicated for each experiment represents approximately 80% of the total number undergoing surgery. The remaining 20% were not included in the analysis because: the injection sites could not be verified due to the loss of the cannulae, the animal did not survive probe implantation, an insufficient sample volume was collected during microdialysis, or complications with high performance liquid chromatography (HPLC) sample analysis occurred.

### Drinking Procedures

After a 2-wk habituation to the home-cage room, animals received access to either water alone (ethanol-naïve; N) or water and 15% v/v ethanol solution

(continuous ethanol; CE) for at least 12 wk before stereotaxic surgery. For microdialysis experiments, an additional group of animals received access to water and 15% v/v ethanol solution for at least 12 wk followed by 30 d with water alone (ethanol-abstinent; Ab). Animals were handled and weighed on weekdays. Weights of the ethanol solution were monitored and recorded during the final 9 wk of drinking. Beginning 14 d before surgery, ethanol access was removed for one hr/d for the CE group. The length ethanol deprivation was incrementally increased during the following 7 d to a final duration of 4 hr/d. Nearly all past operant and free-access ethanol-drinking experiments utilizing P rats used 15% v/v ethanol, a concentration at which P rats display high levels of intake and preference. Ethanol intakes were converted to g/kg/d.

### Chemical Agents

MgCl<sub>2</sub>\*6H<sub>2</sub>O, Na<sub>2</sub>HPO<sub>4</sub>\*7H<sub>2</sub>O, H<sub>3</sub>PO<sub>4</sub>, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), 1-octanesulfonic acid sodium salt (OSA), methanol, NaCl, and D-glucose were obtained from Sigma (St. Louis, MO). KCl, CaCl<sub>2</sub>\*2H<sub>2</sub>O, MgSO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and NaHCO<sub>3</sub> were obtained from Fisher Scientific (Fair Lawn, NJ). The artificial cerebrospinal fluid (aCSF) vehicle for ICSA or microinjection consisted of 120.0 mM NaCl, 4.75 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 25.0 mM NaHCO<sub>3</sub>, 2.5 mM CaCl<sub>2</sub>\*2H<sub>2</sub>O, and 10.0 mM D-glucose. The 2.5 mM concentration of CaCl<sub>2</sub>\*2H<sub>2</sub>O has been commonly used for *in vitro* electrophysiological studies (Bonci and Malenka, 1999; Brodie et al., 1990) and ICSA studies (Ikemoto et al., 1997a; Rodd-Henricks et al., 2000a; 2002a). Cocaine HCl was obtained from the National Institute on Drug Abuse (Bethesda, MD). Cocaine was dissolved into the aCSF solution at the desired concentration and the pH was adjusted to 7.2 to 7.4 using 0.1 N HCl or 0.1 N NaOH prior to use. Microdialysis aCSF consisted of 145.0 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl<sub>2</sub>\*6H<sub>2</sub>O, 1.2 mM CaCl<sub>2</sub>\*2H<sub>2</sub>O, and 2.0 mM Na<sub>2</sub>HPO<sub>4</sub>\*7H<sub>2</sub>O, with the pH adjusted to 7.2 to 7.4 using 0.1 N HCl or 0.1 N NaOH prior to use.

## Histology

At the termination of each experiment, the animals were euthanized with a fatal dose of inhaled carbon dioxide. Bromophenol blue (1%) was injected into the infusion site for 30 s at a rate of 1.0  $\mu\text{l}/\text{min}$ . For microdialysis experiments, bromophenol blue was also perfused through the probes. Brains were quickly removed and immediately frozen at  $-70^{\circ}\text{C}$ . Frozen brains were allowed to equilibrate at  $-15^{\circ}\text{C}$  in a cryostat microtome before being sliced into 40- $\mu\text{m}$  sections. Sections were stained with cresyl violet and examined under a light microscope for precise identification of the injection site using the rat brain atlas of Paxinos and Watson (2005). Additionally, a second individual, blind to the subjects' group assignment, verified all histologies.

### Sensitization of Cocaine Reinforcement within the Nucleus Accumbens Shell Following Chronic Ethanol Drinking

#### Animal Preparation

While under isoflurane anesthesia, a unilateral 22-gauge guide cannula (Plastics One Inc., Roanoke, VA) was stereotaxically implanted into the right hemisphere of each subject, aimed 1.0 mm above the AcbSh from a  $10^{\circ}$  angle to the vertical. Coordinates (Paxinos and Watson, 2005) for placements into the AcbSh were 1.7 mm anterior to bregma (AP +1.7 mm), 2.3 mm lateral to the midline (ML +2.3 mm), and 7.5 mm ventral from the surface of the skull (DV -7.5 mm). Coordinates for placements into the AcbC or ventral to the AcbSh were AP +1.4 mm, ML +2.6 mm, DV -7.0 mm or AP +1.4 mm, ML +2.3 mm, DV -8.0 mm, respectively. In between experimental sessions, a 28-gauge stylet was placed into the 22-gauge guide cannula and extended 0.5 mm beyond the tip of the guide. Rats were single-housed following surgery. Each subject was handled for

at least 5 min daily following the third recovery d. Subjects were not acclimated to the test chamber prior to the commencement of data collection, nor were they previously trained on any operant paradigm.

### Intracranial Self-administration Apparatus

The test chambers (30 x 30 x 26 cm, width x height x depth) were situated in sound-attenuating cubicles (64 x 60 x 50 cm; Coulbourn Instruments, Allentown, PA), which were illuminated by a dim house light during testing. Two identical levers (3.5 x 1.8 cm) were mounted on a single wall of the test chamber, 15 cm above a grid floor, and were separated by 12 cm. Levers were raised to this level to avoid accidental brushing against the lever and to reduce responses as a result of general locomotor activity. Directly above each lever was a row of three different colored cue lights. The light (red) to the far right over the active lever was illuminated during resting conditions. A desktop computer equipped with an operant control system (L2T2 or Graphic State 3.0; Coulbourn Instruments) recorded the data and controlled the delivery of infusate in relation to lever response. An electrolytic microinfusion transducer (EMIT) system developed by Criswell (1977; see Bozarth and Wise, 1980; Goeders and Smith, 1987) was used to control injections of drug or vehicle. Briefly, two platinum electrodes were placed in an airtight drug reservoir (28 mm in length x 6 mm in diameter) equipped with a 28-gauge injection cannula (Plastics One, Roanoke, VA). The electrodes were connected by a spring-coated cable (Plastics One) and swivel (model 205; Mercotac, Carlsbad, CA) to a constant current generator (MNC, Shreveport, LA or A-M Systems, Sequim, WA). A 10  $\mu$ A of quiescent current was used to maintain electrodes in a ready state without ejecting or aspirating any solution. The current generator was calibrated to produce 225  $\mu$ A of current between the electrodes during infusion.

Depression of the active lever delivered the infusion current for five s, which led to the rapid generation of hydrogen gas (raising the pressure inside the airtight cylinder) and, in turn, forced 100 nl of the solution through the injection cannula into the brain. During the 5-s infusion and additional 5-s time-out period, the house light and right cue light (red) were extinguished, and the left cue light (green) over the active lever flashed on and off at 0.5-s intervals. Depression of the inactive lever did not result in any infusion. See Figure 1 for an illustration of the EMIT apparatus. The amount of drug infused is directly related to the amount of hydrogen gas generated, which is directly proportional to the current intensity and duration, as well as the gas constant (Criswell, 1977). The EMIT system was calibrated prior to the experiments and once each month subsequently.

#### General Test Condition

For CE groups, ethanol bottles were removed 4 h before the commencement of ICSA, in order to minimize any appreciable blood ethanol levels. Seven d after surgery, subjects were brought to the testing room, the stylet removed, and the injection tank carefully screwed into place. Rats were placed individually in the test chambers with the injection needles extended 1.0 mm beyond the tip of the guide. To avoid trapping air at the tip of the injection needle, the infusion current was delivered for five s during insertion of the injector, resulting in a single non-contingent administration of drug or vehicle (priming) at the beginning of the session. No shaping technique was used to facilitate the acquisition of lever responses. The L2T2 or Graphic State 3.0 computer program recorded the number of infusions and responses on the active lever. The program also recorded inactive lever responses, which did not produce infusions. Depression of the active lever (FR1 schedule of reinforcement) caused the delivery of a 100-nl bolus of solution over a 5-s period followed by a 5-s time-out period. The program also recorded active lever responses during both the infusion and time-out periods, when responses did not

produce additional infusions. The assignment of active and inactive lever with respect to left or right position was counterbalanced among subjects. However, the active and inactive levers remained the same for each subject throughout the experiment. The duration of each experimental session was 4 hr, and sessions occurred every other day at the same time (approximately 1400-2000 hr).

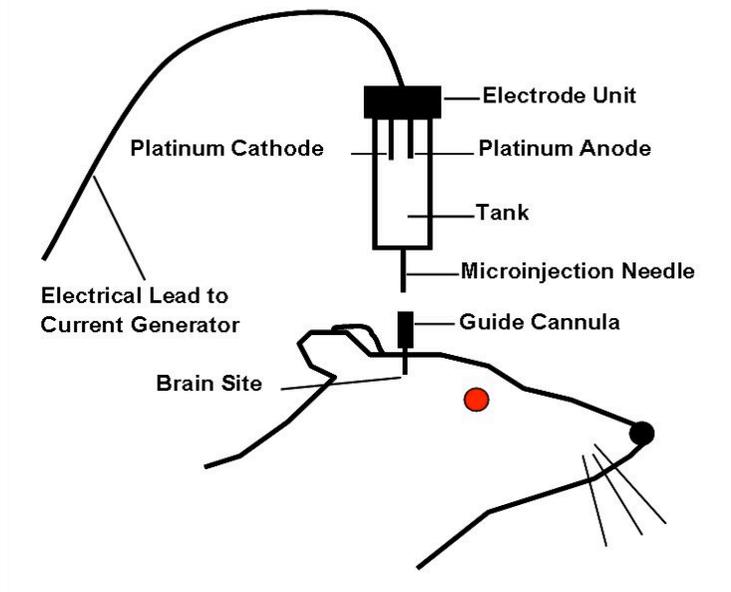


Figure 1. Photograph of rat depressing on the active lever for an injection of infusate (top) and a diagram of the electrolytic microinfusion transducer (EMIT) system used for the intracranial self-administration (ICSA) of cocaine solution (bottom; adapted from Bozarth and Wise, 1980 and Criswell, 1977).

## Experimental Groups

For both the N and CE groups, animals with cannula aimed at the AcbSh were randomly assigned to one of seven groups ( $n = 6-7/\text{group}$ ). Vehicle groups received infusions of aCSF for all eight sessions. All other groups received infusions of 10, 50, 100, 200, 400, or 800 pmol / injection (0.1-8.0 mM) cocaine for the Sessions 1-4 and Session 8. These groups received the vehicle (aCSF) alone for Sessions 5-7. For both N and CE pretreatment, animals with placements aimed dorsolateral (e.g. AcbC) or ventromedial to the AcbSh were assigned to receive infusions of 0.5, 2.0, 4.0, or 8.0 mM cocaine in the experimental protocol described previously. Data from animals assigned to the AcbSh, but with histologically verified placements within the AcbC or ventral regions, were included with data from the planned anatomical controls.

## Statistical Analysis

All data were expressed as mean  $\pm$  standard error of the mean (SEM). The initial analysis consisted of a two factor (pretreatment x dose) ANOVA performed on the average number of reinforcers infused during the first four sessions (acquisition). Further data analysis consisted of a 'pretreatment x dose x session' mixed ANOVA, with a repeated measure of session, performed on the number of active lever responses or infusions in different groups. When significant differences were detected, Tukey's b post-hoc tests were performed to compare the number of active lever responses or infusions among different groups in each session. When within subject differences were detected, post-hoc paired-samples t-tests were used.

Additionally, for each individual group, lever discrimination was determined by 'lever-type (active or inactive) x session' mixed ANOVA, with a repeated measure of session. When differences were detected, post-hoc paired-samples t-tests were used to compare active and inactive lever responding for a

particular session. Lever discrimination is a key factor used to distinguish between reinforcement-contingent behavior and drug-stimulated locomotor activation when animals self-administer an agent with stimulant properties (e.g. ethanol, cocaine, amphetamine).

### Persistent Alteration of Posterior Ventral Tegmental Area Dopamine Neurons to the Stimulating Effects of Cocaine Following Chronic Ethanol Drinking

#### Animal Preparation

While under isoflurane anesthesia, two guide cannulae (Plastics One Inc.) were stereotaxically implanted into the right hemisphere of each subject. One 22-gauge cannula was aimed 1.0 mm above the pVTA or aVTA for microinjection, and one 18-gauge cannula was placed above the ipsilateral AcbSh for microdialysis. Both cannulae were placed at a 10° angle to the vertical. Coordinates (Paxinos and Watson, 2005) for cannula placements into the pVTA, aVTA, and AcbSh were AP -5.7 mm, ML +2.1 mm, DV -8.6 mm; AP -4.8 mm, ML +2.1 mm, DV -8.6 mm; and AP +1.7 mm, ML +2.3 mm, DV -5.5 mm, respectively. Stylets were placed into both guide cannula when no experiments were being conducted. Rats were single-housed following surgery. Following the third recovery day, each subject was placed into the microdialysis chamber for 3 h and handled for at least 5 min daily. Loop-style dialysis probes (active length 1.5 mm, Spectra / Por RC, inner diameter 200 µm, molecular weight cut-off: 13,000 Dalton, Spectrum Laboratories, Inc, Rancho Dominguez, CA) were constructed as previously described (Benveniste and Huttemeier, 1990; Engleman et al. 2003). Six days after surgery, the animal was placed under anesthesia and the loop-style probe was inserted along the rostrocaudal axis into the AcbSh and secured with cranioplastic cement as previously described (Kohl et al., 1998). Microdialysis probes were inserted 12-24 h before the experiment began.

### General Test Condition

As with the ICSA experiments, ethanol access was removed from CE animals 4 h before the collection of baseline samples in order to minimize any appreciable blood ethanol levels. General dialysis procedures were described previously (Engleman et al., 2003; Kohl et al., 1998). Briefly, animals were placed into Plexiglas chambers (20x18x15 cm) open at the top and opaque on all but one side on the seventh day after surgery. The animals were connected with 20-gauge polyethylene tubing (PE-20; Becton Dickson & Co., MD) to a syringe pump (Harvard Apparatus, Holliston, MA). Microdialysis aCSF was perfused through the microdialysis probes at a rate of 1.0  $\mu$ l/min. After a 80-min washout period, four 20-min baseline samples were collected. Then, each rat received microinjections of either cocaine (0.25, 0.5, 1.0, or 2.0 mM) or aCSF for 10 min.

Non-contingent microinjections were accomplished using the EMIT system described above. A 28-gauge injection cannula (Plastics One) connected to an airtight reservoir was inserted into each animal. The reservoir contained two electrodes connected by a spring-coated cable (Plastics One) and swivel (model 205; Mercotac) to a constant current generator (MNC or A-M Systems). This allowed the animals free movement during experiments. Thirty pulse injections were experimentally delivered into the pVTA during the 10-min period. Each pulse infused 100-nl bolus of solution over five s followed by a 15-s time-out with no infusion. In total, 3.0 ml solution was infused into each animal and the injector remained in place for 30 s after infusion to allow pressure equilibration. Six 20-min samples were collected following the initiation of cocaine microinjections. The residence time of the microdialysis probe outlet was 20 min and all analyses were adjusted accordingly. All samples were collected into microfuge vials containing 5.0  $\mu$ l of 0.1 normal perchloric acid, frozen immediately in dry ice, and stored at -70°C. A previous study indicated no degradation of DA up to one month using this procedure (Campbell and McBride, 1995).

## Experimental Groups

For all three pretreatment conditions (N, CE, and Ab), animals with microinjection cannula aimed at the pVTA were randomly assigned to one of five groups ( $n = 5-7/\text{dose}$ ) prior to surgery. Subjects received microinjections of a single dose of cocaine (25, 50, 100, or 200 pmol / injection [0.25-2.0 mM]) or aCSF. There were 14 total pVTA groups, as there was not a 1.0 mM cocaine group for the Ab pretreatment. For the N and CE pretreatments, animals with microinjection cannula aimed at the aVTA were randomly assigned to one of three groups ( $n = 3-5/\text{dose}$ ). Subjects received microinjections of a single concentration of cocaine (0.5 or 2.0 mM) or aCSF.

## Microdialysis Sample Analysis

DA concentrations were analyzed with a reversed-phase HPLC with electrochemical detection as described by Engleman et al. (2006). Briefly, samples were loaded into a 10- $\mu\text{l}$  loop and injected into an analytical column (BDS Hypersil C18 pioneer, 100 mm X 1 mm, Thermo Fisher Scientific, Waltham, MA). The mobile phase (50 mM  $\text{H}_3\text{PO}_4$ , 0.1 mM EDTA, 0.6mM OSA, 12.8 mM KCl, 10% v/v methanol, pH 6.0) was delivered by an ESA 582 solvent delivery system (Chelmsford, MA). DA was detected by a 3-mm glassy carbon microelectrochemical flow cell (VT-03, Antec-Leyden, Palm Bay, FL) and an amperometric detector at a potential of +400 mV and a sensitivity set at 100 pA/V (Decade II, Antec-Leyden). The outputs from the detector were recorded by a chromatography data analysis system (ChromPerfect 4.4.0, Justice Innovations, Inc., Palo Alto, CA). The lower limit for DA detection was approximately 0.1 nM.

## Statistical Analysis

All data were expressed as mean  $\pm$  SEM. Microdialysis data were normalized and expressed as a percentage of basal values to correct for baseline variability. Basal dialysate DA values for each subject were calculated as the mean of three determinations prior to the injection of cocaine. Data analysis consisted of mixed ANOVAs, with a repeated measure of time, performed on the percent baseline DA level. A 'pretreatment' one-way ANOVA was performed on the average basal levels of extracellular DA. Additionally, the values of area under the curve (AUC) for each group were calculated using GraphPad Prism 4.0 software (La Jolla, CA) and analyzed with a 'pretreatment x dose' two-way ANOVA. If significant differences were detected with ANOVAs, post-hoc Tukey's b tests were performed to determine individual differences between groups. When within subject differences were detected, post-hoc paired-samples t-tests were used. Statistical significance was set at  $p < 0.05$ .

### 3. RESULTS

#### Sensitization of Cocaine Reinforcement within the Nucleus Accumbens Shell Following Chronic Ethanol Drinking

##### Histology

Figure 2 shows representative placements of injection sites within the AcbSh, AcbC, and areas ventral to the AcbSh. Only animals completing all eight experimental sessions and with correct placements within one of these sites were included for analysis and represent approximately 85% of animals undergoing stereotaxic surgery. A total of 122 animals completed the training procedure. Ninety rats had placements within the medial portion of the AcbSh, while no animals had placements within the ventrolateral AcbSh. As described previously, the dorsolateral portion of the AcbSh is more similar to the AcbC than to the medial AcbSh in regards to afferent and efferent connectivity, locomotor response to locally applied stimulants, and cocaine ICSA (Ikemoto, 2002, 2003, 2007; Ikemoto et al., 2005). Thirty-two rats had placements outside the AcbSh but within the AcbC or regions ventral to the AcbSh. Nine animals from the N group (0.5 mM,  $n = 2$ ; 2.0 mM,  $n = 4$ ; 8.0 mM,  $n = 3$ ) and eight animals from the CE group (0.5 mM,  $n = 2$ ; 2.0 mM,  $n = 3$ ; 4.0 mM,  $n = 3$ ) had placements within regions ventral to the AcbSh. Ten animals from the N group (0.5 mM,  $n = 4$ ; 4.0 mM,  $n = 2$ ; 8.0 mM,  $n = 4$ ) and five animals from the CE group (0.5 mM,  $n = 2$ ; 4.0 mM,  $n = 3$ ) had placements within the AcbC.

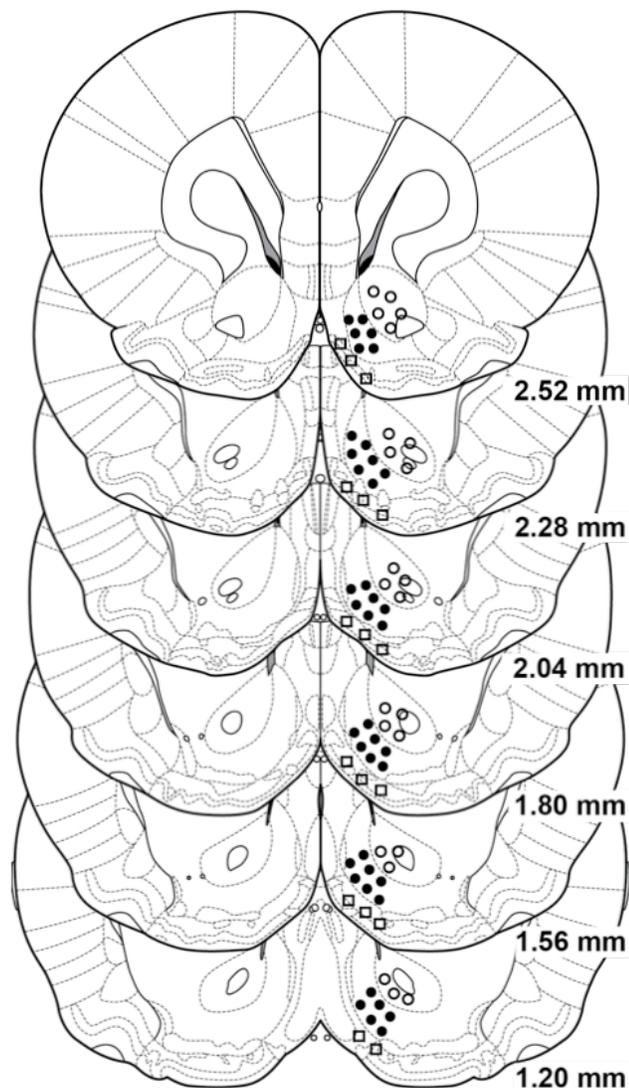


Figure 2. Illustration depicts representative, non-overlapping placements of injection sites in the forebrain for the self-infusion of cocaine solutions or aCSF in P rats. The filled circles represent placements of injection sites within the AcbSh. The open circles represent placements of injection sites within the AcbC. The open squares represent placements of injection sites ventral to the AcbSh.

### Ethanol Intake for Chronic Ethanol-drinking Rats

Average daily ethanol intakes for the P rats with chronic continuous ethanol-drinking experience (CE) were approximately 5-6 g/kg/d for the first wk ethanol consumptions were measured. The final wk before surgery, the average

ethanol intake for CE animals was  $6.4 \pm 0.3$  g/kg/d. During the 4-d period following surgery, there was a modest decrease in mean ethanol intake ( $5.6 \pm 0.8$  g/kg/d). Ethanol intakes returned to pre-surgery baseline levels and were stable at  $6.8 \pm 0.5$  g/kg/d for the 2-d period before ICOSA testing began and the 15-d period of ICOSA experiments. Ethanol intakes were in line with previous studies using P rats given long-term, concurrent access to water and 15% ethanol (Rodd et al., 2005b,c; Rodd-Henricks et al., 2000b).

#### Acquisition of Cocaine Self-infusion: Sessions 1-4

Cocaine concentrations between 0 and 8.0 mM (0-800 pmol / 100 nl) were tested to determine the response contingent behaviors of female P rats with (CE) or without (N) previous ethanol-drinking experience with injection sites within the AcbSh. Condensing the analysis to the average number of infusions received during the four acquisition sessions revealed significant main effects of pretreatment ( $F(1,76) = 6.7, p < 0.01$ ) and dose ( $F(6,76) = 8.9, p < 0.001$ ), but not the 'pretreatment x dose' interaction ( $F(1,79) = 1.1, p = 0.39$ ). For N rats, there was a significant effect of cocaine dose on the number of self-infusions ( $F(6,37) = 4.7, p < 0.001$ ). Post hoc comparisons (Tukey's b) indicated the 2.0, 4.0, and 8.0 mM cocaine groups received significantly more infusions than the aCSF and 0.1 mM cocaine groups ( $p < 0.05$ ; Figure 3). For CE rats, there was a significant effect of cocaine dose on the number of self-infusions ( $F(6,39) = 5.3, p < 0.001$ ). Post hoc comparisons (Tukey's b) indicated animals the 0.5, 1.0, 2.0, 4.0, or 8.0 mM cocaine groups received significantly more infusions than the aCSF and 0.1 mM cocaine groups ( $p < 0.05$ ). Maintaining a constant cocaine dose during analysis allowed for the direct comparison of infusions received during acquisition between N and CE animals at each dose of cocaine tested. At a concentration of 0.5 mM cocaine, CE rats received significantly more infusions than N rats ( $p < 0.05$ ).

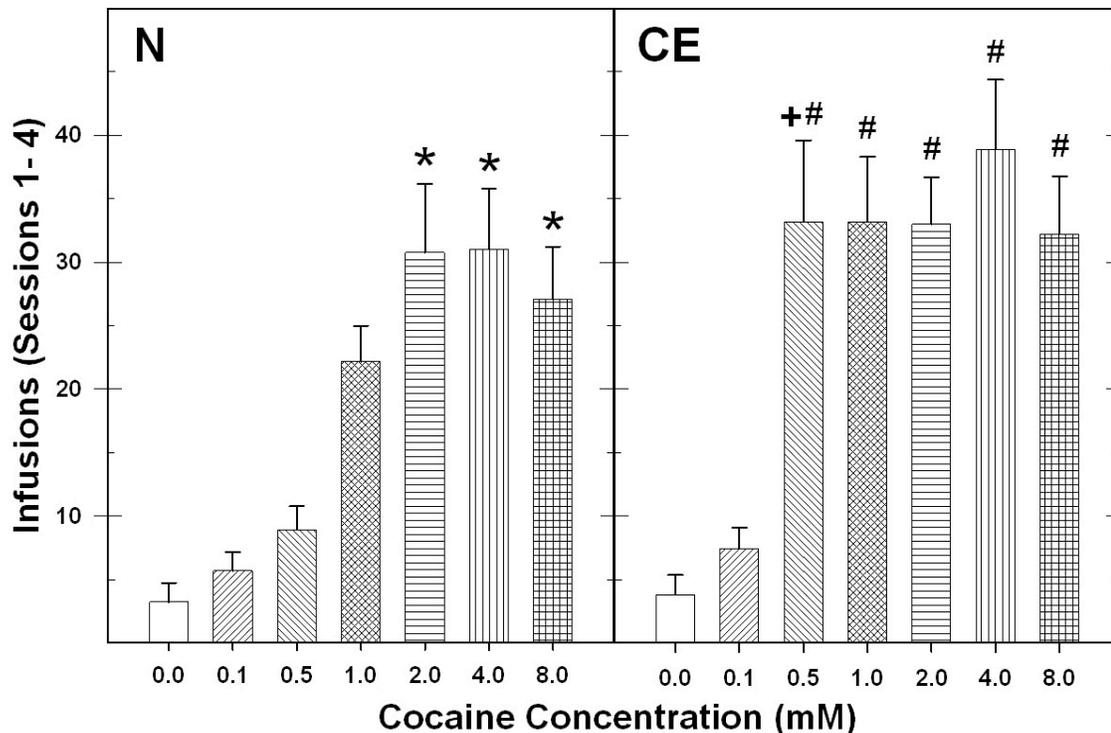


Figure 3. Dose-response effects for the acquisition of self-infusion of 0-8.0 mM cocaine into the AcbSh of P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE). Concentration of 0 mM is aCSF only. The number of infusions was averaged over Sessions 1-4 for each animal. \*  $p < 0.05$  (Tukey's b), significantly greater infusions than 'N-0' group; #  $p < 0.05$  (Tukey's b), significantly greater infusions than 'CE-0' and 'CE-0.1' groups; +  $p < 0.05$ , significantly greater infusions than the 'N-0.5' group. Data are the means  $\pm$  SEM. ( $n = 6$  to  $8$ /dose/pretreatment)

Overall analysis of the number of self-infusions received across the eight sessions revealed significant effects of session ( $F(7,70) = 22.9$ ,  $p < 0.001$ ), pretreatment ( $F(1,76) = 19.8$ ,  $p < 0.001$ ) and dose ( $F(6,76) = 10.7$ ,  $p < 0.001$ ), as well as significant interaction effects for 'session x pretreatment' ( $F(7,70) = 2.5$ ,  $p < 0.05$ ) and 'session x dose' ( $F(42,450) = 1.9$ ,  $p < 0.001$ ). Holding pretreatment and session constant, the number of infusions was examined for the final acquisition session, the three extinction sessions, and the reinstatement session (Sessions 4-8). Post hoc analyses were also conducted to compare the number of infusions received during each session for each dose of cocaine tested. For N

rats, there was a significant effect of cocaine dose for Session 4 ( $F(6,37) = 3.5$ , both  $p < 0.01$ ). The 1.0, 2.0, 4.0, and 8.0 mM cocaine groups of N animals received more self-infusions than the aCSF group (Tukey's b,  $p < 0.05$ ; Figure 4). CE rats also displayed a significant effect of cocaine dose for Session 4 ( $F(6,39) = 3.3$ ,  $p < 0.01$ ). The 0.5, 1.0, 2.0, 4.0 and 8.0 mM cocaine groups of CE animals received more self-infusions than the aCSF group ( $p < 0.05$ ; Figure 5).

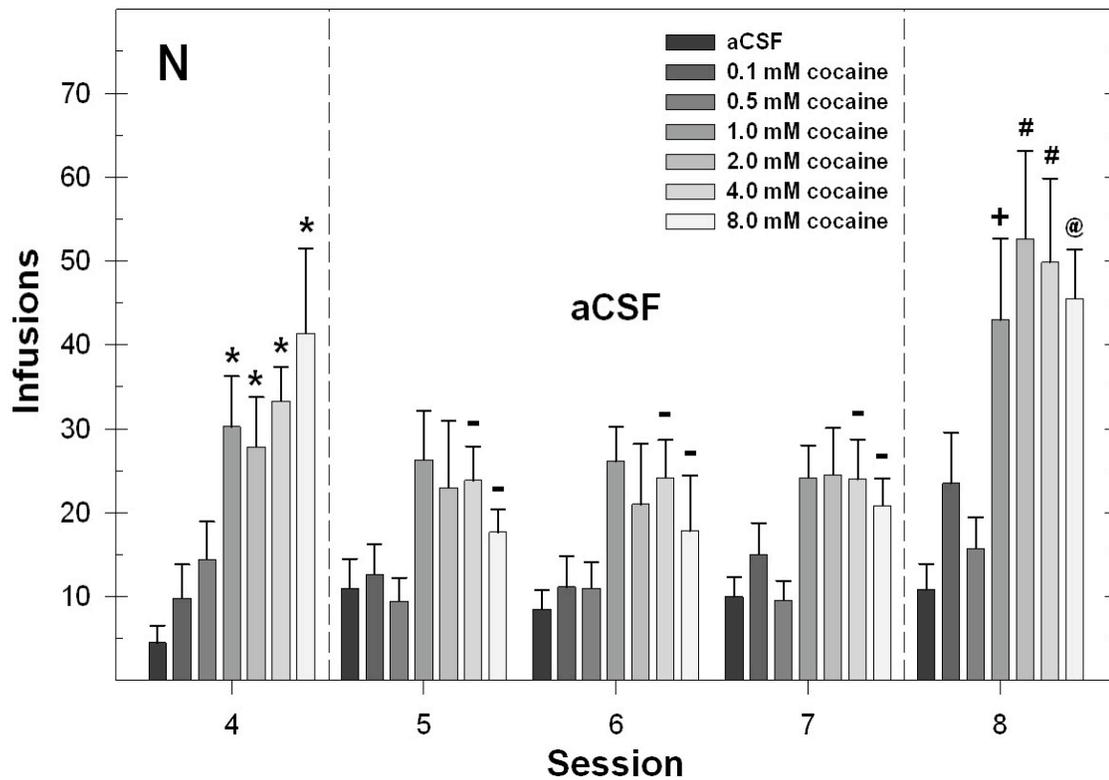


Figure 4. Dose-response effects for the extinction and reinstatement of self-infusion of 0.1-8.0 mM cocaine or aCSF into the AcbSh of P rats with no ethanol-drinking experience (N). \*  $p < 0.05$  (Tukey's b), significantly greater infusions than 'N-aCSF' group for the same session; -  $p < 0.05$ , significantly fewer infusions than Session 4 for same dose of cocaine; +  $p < 0.05$ , significantly greater infusions than Session 7 for same dose of cocaine; @  $p < 0.05$ , significantly greater infusions than 'N-aCSF' group for the same session and than Session 7 for same dose of cocaine; #  $p < 0.05$ , significantly greater infusions than 'N-aCSF' group for the same session and than Sessions 4 and 7 for same dose of cocaine. Data are the means  $\pm$  SEM. ( $n = 6$  to  $8$ /dose/pretreatment)

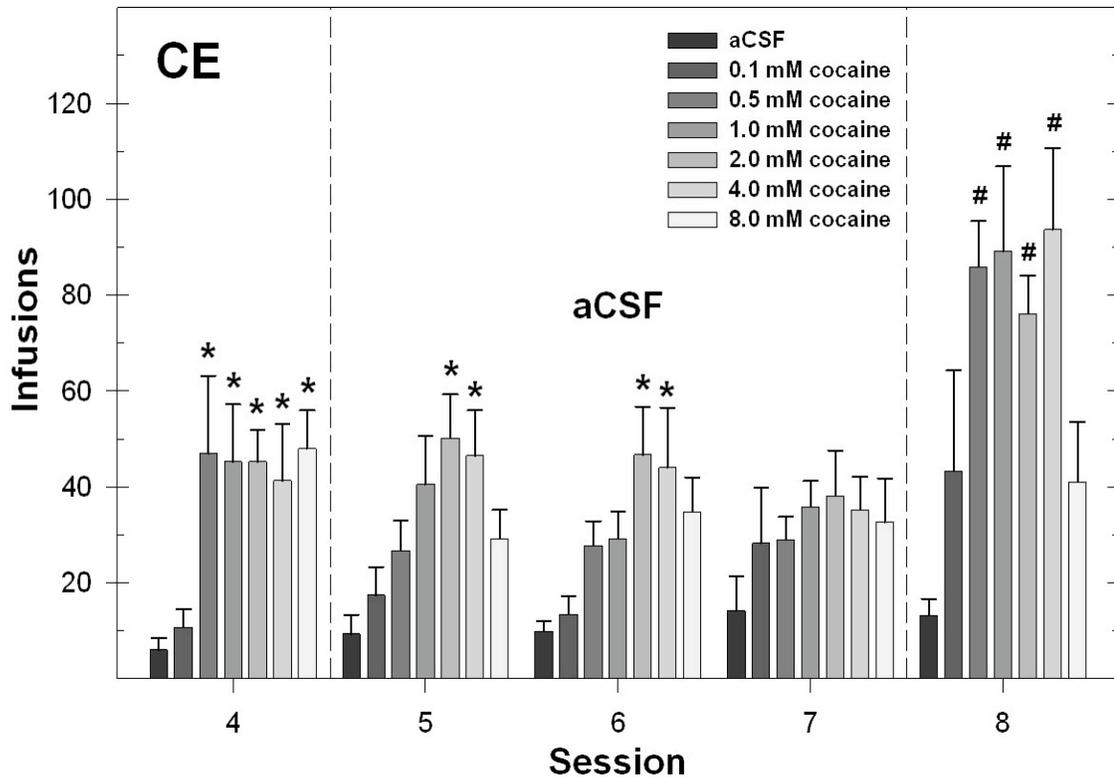


Figure 5. Dose-response effects for the extinction and reinstatement of self-infusion of 0.1-8.0 mM cocaine or aCSF into the AcbSh of P rats with continuous ethanol-drinking experience (CE). \*  $p < 0.05$  (Tukey's b), significantly greater infusions than 'CE-aCSF' group for the same session; #  $p < 0.05$ , significantly greater infusions than 'CE-aCSF' group for the same session and than Sessions 4 and 7 for same dose of cocaine. Data are the means  $\pm$  SEM. ( $n = 6$  to 8/dose/pretreatment)

#### Extinction of Cocaine Self-infusion: Sessions 5-7

When aCSF was substituted for cocaine during Sessions 5-7, there was not a significant effect of initial cocaine dose on the number of infusions received by N animals for any Session 5-7 (all  $F(6,37) < 2.1$ , all  $p$ -values  $> 0.08$ ). However, N animals given 0.1, 0.5, 1.0, or 2.0 mM cocaine did not receive significantly fewer infusions during any extinction session compared to Session 4 (all  $p$ -values  $> 0.05$ ; Figure 4). On the other hand, N animals initially self-infusing 4.0 or 8.0 mM cocaine received significantly fewer infusions during each of the

three extinction sessions compared to Session 4 (all  $p$ -values  $< 0.05$ ). The substitution of aCSF for cocaine produced a different effect on self-infusion for CE animals compared to their N counterparts.

During Sessions 5 and 6, CE animals displayed a significant effect of initial cocaine dose on self-infusions (both  $F(6,39) > 3.9$ , both  $p$ -values  $< 0.01$ ). During both Sessions 5 and 6, the 2.0 and 4.0 mM groups received more self-infusions than the aCSF group ( $p < 0.05$ ; Figure 5). During Session 7, there was not a significant effect of dose on the number of infusions received ( $F(6,39) = 0.9$ ,  $p = 0.50$ ). In contrast from the N groups, no CE groups received significantly fewer infusions during any of the three extinction sessions compared to Session 4 at any cocaine dose tested (all  $p$ -values  $> 0.05$ ; Figure 5).

#### Reinstatement of Cocaine Self-infusion: Session 8

When access to cocaine self-infusion was restored during Session 8, the 1.0, 2.0, 4.0, and 8.0 mM cocaine groups of N animals received a greater number of infusions compared to Session 7 (all  $p$ -values  $< 0.05$ ; Figure 4). The 0.5, 1.0, 2.0, and 4.0 mM cocaine groups of CE animals received a greater number of infusions during Session 8 compared to Session 7 (all  $p$ -values  $< 0.05$ ; Figure 5). To clarify, reinstatement in this case is defined as the return of the availability of reinforce following a period of unavailability (Flaherty, 1985). This is a distinct concept from 'reinstatement of responding', the animal model of drug-induced seeking (Shaham et al., 2003).

Analysis revealed significant main effects of pretreatment ( $F(1,76) = 20.1$ ,  $p < 0.001$ ) and dose ( $F(6,76) = 6.0$ ,  $p < 0.001$ ), as well as the 'pretreatment x dose' interaction ( $F(1,76) = 2.4$ ,  $p < 0.05$ ). For N rats, there was a significant effect of cocaine dose on the number of self-infusions ( $F(6,37) = 5.1$ ,  $p < 0.001$ ). Post hoc comparisons (Tukey's b) for reinstatement indicated the 1.0, 2.0, 4.0, and 8.0 mM cocaine groups of N animals received more infusions than the aCSF group ( $p < 0.05$ ; Figures 4 & 6). Additionally, the 2.0 and 4.0 mM cocaine

received more infusions than the 0.5 mM cocaine group ( $p < 0.05$ ). For CE rats, there was a significant effect of cocaine dose on the number of self-infusions ( $F(6,39) = 4.1$ ,  $p < 0.005$ ). Post hoc comparisons (Tukey's b) indicated the 0.5, 1.0, 2.0, or 4.0 mM cocaine groups of CE animals received more infusions than the aCSF group ( $p < 0.05$ ; Figures 5 & 6). Comparison of infusions received by N and CE rats during Session 8 revealed CE rats received significantly more infusions than N rats at concentrations of 0.5, 1.0, 2.0 and 4.0 mM cocaine (all  $p$ -values  $< 0.05$ ; Figure 6).

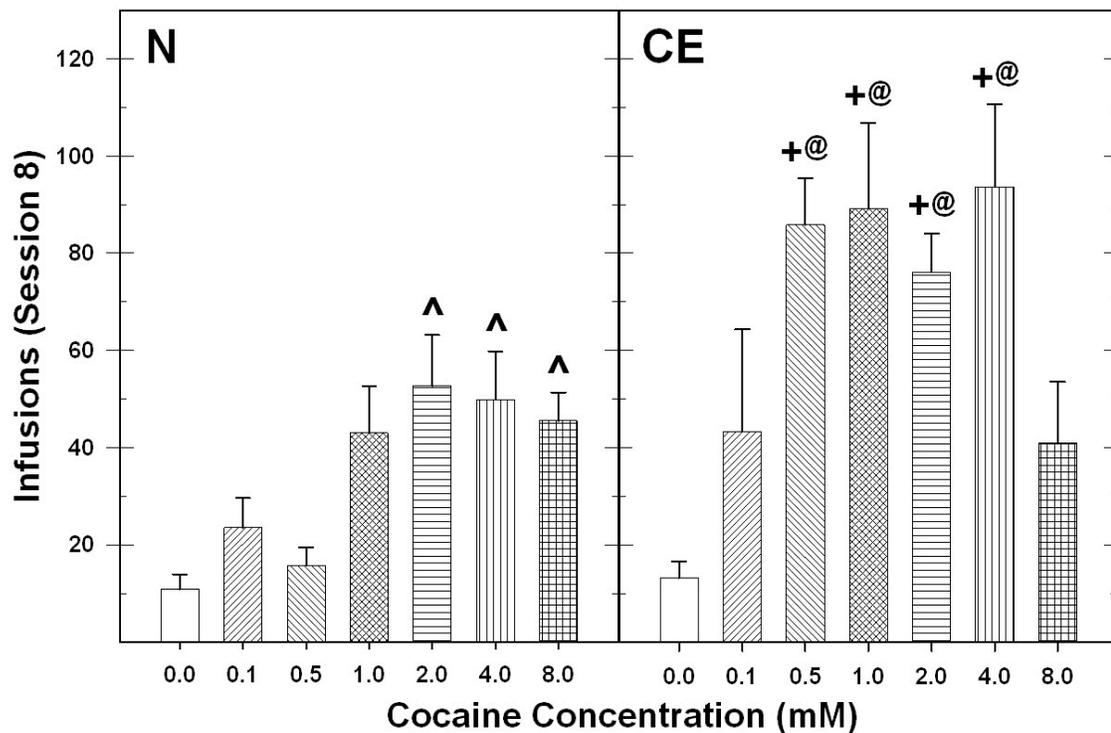


Figure 6. Dose-response effects for the reinstatement of self-infusion of 0-8.0 mM cocaine into the AcbSh of P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE). Concentration of 0 mM is aCSF only. The number of infusions was obtained from Session 8 for each animal. \*  $p < 0.05$  (Tukey's b), significantly greater infusions than 'N-0' group; @  $p < 0.05$  (Tukey's b), significantly greater infusions than 'CE-0' group; +  $p < 0.05$ , significantly greater infusions compared to the N group with the same cocaine dose. Data are the means  $\pm$  SEM. ( $n = 6$  to  $8$ /dose/pretreatment)

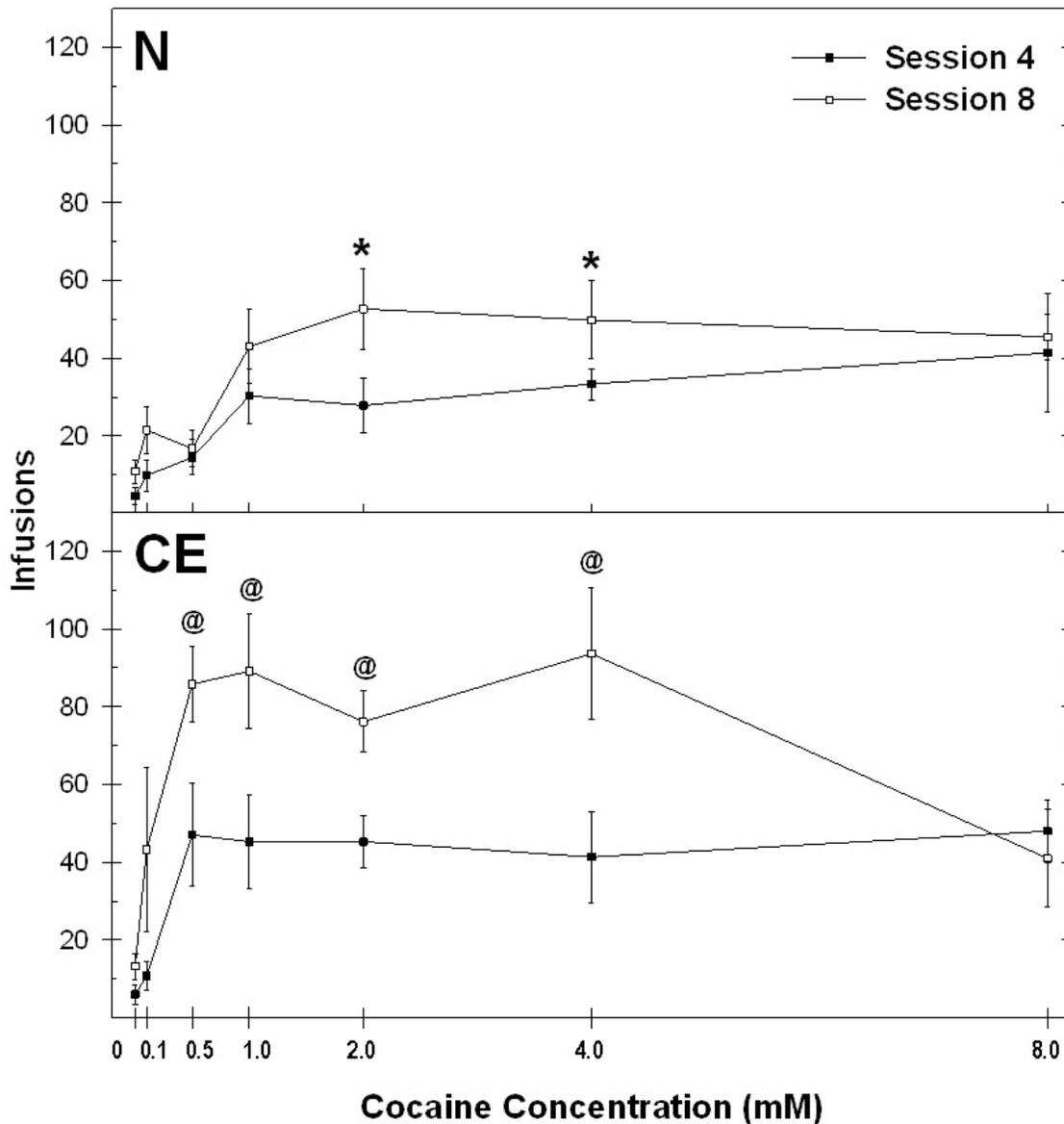


Figure 7. Comparison of infusions of 0-8.0 mM cocaine into the AcbSh between Session 8 (■) and Session 4 (□) for P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE). Concentration of 0 mM is aCSF only. \*  $p < 0.05$ , significantly greater infusions for Session 8 compared to Session 4 for the same pretreatment condition and cocaine dose; @  $p < 0.05$ , significantly greater infusions for Session 8 compared to Session 4 for the same pretreatment condition and cocaine dose and compared to the corresponding N group. Data are the means  $\pm$  SEM. ( $n = 6$  to  $8$  /dose/pretreatment)

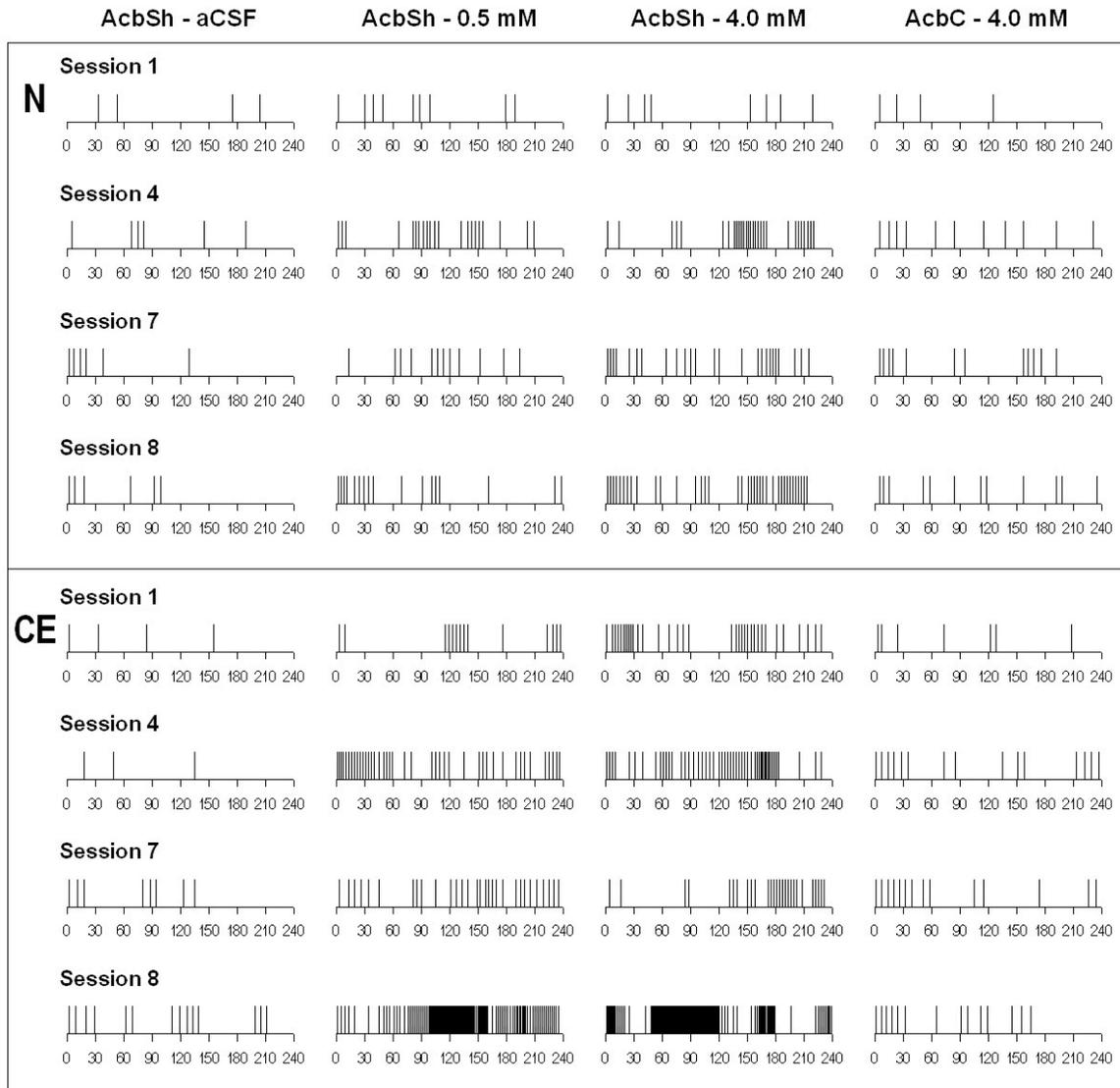


Figure 8. Representative infusion pattern for P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE) self-administering either 0.5 or 2.0 mM cocaine or aCSF into the AcbSh or AcbC. Each line represents one infusion. The first row of traces represents the initial test session. The second row of traces represents the final session of acquisition (Session 4). The third row of traces represents the final session of extinction (Session 7). The fourth row of traces represents reinstatement (Session 8). ( $n = 6$  to  $8$  /dose/pretreatment)

Comparison of the number of self-infusions received during Session 8 compared to Session 4 (Figure 7) revealed significant effects of session ( $F(1,76) = 39.7, p < 0.001$ ), pretreatment ( $F(1,76) = 18.4, p < 0.001$ ), and dose ( $F(6,76) = 7.6, p < 0.001$ ), as well as significant interactions for 'session x pretreatment' ( $F(1,76) = 7.6, p < 0.01$ ) and 'session x dose' ( $F(6,76) = 2.3, p < 0.05$ ). For N rats, the 2.0 and 4.0 mM cocaine groups received a significantly greater number of infusions during Session 8 compared to Session 4 ( $p < 0.05$ ). For CE rats, the 0.5, 1.0, 2.0, and 4.0 mM cocaine groups received a significantly greater number of infusions during Session 8 compared to Session 4 (1.0 and 2.0 mM:  $p < 0.005$ ; 0.5 and 4.0 mM:  $p < 0.05$ ).

#### Lever Responding of Ethanol-naïve Rats

Overall analysis of the number of active lever responses across all eight sessions (Figures 9-11) revealed significant main effects of session ( $F(7,70) = 22.3, p < 0.001$ ), pretreatment ( $F(1,80) = 20.0, p < 0.001$ ) and dose ( $F(6,80) = 12.1, p < 0.001$ ), as well as a significant interaction effect for 'session x pretreatment' ( $F(7,70) = 3.4, p < 0.005$ ).

For rats with no ethanol-drinking experience (N), were significant main effects of session ( $F(7,31) = 11.1, p < 0.001$ ) and dose ( $F(6,37) = 6.3, p < 0.001$ ), as well as a significant 'session x dose' interaction ( $F(42,216) = 1.6, p < 0.05$ ). For the 'session x dose' interaction, individual ANOVAs were performed for each session to determine the dose effect. Analysis revealed a significant effect of dose during each of the initial four sessions (acquisition; all  $F(6,37) > 2.6$ , all  $p$ -values  $< 0.03$ ). During Session 1, the 1.0 and 2.0 mM cocaine groups responded more on the active lever than the aCSF and 0.1 mM cocaine groups ( $p < 0.05$ , Tukey's b). During Session 2, the 2.0, 4.0, and 8.0 mM cocaine groups responded more on the active lever than the aCSF group, and the 2.0 mM group responded more on the active lever than the 0.1 and 0.5 mM cocaine groups ( $p < 0.05$ , Tukey's b). During Sessions 3 and 4, the 1.0, 2.0, 4.0, and 8.0 mM cocaine

groups responded more on the active lever than the aCSF group and the 0.1 and 0.5 mM cocaine groups ( $p < 0.05$ , Tukey's b).

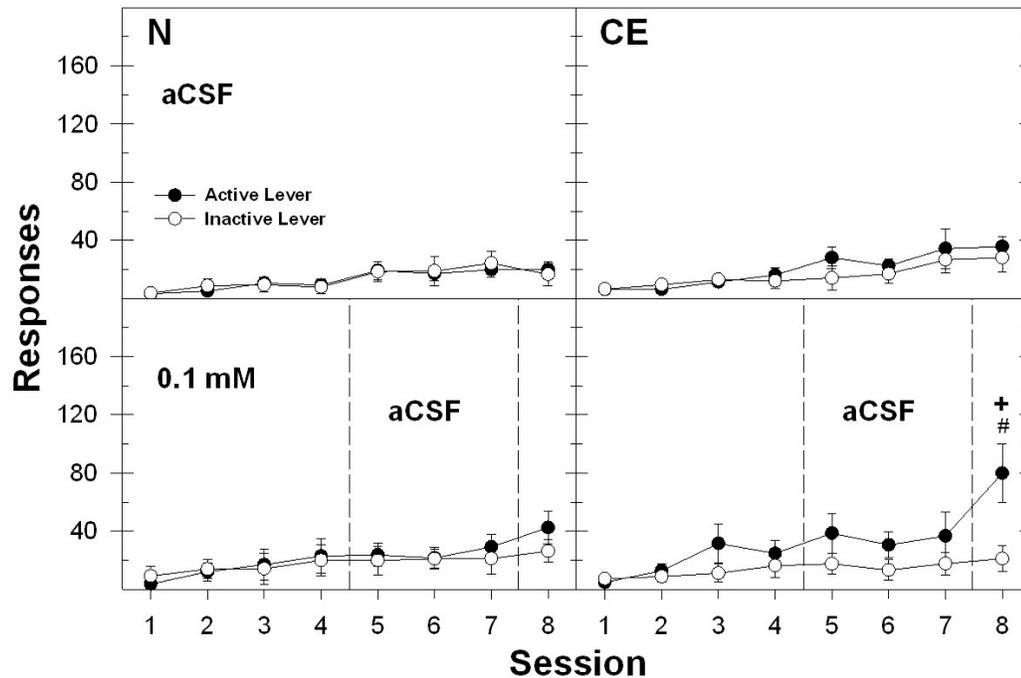


Figure 9. Responses on the active and inactive levers for the self-infusion of 0.1 mM cocaine or aCSF into the AcbSh of P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE) for four acquisition sessions, three extinction sessions, and one reinstatement session. #  $p < 0.05$ , significantly greater active lever responses during Session 8 compared to inactive lever responses for Session 8 and compared to active lever responses for Sessions 4 and 7; +  $p < 0.05$ , significantly greater active lever responses for CE rats compared to active lever responses for N rats at the same cocaine dose during the same session. Data are the means  $\pm$  SEM. ( $n = 6$  to 8 /dose/pretreatment)

During the first session of extinction (Session 5) there was no significant effect of dose on the level of active lever responding ( $F(6,37) = 2.0$ ,  $p = 0.08$ ). However, there was a significant effect of dose on active lever responding during Sessions 6 and 7 (both  $F(6,37) > 4.4$ ,  $p < 0.005$ ). Post hoc analysis (Tukey's b) revealed only the 1.0 mM cocaine group responded more on the active lever compared to the aCSF group during Session 6 or 7 (Figures 9 and 10;  $p < 0.05$ ).

The aCSF group and the 0.1, 0.5, and 1.0 mM cocaine groups did not display significantly different levels of active lever responding during Session 5, 6, or 7 compared to Session 4 (all  $p$ -values  $> 0.24$ ). The 2.0 mM cocaine group displayed reduced active lever responding during Session 6 (but not Session 5 or 7) compared to Session 4 ( $p < 0.05$ ). The 4.0 and 8.0 mM cocaine groups displayed reduced active lever responding during Sessions 5, 6, and 7 compared to Session 4 (all  $p$ -values  $< 0.05$ ).

When cocaine access for N rats was reinstated during Session 8, there was a significant effect of dose on the level of active lever responding ( $F(6,37) = 4.0$ ,  $p < 0.005$ ). The 1.0, 2.0, 4.0, and 8.0 mM cocaine groups responded more on the active lever than the aCSF group and the 0.1 and 0.5 mM cocaine groups ( $p < 0.05$ , Tukey's  $b$ ). However, none of the seven N groups responded more on the active lever during Session 8 compared to Session 4 (Figures 9-11; all  $p$ -values  $> 0.20$ ). Additionally, the number of active lever responses during Session 8 compared to Session 7 was significantly greater the 2.0, 4.0, and 8.0 mM cocaine groups (all  $p$ -values  $< 0.04$ ), but not for the aCSF group and the 0.1, 0.5, and 1.0 mM cocaine groups (all  $p$ -values  $> 0.06$ ).

For N rats, the aCSF group and the 0.1 and 0.5 mM cocaine groups did not show significant effects of lever-type (all  $F$ -values  $< 2.1$ , all  $p$ -values  $> 0.20$ ) or 'lever-type x session' interaction (all  $F$ -values  $< 1.4$ , all  $p$ -values  $> 0.24$ ) on lever responding (Figures 9 and 10). The 2.0, 4.0, and 8.0 mM cocaine groups displayed significant effects of both lever-type (all  $F$ -values  $> 13.3$ , all  $p$ -values  $< 0.02$ , Figure 11) and 'lever-type x session' interaction (all  $F$ -values  $> 3.2$ , all  $p$ -values  $< 0.01$ ). During Sessions 2, 3, 4, and 8, the 2.0, 4.0, and 8.0 mM cocaine groups responded significantly more on the active lever than the inactive lever (all  $p$ -values  $< 0.04$ ; Figure 11). The 1.0 mM cocaine group showed a marginally significant effect on lever responding of lever-type ( $F(1,6) = 4.7$ ,  $p = 0.07$ ), but not of a 'lever-type x session' interaction ( $F(7,42) = 0.9$ ,  $p = 0.49$ ). Further analysis revealed significantly greater active versus inactive lever responding for the 1.0 mM cocaine group only during Session 8 ( $p < 0.05$ , Figure 10).

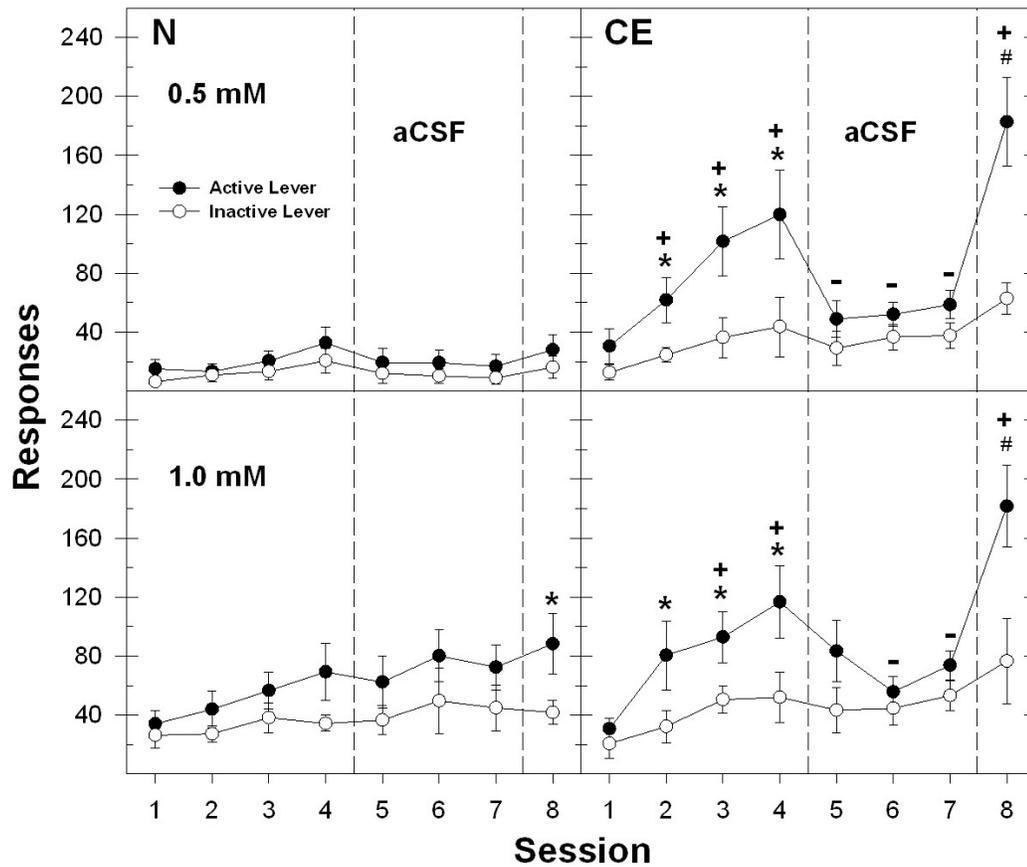


Figure 10. Responses on the active and inactive levers for the self-infusion of 0.5 or 1.0 mM cocaine into the AcbSh of P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE) for four acquisition sessions, three extinction sessions, and one reinstatement session. \*  $p < 0.05$ , significantly greater active lever responses compared to inactive lever responses for the same session; -  $p < 0.05$ , significantly fewer active lever responses during a particular extinction session compared to active lever responses during Session 4; #  $p < 0.05$ , significantly greater active lever responses during Session 8 compared to inactive lever responses for Session 8 and compared to active lever responses for Sessions 4 and 7; +  $p < 0.05$ , significantly greater active lever responses for CE rats compared to active lever responses for N rats at the same cocaine dose. Data are the means  $\pm$  SEM. ( $n = 6$  to  $8$  /dose/pretreatment)

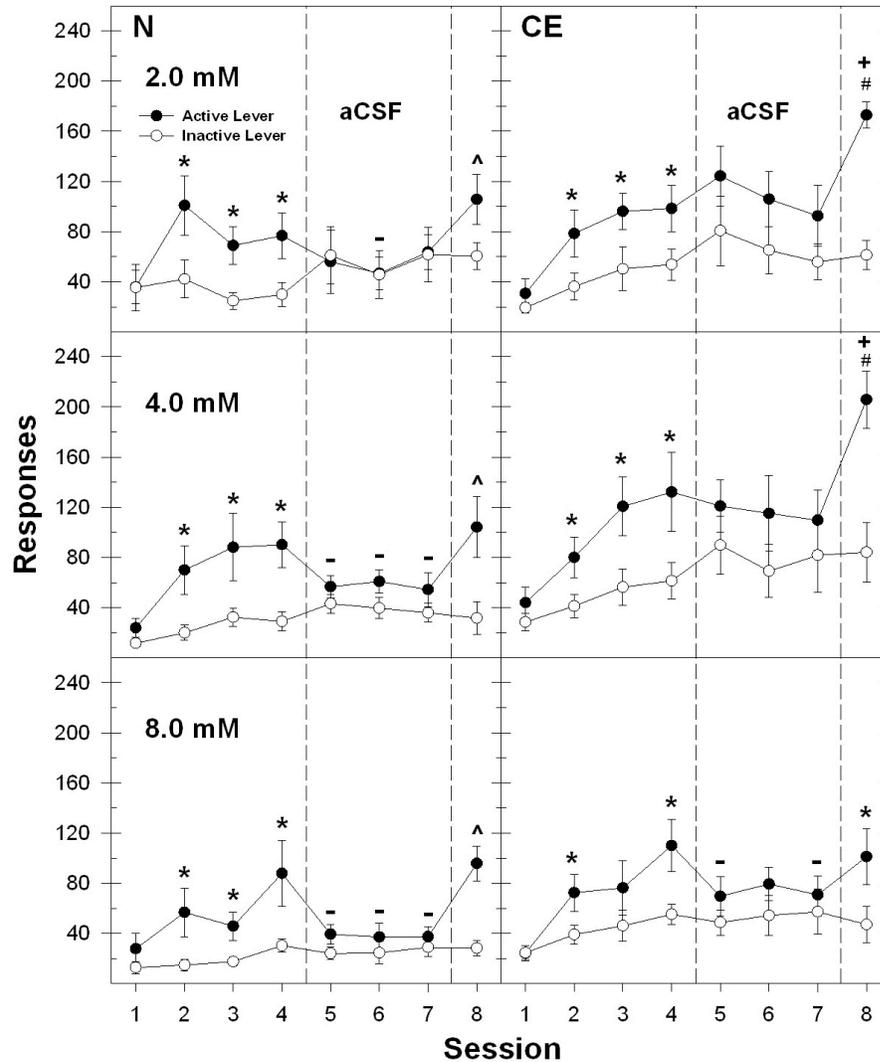


Figure 11. Responses on the active and inactive levers for the self-infusion of 2.0, 4.0 or 8.0 mM cocaine into the AcbSh of P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE) for four acquisition sessions, three extinction sessions, and one reinstatement session. \*  $p < 0.05$ , significantly greater active lever responses compared to inactive lever responses for the same session; -  $p < 0.05$ , significantly fewer active lever responses during a particular extinction session compared to active lever responses during Session 4; ^  $p < 0.05$ , significantly greater active lever responses during Session 8 compared to inactive lever responses for Session 8 and compared to active lever responses for Session 7; #  $p < 0.05$ , significantly greater active lever responses during Session 8 compared to inactive lever responses for Session 8 and compared to active lever responses for Sessions 4 and 7; +  $p < 0.05$ , significantly greater active lever responses for CE rats compared to active lever responses for N rats at the same cocaine dose. Data are the means  $\pm$  SEM. ( $n = 6$  to  $8$  /dose/pretreatment)

## Lever Responding of Ethanol-drinking Rats

For rats with continuous ethanol-drinking experience (CE), there were significant main effects of session ( $F(7,33) = 15.9, p < 0.001$ ) and dose ( $F(6,39) = 7.1, p < 0.001$ ), and a significant 'session x dose' interaction ( $F(42,228) = 1.5, p < 0.05$ ). For the 'session x dose' interaction, individual ANOVAs were performed for each session to determine the dose effect. Analysis revealed a significant effect of dose during Sessions 1-4 (all  $F(6,39) > 2.6$ , all  $p$ -values  $< 0.03$ ). During Session 1, only the 4.0 mM cocaine group responded more on the active lever than the aCSF group ( $p < 0.05$ , Tukey's b). During Session 2, the 1.0, 2.0, 4.0, and 8.0 mM cocaine groups responded more on the active lever than the aCSF group ( $p < 0.05$ , Tukey's b). During Sessions 3 and 4, the 0.5, 1.0, 2.0, 4.0, and 8.0 mM cocaine groups responded more on the active lever than the aCSF group, and 4.0 mM group responded more on the active lever than the 0.1 mM cocaine group ( $p < 0.05$ , Tukey's b).

During Sessions 5 and 6, there was a significant effect of cocaine dose on the level of active lever responding (both  $F(6,39) > 4.6$ , both  $p$ -values  $< 0.005$ ). During both Sessions 5 and 6, the 2.0 and 4.0 mM cocaine groups responded more on the active lever than aCSF group and the 0.1 mM cocaine group ( $p < 0.05$ , Tukey's b; Figures 9 and 11). On the other hand, there was not a significant effect of cocaine dose on the level of active lever responding during Session 7 ( $F(6,39) = 2.0, p = 0.09$ ). The aCSF and 0.1 mM cocaine groups did not have significantly different levels of active lever responding during Session 5, 6, or 7 compared to Session 4 (all  $p$ -values  $> 0.18$ ). The 0.5, 1.0, and 8.0 mM cocaine groups showed reduced active lever responding during at least two of Sessions 5-7 compared to Session 4 (all  $p$ -values  $< 0.05$ ; Figures 10 and 11). On the other hand, the 2.0 and 4.0 mM cocaine groups did not show reduced active lever responding during Session 5, 6, or 7 compared to Session 4 (all  $p$ -values  $> 0.08$ ).

When cocaine access for CE animals was reinstated during Session 8, there was a significant effect of dose on the level of active lever responding

( $F(6,39) = 5.2, p < 0.001$ ). The 0.1, 0.5, 1.0, 2.0, 4.0, and 8.0 mM cocaine groups responded more on the active lever than the aCSF group ( $p < 0.05$ , Tukey's *b*). The 0.1, 0.5, 1.0, 2.0, and 4.0 mM cocaine groups responded more on the active lever during Session 8 compared to both Sessions 4 and 7 (all  $p$ -values  $< 0.04$ ; Figures 11 and 12). The 8.0 mM cocaine group, however, did not respond more on the active lever during Session 8 compared to either Session 4 or 7 (both  $p$ -values  $> 0.08$ ).

For CE rats, the aCSF group did not show significant effects of lever-type ( $F(1,5) = 0.1, p = 0.90$ ) or 'lever-type x session' interaction ( $F(7,35) = 0.6, p = 0.75$ ) on lever responding (Figure 9). The 0.5, 1.0, 2.0, 4.0, and 8.0 mM cocaine groups displayed significant effects of both lever-type (all  $F$ -values  $> 12.1$ , all  $p$ -values  $< 0.02$ ) and 'lever-type x session' interaction (all  $F$ -values  $> 2.8$ , all  $p$ -values  $< 0.02$ ). During Sessions 2, 3, 4, and 8, the 0.5, 1.0, 2.0, and 4.0 mM cocaine groups responded more on the active lever than the inactive lever (all  $p$ -values  $< 0.04$ ; Figures 10 and 11). The 8.0 mM cocaine group responded more on the active lever than the inactive lever during Sessions 2, 4, and 8 (all  $p$ -values  $< 0.03$ ). The 0.1 mM cocaine group showed marginally significant effects on lever responding of lever-type ( $F(1,6) = 5.0, p = 0.06$ ) and of a 'lever-type x session' interaction ( $F(7,42) = 2.1, p = 0.06$ ). Further analysis revealed significantly greater active versus inactive lever responding for the 0.1 mM cocaine group only during Session 8 ( $p < 0.05$ , Figure 10).

A comparison of active lever responding between N and CE animals during each session at each dose of cocaine revealed significant differences during acquisition and reinstatement. During Sessions 3 and 4, the 0.5 and 1.0 mM cocaine groups of CE animals responded more on the active lever compared to N animals receiving the same dose (all  $p$ -values  $< 0.03$ ; Figure 10). Additionally, the 0.5 mM cocaine group of CE animals responded more during Session 2 on the active lever compared to N animals given the same dose ( $p < 0.05$ ). During Session 8, the 0.1, 0.5, 1.0, 2.0, and 4.0 mM cocaine groups of CE

animals responded more on the active lever compared to N animals receiving the same dose (all  $p$ -values  $< 0.05$ ; Figures 10 and 11).

### Infusions and Lever Responding of Anatomical Controls

Animals with cannula implanted in the AcbC or ventral to the AcbSh self-administering 0.5-8.0 mM cocaine received a comparable number of infusions during Sessions 1-4 as those administering aCSF or 0.1 mM cocaine into the AcbSh (Figure 12). Analyzing the average number of infusions received by the 32 anatomical control animals during the four acquisition sessions revealed no significant main effects or interactions for site, pretreatment, or dose (all  $F$ -values  $< 2.5$ , all  $p$ -values  $> 0.13$ ). Collapsing the data across dose, the average number of infusions received for the first four sessions were  $6.1 \pm 0.9$  and  $5.6 \pm 1.2$  for N animals and  $10.8 \pm 1.9$  and  $5.8 \pm 0.8$  for CE animals with placements within the AcbC or ventral to the AcbSh, respectively. Overall, cannula placements surrounding the AcbSh did not support the acquisition of cocaine self-infusion at any of the doses tested for either pretreatment condition.

AcbC and ventral groups self-administering 0.5-8.0 mM cocaine received a comparable number of infusions during Session 8 as those administering aCSF or 0.1 mM cocaine into the AcbSh (Figure 12). Analyzing the average number of infusions received by the 32 anatomical control animals during Session 8 revealed no significant main effects or interactions for site, pretreatment, or dose (all  $F$ -values  $< 3.3$ , all  $p$ -values  $> 0.07$ ). The average number of infusions received for the Session 8 was  $17.6 \pm 4.9$  and  $12.1 \pm 2.3$  for N animals and  $16.8 \pm 2.6$  and  $16.8 \pm 4.9$  for CE animals with placements within the AcbC or ventral to the AcbSh, respectively.

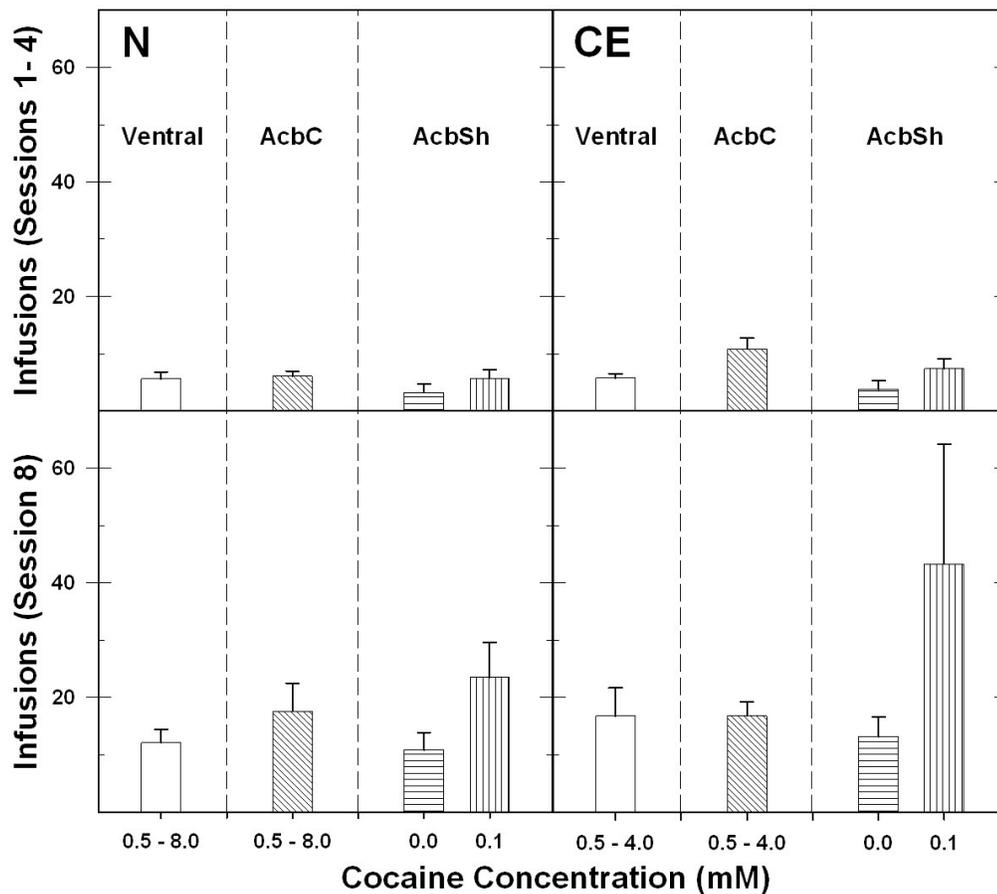


Figure 12. Dose-response effects for the acquisition and reinstatement of self-infusion of 0.5-8.0 mM cocaine into the AcbC or regions ventral to the AcbSh of P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE). Concentration of 0 mM is aCSF only. For acquisition, the number of infusions was averaged over Sessions 1-4 for each animal. For reinstatement, the number of infusions was obtained from Session 8 for each animal. Data was collapsed across dose into groups with placements within the AcbC or ventral to the AcbSh. Infusion data for rats given 0.1 nM cocaine or aCSF into the AcbSh is presented for comparison. Data are the means  $\pm$  SEM. ( $n = 5$  to 10/pretreatment for AcbC or ventral to AcbSh)

Analyzing the number of active lever responses by the control animals across the eight sessions revealed a significant effect of session ( $F(7,15) = 5.7$ ,  $p < 0.005$ ) but no other significant main effects or interactions for site, pretreatment, or dose (all  $F$ -values  $< 1.4$ , all  $p$ -values  $> 0.12$ ). Anatomical control

animals performed a comparable number of active lever responses for each session as those administering aCSF into the AcbSh (Figure 13). Comparison of active and inactive lever responding revealed no significant main effects or interactions of lever-type, session, or dose for each of the four groups (N or CE animals with placements within the AcbC or the region ventral to the AcbSh; all  $F$ -values  $< 3.3$ , all  $p$ -values  $> 0.07$ ).

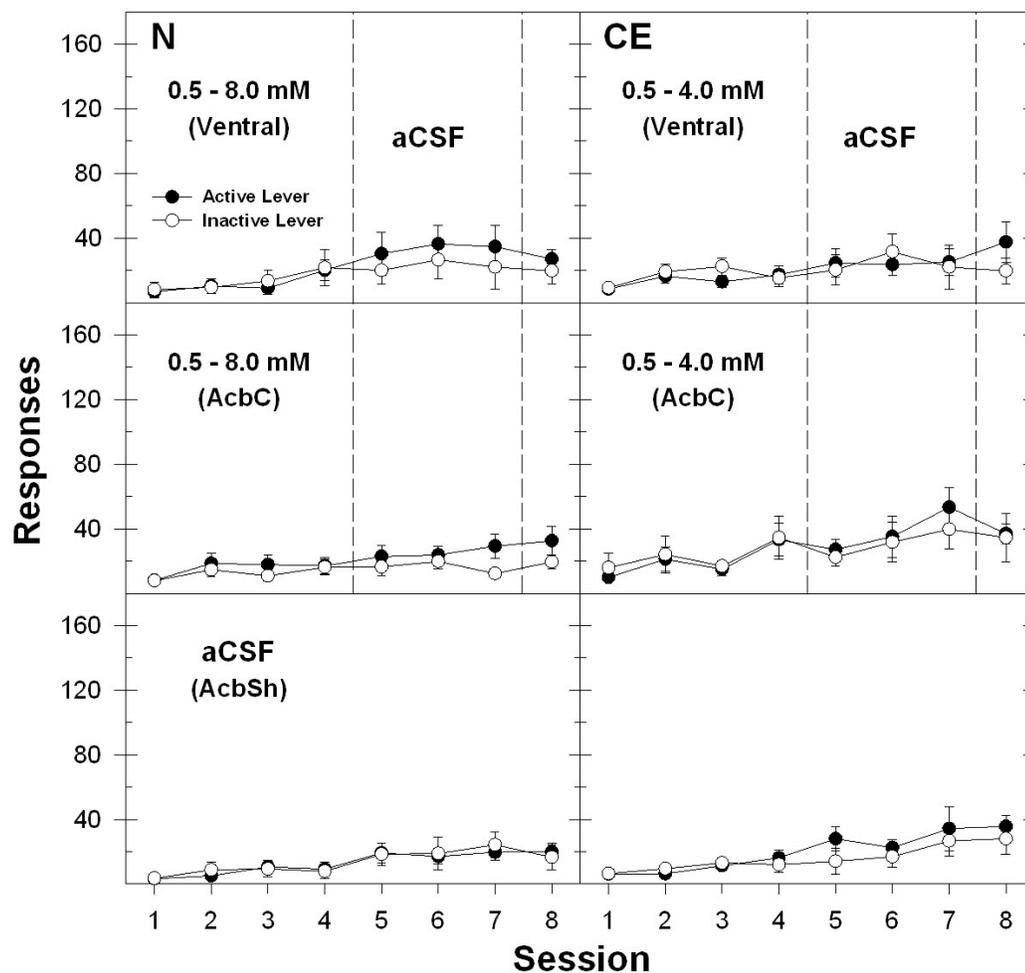


Figure 13. Responses on the active and inactive levers for the self-infusion of 0.5-8.0 mM cocaine into the AcbC or regions ventral to the AcbSh of P rats with no ethanol-drinking experience (N) or continuous ethanol-drinking experience (CE) for four acquisition sessions, three extinction sessions, and one reinstatement session. Lever responses of rats self-infusing aCSF into the AcbSh are presented for comparison. Data are the means  $\pm$  SEM. ( $n = 5$  to 10/pretreatment for AcbC or ventral to AcbSh)

Persistent Alteration of Posterior Ventral Tegmental Area Dopamine Neurons to the Stimulating Effects of Cocaine Following Chronic Ethanol Drinking

Histology

Figure 14 shows representative placements of microinjection sites and microdialysis probes, respectively. The primary analysis included animals with verified placements within both the pVTA and AcbSh. The pVTA was defined as the VTA region at the level of the interpeduncular nucleus, coronal sections at 5.3 to 6.1 posterior to bregma. The aVTA was defined as the VTA region at the level of the mammillary body, coronal sections at 4.8 to 5.2 posterior to bregma. Microinjection sites outside of the VTA included neuroanatomical controls within adjacent regions: dorsomedial (rostral linear nucleus of the raphe), dorsolateral (red nucleus), ventrolateral (medial lemniscus and SN), and ventromedial (interpeduncular nucleus). Probe placements with at least 75% of active membrane area located within the AcbSh sub-region were classified as AcbSh. A number of probe placements spanned portions of the AcbC, OT, or VP. Animals with placements within both the pVTA or aVTA and AcbSh represented approximately 75% of rats undergoing stereotaxic surgery. A total of 149 animals completed the microinjection-microdialysis protocols with sufficient dialysate volumes for analysis. Groups with probe placements within the AcbSh and microinjection placements within the pVTA, aVTA, or outside the VTA were composed of 82, 25, and 36 animals, respectively. There were an additional six animals with injection placements within the pVTA and probe placements with at least 30% membrane area within the dorsal striatum.

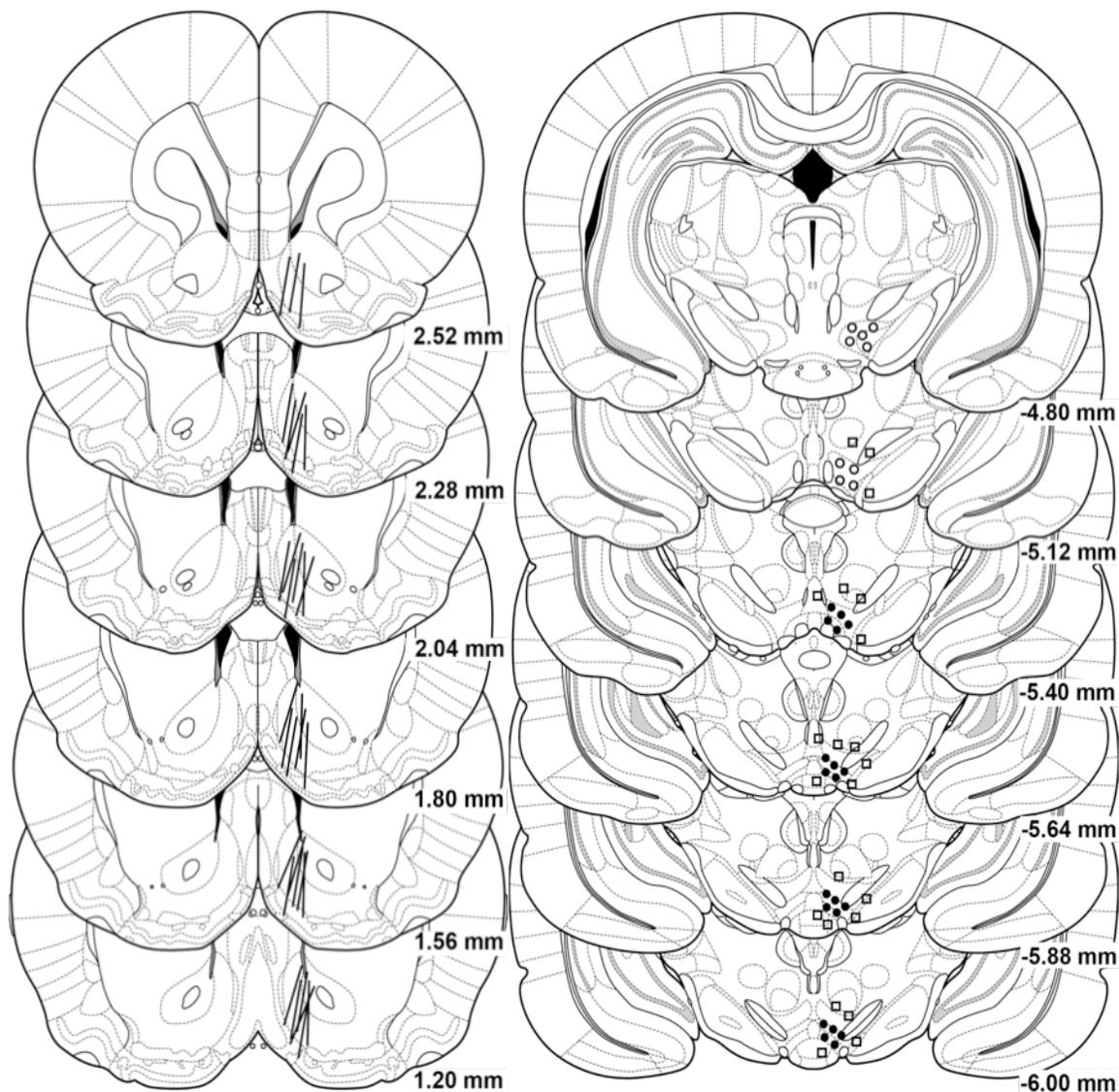


Figure 14. Illustration depicts representative, non-overlapping placements of microdialysis probes in the forebrain (left) and microinjections sites in the midbrain (right) of P rats. The lines represent the 1.5mm length of microdialysis probes in the AcbSh. The filled circles represent placements of injection sites within the pVTA. The open circles represent placements of injection sites within the aVTA. The open squares represent placements of injection sites in regions adjacent to the VTA.

## Ethanol Intake for Ethanol-drinking and Ethanol-abstinent Rats

Average daily ethanol intakes for the P rats with continuous ethanol-drinking experience (CE) were approximately 5-6 g/kg/d for the first wk ethanol consumptions were measured. The final wk before surgery, the average ethanol intake for CE animals was  $6.6 \pm 0.5$  g/kg/d. During the 4-d period following surgery, there was a modest decrease in mean ethanol intake ( $5.7 \pm 0.7$  g/kg/d). Ethanol intakes returned to pre-surgery baseline levels for the 2-d period before microinjection-microdialysis experiments. Average daily ethanol intakes for the P rats with continuous ethanol-drinking experience followed by a period of abstinence (Ab) were approximately 5-6 g/kg/d for the first wk ethanol consumption was measured. The final wk before abstinence, the average ethanol intake for Ab animals was  $6.3 \pm 0.4$  g/kg/d. Ethanol intakes were in line with previous studies using P rats given long-term, concurrent access to water and 15% ethanol (Rodd et al., 2005b,c; Rodd-Henricks et al., 2000b).

## Temporal Pattern of the Stimulating Effects of Cocaine on Posterior Ventral Tegmental Area Dopamine Neurons

There was no significant difference in the basal extracellular DA levels in the AcbSh of animals resulting from drinking experience ( $F(2,79) = 1.4$ ,  $p = 0.25$ ). Basal DA levels were  $1.15 \pm 0.06$  nM,  $1.11 \pm 0.06$  nM, and  $1.01 \pm 0.07$  nM for N, CE, and Ab animals, respectively. These levels are within the range of Acb basal extracellular DA levels previously reported for P rats using traditional microdialysis (Ding et al., 2009a; Engleman et al., 2000; Melendez et al., 2002). The reported values are not corrected for *in vivo* probe recovery, and definitive differences between groups in extracellular DA levels could not be determined without conducting quantitative (e.g. zero-net-flux) microdialysis experiments.

Analysis of percent baseline DA levels revealed significant main effects of time ( $F(7,62) = 17.1$ ,  $p < 0.001$ ), pretreatment ( $F(2,68) = 4.7$ ,  $p < 0.05$ ), and dose

( $F(4,68) = 51.8, p < 0.001$ ). Significant interaction effects for 'pretreatment x dose' ( $F(7,68) = 35.3, p < 0.001$ ), 'time x pretreatment' ( $F(14,126) = 2.2, p < 0.01$ ), 'time x dose' interaction ( $F(28,260) = 4.2, p < 0.001$ ), and 'time x pretreatment x dose' ( $F(49,476) = 3.2, p < 0.001$ ) were also found.

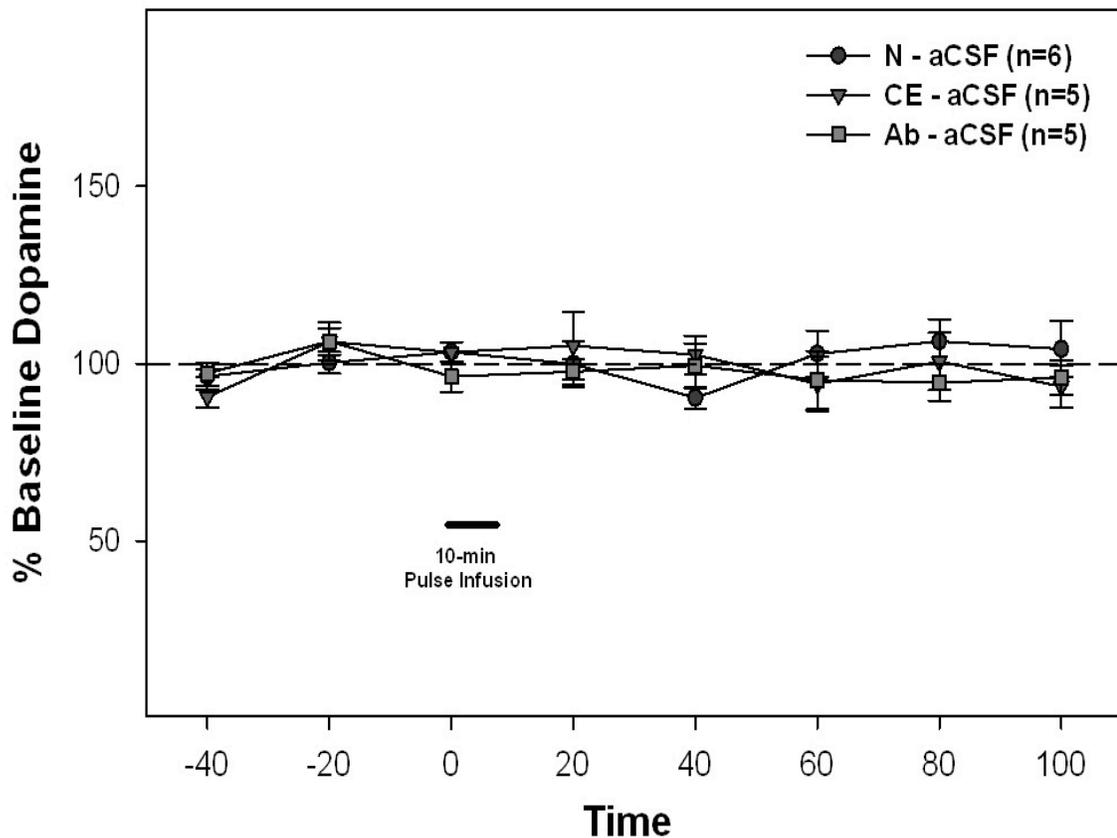


Figure 15. Time course of extracellular DA levels in the AcbSh of P rats before and after the microinjection of aCSF into the pVTA. N: no ethanol-drinking experience; CE: continuous ethanol-drinking experience; Ab: chronic ethanol-drinking experience followed by abstinence. Data are the means  $\pm$  SEM.

## Nucleus Accumbens Shell Dopaminergic Response After Injection of Artificial Cerebrospinal Fluid or 0.25 mM Cocaine into the Posterior Ventral Tegmental Area

Figure 15 shows injection of aCSF into the pVTA did not significantly alter baseline levels of extracellular DA regardless of pretreatment. Injection of 0.25 mM cocaine into the pVTA of N, CE, or Ab rats increased extracellular DA levels within the AcbSh with a relative magnitude as follows: Ab > CE > N (Figure 16). For N animals, DA levels were not elevated to a significant extent (peak =  $131 \pm 14\%$  of baseline, all  $p > 0.06$ ). For CE animals, DA levels gradually increased within 20 min after cocaine injection ( $127 \pm 11\%$  of baseline), reached peak levels 20 min later ( $201 \pm 28\%$ ,  $p < 0.05$ ), then gradually returned to baseline levels at 100 min after cocaine injection. For Ab animals, DA levels sharply increased within 20 min after cocaine injection ( $195 \pm 13\%$  of baseline,  $p < 0.005$ ), reached a peak 20 min later ( $232 \pm 11\%$ ,  $p < 0.001$ ), then gradually returned to baseline levels at 100 min after cocaine injection.

At 20 min after 0.25 mM cocaine injection, DA levels were significantly greater ( $F(2,14) = 8.7$ ,  $p < 0.005$ ) for Ab animals compared to both N and CE animals ( $p < 0.005$ ; Figure 16). DA levels were significantly greater ( $F(2,14) = 11.3$ ,  $p < 0.005$ ) for CE and Ab animals compared to N animals at 40 min post-injection ( $p < 0.01$ ). At 60 min after injection, DA levels were significantly different between all groups ( $F(2,14) = 13.1$ ,  $p < 0.001$ ). DA levels at 60 min post-injection were greater for Ab animals compared to both CE and N animals ( $p < 0.05$ ) and greater for CE animals compared to N animals ( $p < 0.05$ ). At 80 min after injection, DA levels were significantly greater ( $F(2,14) = 4.5$ ,  $p < 0.05$ ) for Ab animals compared to N animals ( $p < 0.05$ ).

## Nucleus Accumbens Shell Dopaminergic Response After Injection of 0.5 mM Cocaine into the Posterior Ventral Tegmental Area

Figure 17 represents the effects of 0.5 mM cocaine injection into the pVTA on extracellular DA levels in the AcbSh of N, CE, or Ab animals. Injection of 0.5 mM cocaine into the pVTA increased DA levels within the AcbSh for N and (to a greater extent) CE animals. Conversely, injection of 0.5 mM cocaine into the pVTA of Ab animals did not produce a significant change in DA levels within the AcbSh. For N animals, DA levels gradually increased within 20 min after cocaine injection ( $119 \pm 6\%$  of baseline), reached peak levels 20 min later ( $168 \pm 10\%$ ,  $p < 0.005$ ), then gradually returned to baseline levels at 80 min after cocaine injection. For CE animals, DA levels sharply increased to a peak within 20 min after cocaine injection ( $324 \pm 51\%$  of baseline,  $p < 0.01$ ) and then gradually returned to baseline levels at 80 min after injection. For Ab animals, DA levels immediately decreased 20 min after cocaine injection ( $79\% \pm 7$  of baseline, n.s.  $p > 0.05$ ) and then gradually returned to baseline levels at 80 min after injection.

At 20 min after 0.5 mM cocaine injection, DA levels were significantly greater ( $F(2,15) = 19.8$ ,  $p < 0.001$ ) for CE animals compared to both N and Ab animals ( $p < 0.001$ ; Figure 17). DA levels were significantly different between all groups ( $F(2,15) = 16.9$ ,  $p < 0.001$ ) 40 min after injection. DA levels at 40 min post-injection were greater for CE animals compared to both N and Ab animals ( $p < 0.05$ ) and greater for N animals compared to Ab animals ( $p < 0.05$ ). At 60 min after injection, DA levels were significantly greater ( $F(2,15) = 9.8$ ,  $p < 0.005$ ) for CE and N animals compared to Ab animals ( $p < 0.05$ ). At 100 min post-injection, DA levels were significantly greater ( $F(2,15) = 4.7$ ,  $p < 0.05$ ) for N animals compared to CE and Ab animals ( $p < 0.05$ ).

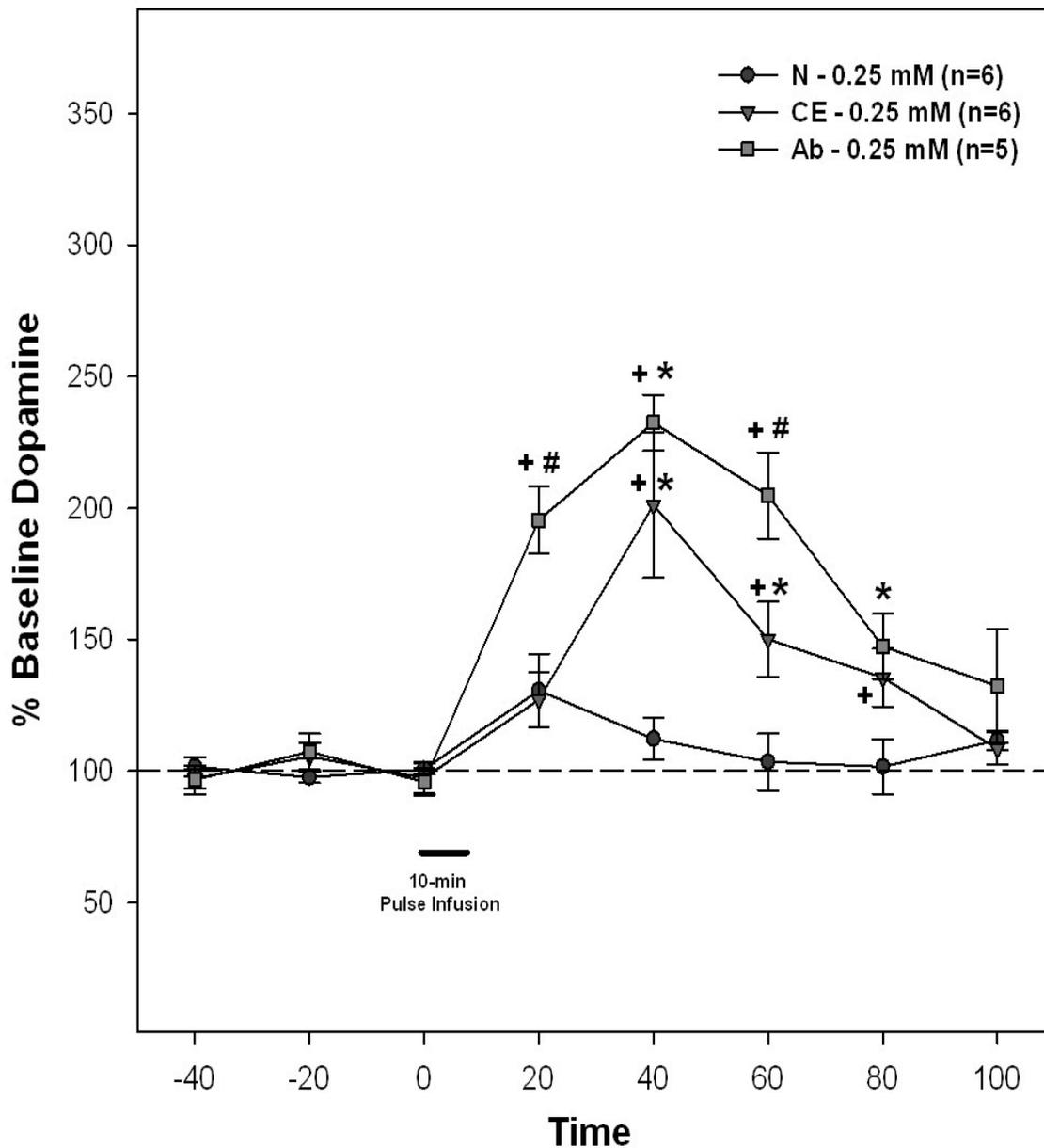


Figure 16. Time course of extracellular DA levels in the AcbSh of P rats before and after the microinjection of 0.25 mM cocaine into the pVTA. N: no ethanol-drinking experience; CE: continuous ethanol-drinking experience; Ab: chronic ethanol-drinking experience followed by abstinence. +  $p < 0.05$ , significantly greater than baseline levels; #  $p < 0.05$  (Tukey's b), significantly greater than all other groups; \*  $p < 0.05$  (Tukey's b), significantly greater than the 'N-0.25' group. Data are the means  $\pm$  SEM.

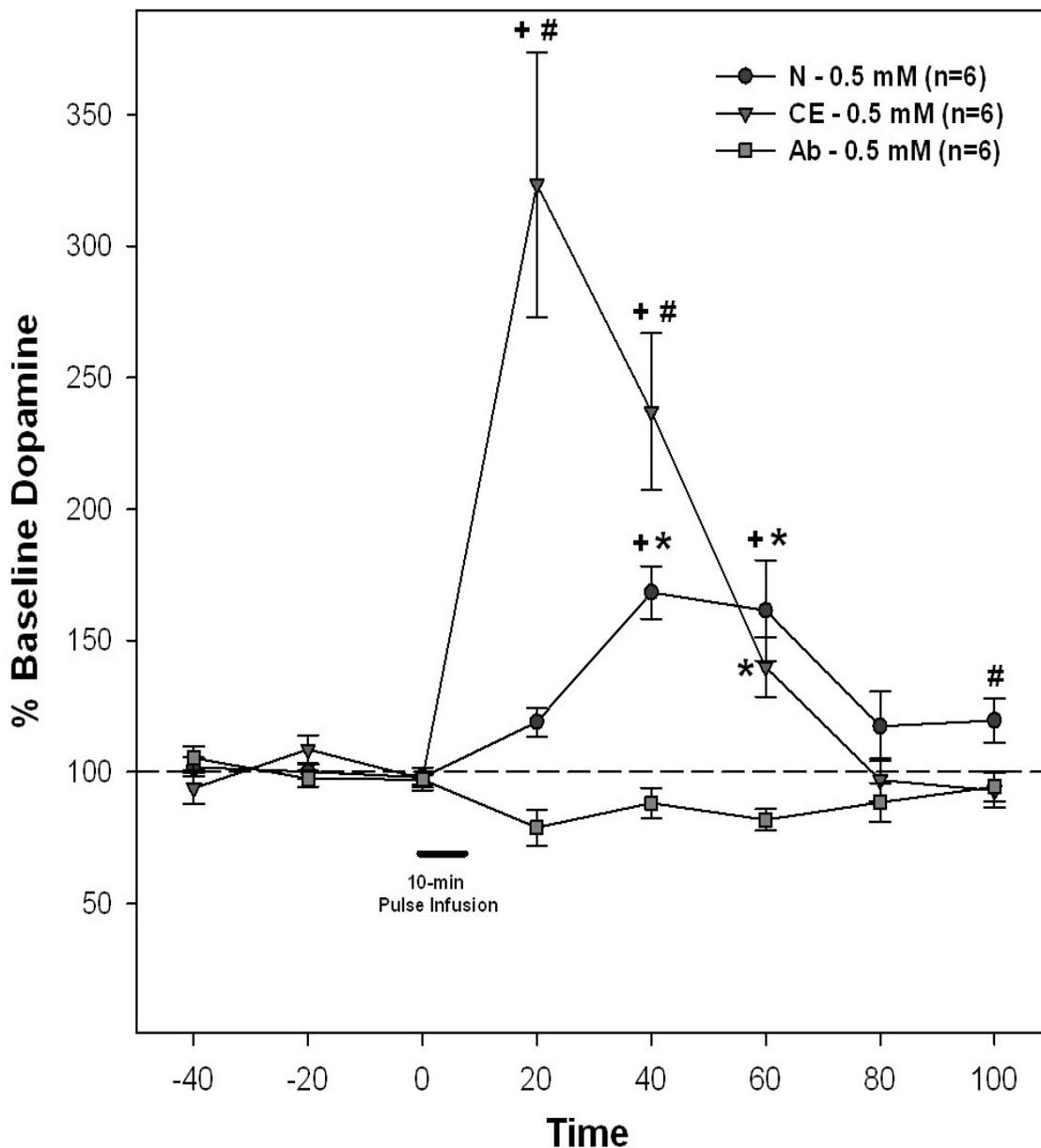


Figure 17. Time course of extracellular DA levels in the AcbSh of P rats before and after the microinjection of 0.5 mM cocaine into the pVTA. N: no ethanol-drinking experience; CE: continuous ethanol-drinking experience; Ab: chronic ethanol-drinking experience followed by abstinence. +  $p < 0.05$ , significantly different from baseline levels; #  $p < 0.05$  (Tukey's b), significantly greater than all other groups; \*  $p < 0.05$  (Tukey's b), significantly greater than 'Ab-0.5' group. Data are the means  $\pm$  SEM.

### Nucleus Accumbens Shell Dopaminergic Response After Injection of 1.0 mM Cocaine into the Posterior Ventral Tegmental Area

For the concentration of 1.0 mM cocaine, data was obtained from N and CE animals, but not for Ab animals (Figure 18). Injection of 1.0 mM cocaine into the pVTA increased DA levels within the AcbSh of N animals. On the other hand, injection of 1.0 mM cocaine into the pVTA of CE animals decreased AcbSh DA levels. For N animals, DA levels increased within 20 min after cocaine injection ( $144 \pm 10\%$  of baseline,  $p < 0.01$ ), reached peak levels 20 min later ( $164 \pm 7\%$ ,  $p < 0.001$ ), then gradually returned to baseline levels at 80 min after cocaine injection. For CE animals, DA levels decreased 20 min after cocaine injection ( $69 \pm 5\%$  of baseline,  $p < 0.01$ ), reached a minimum level 20 min later ( $56 \pm 4\%$ ,  $p < 0.001$ ), then gradually returned to baseline levels at 100 min after injection. At 20, 40, and 60 min after cocaine injection, DA levels were significantly greater for N compared to Ab animals (all  $t$ -values  $> 4.2$ ,  $p$ -values  $< 0.005$ ).

### Nucleus Accumbens Shell Dopaminergic Response After Injection of 2.0 mM Cocaine into the Posterior Ventral Tegmental Area

Figure 19 represents the effects of 2.0 mM cocaine injection into the pVTA on extracellular DA levels in the AcbSh of N, CE, or Ab animals. Injection of 2.0 mM cocaine into the pVTA increased DA levels within the AcbSh in N animals. In contrast, injection of 2.0 mM cocaine into the pVTA of CE or Ab animals reduced AcbSh DA levels. For N animals, DA immediately increased to peak levels 20 min after cocaine injection ( $161 \pm 11\%$  of baseline,  $p < 0.05$ ) and quickly returned to baseline levels 20 min later. For CE animals, DA levels decreased 20 min after cocaine injection ( $75 \pm 4\%$  of baseline,  $p < 0.01$ ) and remained at this level throughout 100 min post-injection. While still depressed, DA levels for CE animals were not significantly different from baseline at 80 or 100 min after cocaine injection (both  $p$ -values  $> 0.07$ ). For Ab animals, DA levels decreased 20

min after cocaine injection ( $76 \pm 4\%$ ,  $p < 0.05$ ), reached a minimum level 20 min later ( $6\% \pm 6\%$ ,  $p < 0.01$ ), then gradually returned to baseline levels at 100 min after injection.

At both 20 min ( $F(2,17) = 46.4$ ,  $p < 0.001$ ) and 40 min ( $F(2,17) = 12.0$ ,  $p < 0.001$ ) after 2.0 mM cocaine injection, DA levels were significantly greater for N animals compared to both CE and Ab animals (both  $p$ -values  $< 0.005$ ). DA levels remained significantly greater for N animals compared to both CE and Ab animals ( $p < 0.05$ ) at 60 min ( $F(2,17) = 5.8$ ,  $p < 0.05$ ) after cocaine injection. In addition to the time-course analysis, post-injection peak dopaminergic response and total AUC were analyzed to succinctly demonstrate the dose-response effects of cocaine microinjection in N, CE, and Ab animals.

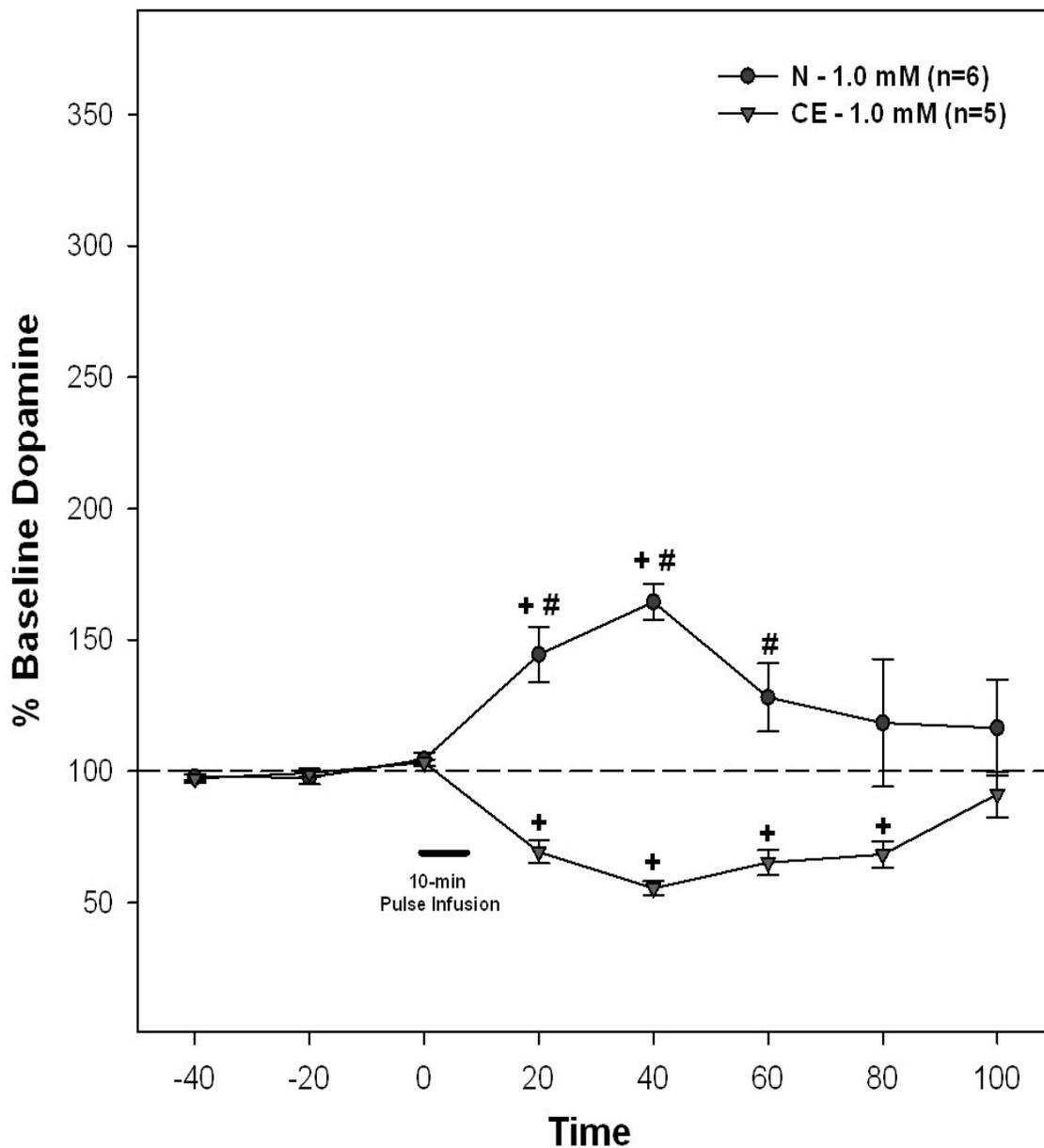


Figure 18. Time course of extracellular DA levels in the AcbSh of P rats before and after the microinjection of 1.0 mM cocaine into the pVTA. N: no ethanol-drinking experience; CE continuous ethanol-drinking experience. +  $p < 0.05$ , significantly different from baseline levels; #  $p < 0.05$ , significantly greater than 'CE-1.0' group. Data are the means  $\pm$  SEM.

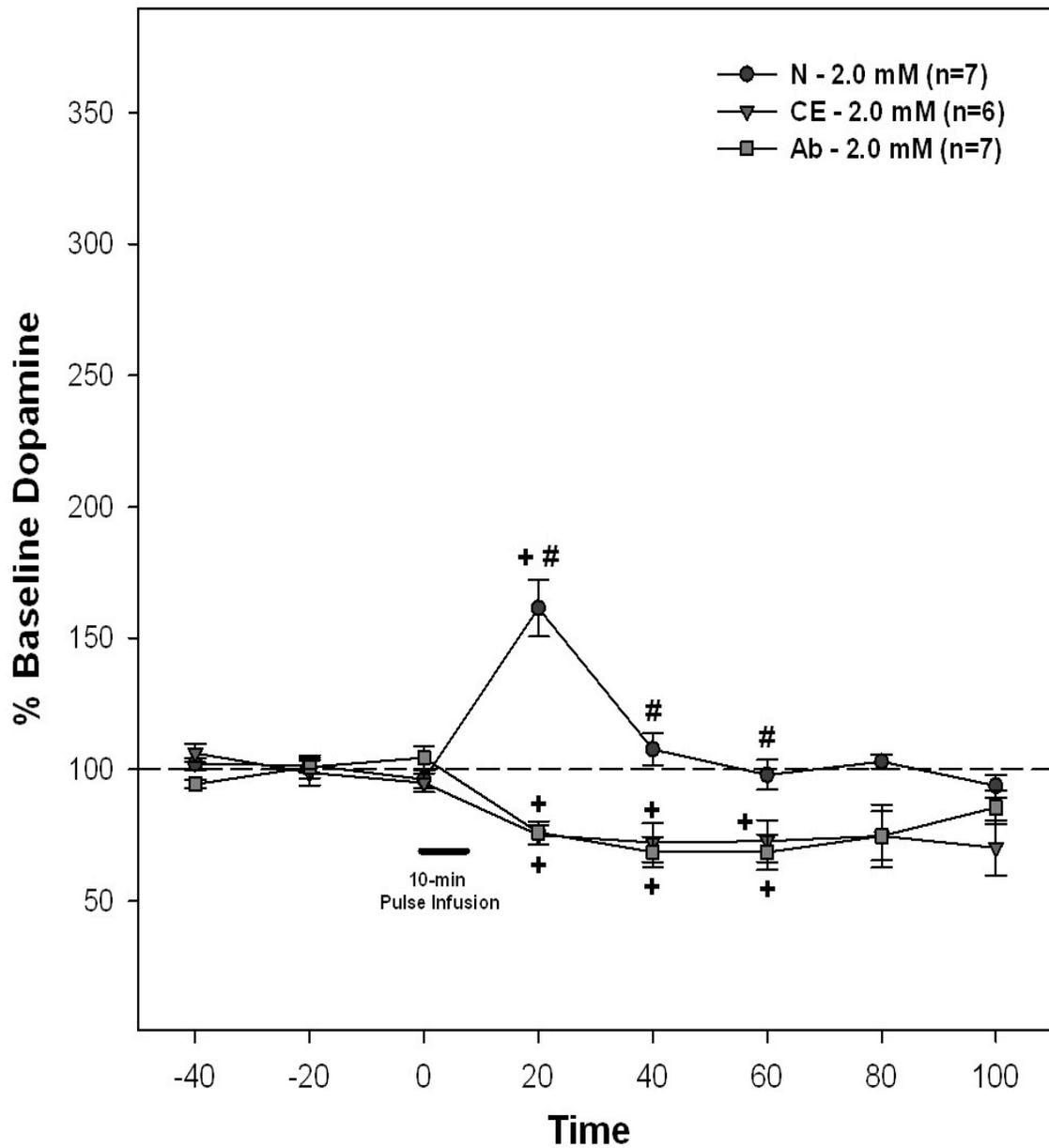


Figure 19. Time course of extracellular DA levels in the AcbSh of P rats before and after the microinjection of 2.0 mM cocaine into the pVTA. N: no ethanol-drinking experience; CE: continuous ethanol-drinking experience; Ab: chronic ethanol-drinking experience followed by abstinence. +  $p < 0.05$ , significantly different from baseline levels; #  $p < 0.05$  (Tukey's b), significantly greater than all other groups. Data are the means  $\pm$  SEM.

### Comparison of Maximal Dopaminergic Response

The maximum or minimum peak dopaminergic response compared to baseline for each animal was determined. The maximal values were used for animals with a net increase in dopaminergic response and minimal values were for animals with a net decrease in dopaminergic response. Analysis of the peak post-injection percent baseline DA levels revealed significant effects of pretreatment ( $F(2,68) = 10.5, p < 0.001$ ) and dose ( $F(4,68) = 29.8, p < 0.001$ ) along with a 'pretreatment x dose' interaction ( $F(7,68) = 25.6, p < 0.001$ ).

For N animals, pVTA injection of 0.5 or 1.0 mM cocaine produced greater peak AcbSh DA levels compared to injection of 0.25 mM cocaine or aCSF ( $p < 0.05$ ). Injection of 2.0 mM cocaine resulted in greater peak DA levels compared to injection of aCSF ( $p < 0.05$ ). For CE animals, injection of 0.25 or 0.5 mM cocaine resulted in a significant dose-dependent increase in peak DA levels compared to injection of 1.0 or 2.0 mM cocaine or aCSF ( $p < 0.01$ ). For Ab animals, injection of 0.25 mM cocaine resulted in greater peak DA levels compared to injection of 0.5 or 2.0 mM cocaine or aCSF ( $p < 0.001$ ).

There was no significant effect of pretreatment condition on the peak AcbSh DA levels of animals injected with aCSF ( $F(2,13) = 0.3, p = 0.75$ ). Injection of 0.25 mM cocaine resulted in greater peak DA levels in CE and Ab animals compared to N animals ( $p < 0.05$ ). Injection of 0.5 mM cocaine resulted in a significant 'pretreatment'-dependent differences in peak DA levels with CE > N > Ab ( $p < 0.01$ ). Injection of 1.0 mM cocaine produced greater peak DA levels for N animals compared to CE animals ( $p < 0.001$ ). Lastly, injection of 2.0 mM cocaine resulted in greater peak DA levels for N animals compared to CE or Ab animals ( $p > 0.001$ ). While comparison of peak DA levels is a useful measure, AUC analysis provides a summation of both the magnitude and the shape across time of the post-injection dopaminergic response.

### Comparison of Area Under the Time-response Curve

Figure 20 represents the AUC values from each group. Briefly, these values were calculated as the sum of individual areas (determined via the trapezoidal method) between each data point for each individual animal. Analysis of the AUC values revealed significant main effects of pretreatment ( $F(2,68) = 4.8, p < 0.05$ ) and dose ( $F(4,68) = 55.2, p < 0.001$ ), as well as a significant effect of the 'pretreatment x dose' interaction ( $F(7,68) = 40.1, p < 0.001$ ). For N animals, injection of 0.5 or 1.0 mM cocaine into the pVTA resulted in significantly greater increases in AUC values compared to injection of 0.25 or 2.0 mM cocaine or aCSF ( $p < 0.05$ ). For CE animals, injection of 0.25 or 0.5 mM cocaine resulted in a significant dose-dependent increase in AUC values compared to aCSF injection ( $p < 0.005$ ). On the other hand, injection of 1.0 or 2.0 mM cocaine for CE animals resulted in a significant reduction in AUC values compared to injection of 0.25 or 0.5 mM cocaine or aCSF ( $p < 0.05$ ). For Ab animals, injection of 0.25 mM cocaine resulted in a significantly greater AUC value compared to injection of 0.5 or 2.0 mM cocaine or aCSF ( $p < 0.001$ ). Conversely, injection of 2.0 mM cocaine for Ab animals resulted in a significantly smaller AUC value compared to aCSF injection ( $p < 0.01$ ).

There were no significant differences in AUC values across pretreatment of animals injected with aCSF ( $F(2,13) = 0.2, p = 0.82$ ). Injection of 0.25 mM cocaine resulted in a significant 'pretreatment'-dependent increase of AUC values with  $Ab > CE > N$  ( $p < 0.005$ ). Injection of 0.5 mM cocaine resulted in a significant 'pretreatment'-dependent differences of AUC values with  $CE > N > Ab$  ( $p < 0.005$ ). Injection of 1.0 mM cocaine resulted in a significantly greater AUC value for N animals compared to CE animals ( $p < 0.001$ ). Lastly, injection of 2.0 mM cocaine resulted in a significantly greater AUC value for N animals compared to CE or Ab animals ( $p > 0.001$ ).

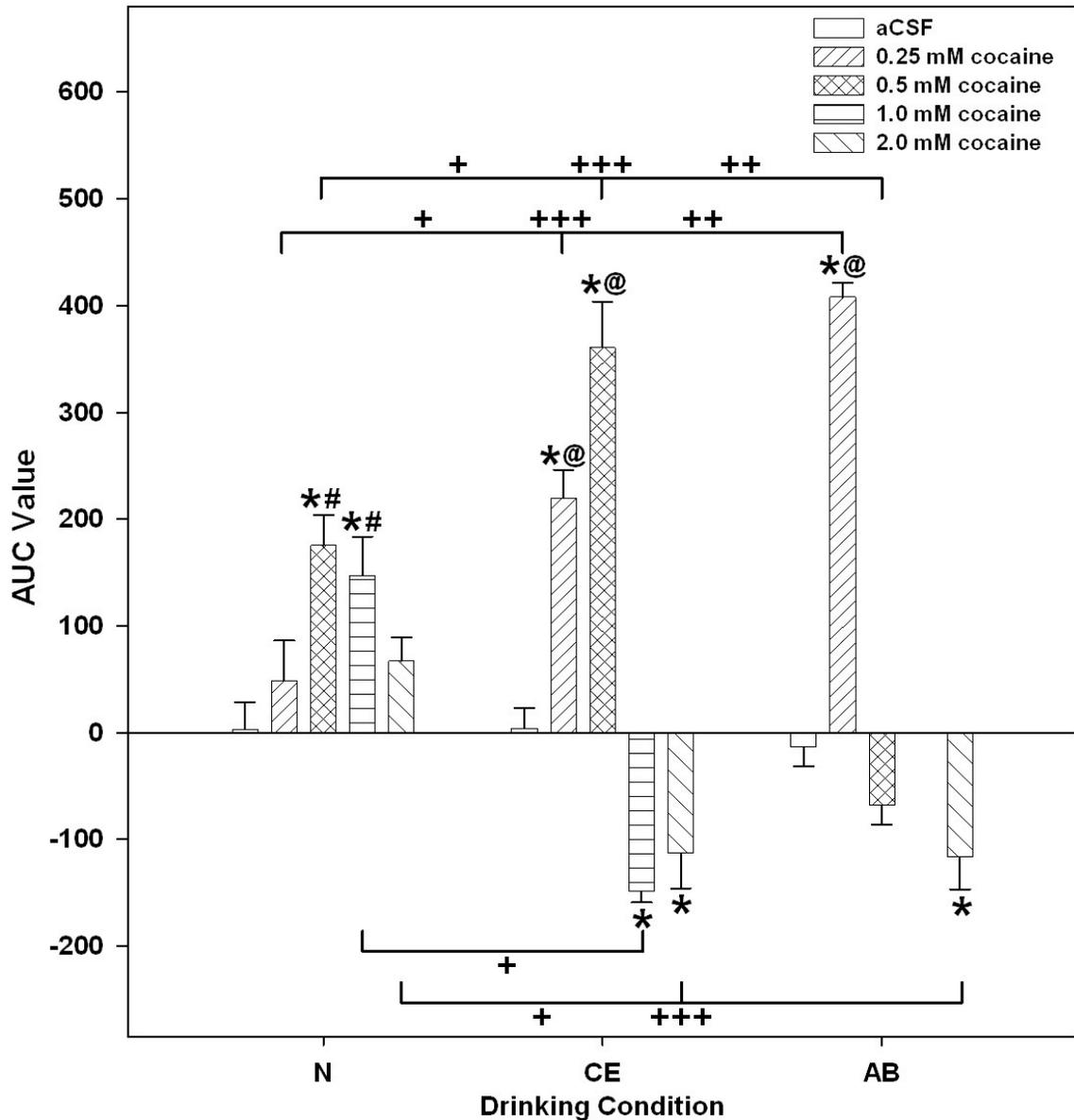


Figure 20. Area under the curve (AUC) analysis for the dose-response effect of microinjection of 0-2.0 mM cocaine into the pVTA on DA in the AcbSh following: no ethanol-drinking experience (N), continuous ethanol-drinking experience (CE), or chronic ethanol-drinking experience followed by a period of protracted abstinence (Ab). \*  $p < 0.05$  (Tukey's b), significantly different from aCSF group with the same pretreatment. #  $p < 0.05$  (Tukey's b), significantly greater than 'N-0.5' and 'N-2.0' groups. @  $p < 0.05$  (Tukey's b), significantly different than all other cocaine doses within the same pretreatment. +  $p < 0.05$  (Tukey's b), significant difference between N and CE groups at the same dose of cocaine. ++  $p < 0.05$  (Tukey's b), significant difference between CE and Ab groups at the same dose of cocaine. +++  $p < 0.05$  (Tukey's b), significant difference between N and Ab groups at the same dose of cocaine. Data are the means  $\pm$  SEM.

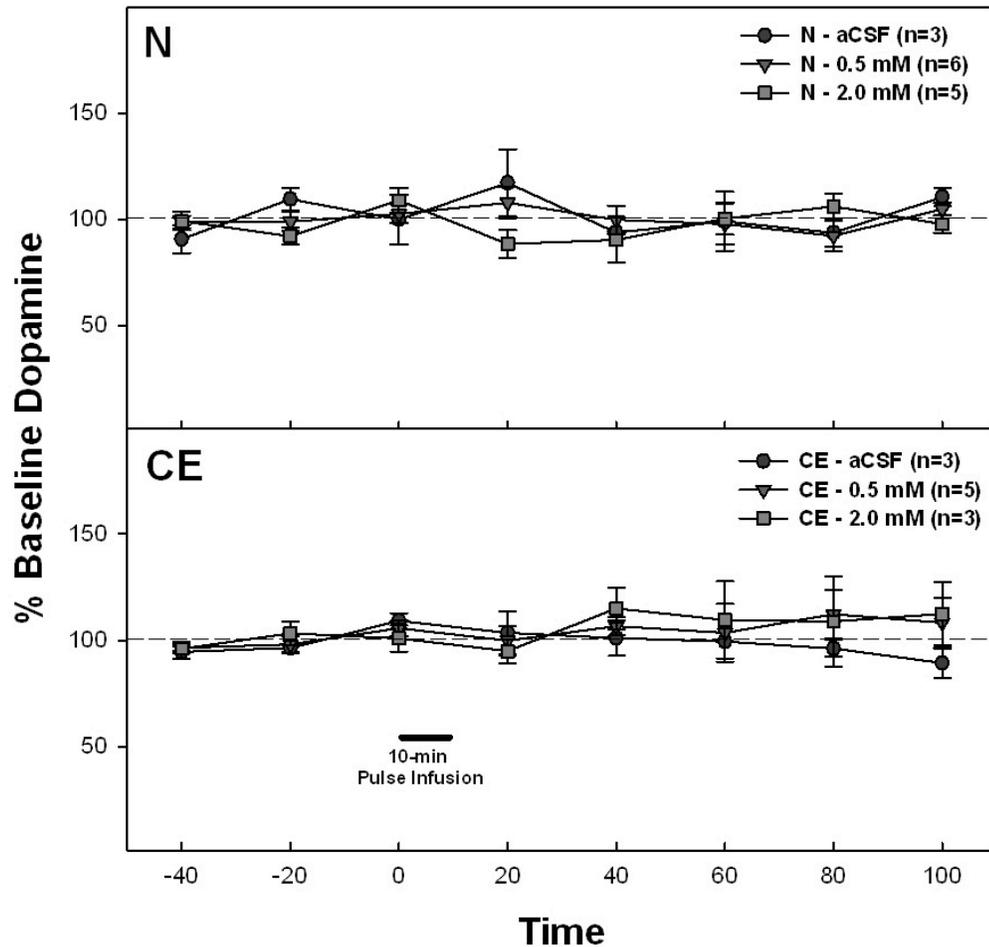


Figure 21. Time course of extracellular DA levels in the AcbSh of P rats before and after the microinjection of 0.5, 1.0, or 2.0 mM cocaine or aCSF into the aVTA. N: no ethanol-drinking experience; CE: chronic ethanol-drinking experience. Data are the means  $\pm$  SEM.

#### Nucleus Accumbens Shell Dopaminergic Response After Injection of Cocaine into the Anterior Ventral Tegmental Area or Regions Adjacent to the Ventral Tegmental Area

Figure 21 represents the effects of microinjections of 0.5 or 2.0 mM cocaine or aCSF into the aVTA on extracellular DA levels in the AcbSh of N or CE animals, respectively. There were no significant differences in the basal extracellular DA levels in the AcbSh resulting from drinking experience ( $t(23) =$

0.7,  $p = 0.50$ ). Basal DA levels were  $1.09 \pm 0.09$  nM and  $1.18 \pm 0.08$  nM for N and CE groups, respectively. There were no significant effects of pretreatment, time, dose, or any interaction terms (all  $F$ -values  $< 1.4$ , all  $p$ -values  $> 0.28$ ).

Figure 22 represents the effects of 0.25-2.0 mM cocaine or aCSF into areas adjacent to the VTA. These injection sites included placements within the red nucleus, rostral linear nucleus, interpeduncular nucleus, medial lemniscus, or SN. Analysis revealed no significant effects of pretreatment, time, injection site, dose, or any interaction terms (all  $F$ -values  $< 2.0$ , all  $p$ -values  $> 0.16$ ). As such, data was collapsed across injection site and dose. There were also six animals with injector placements within the pVTA and at least 30% of the microdialysis probe area within the dorsal striatum. Basal extracellular DA levels within the dorsal striatum for these animals were  $4.63 \pm 0.45$  nM and no significant changes in DA levels were observed after cocaine microinjection ( $F(7,14) = 0.4$ ,  $p = 0.64$ ).

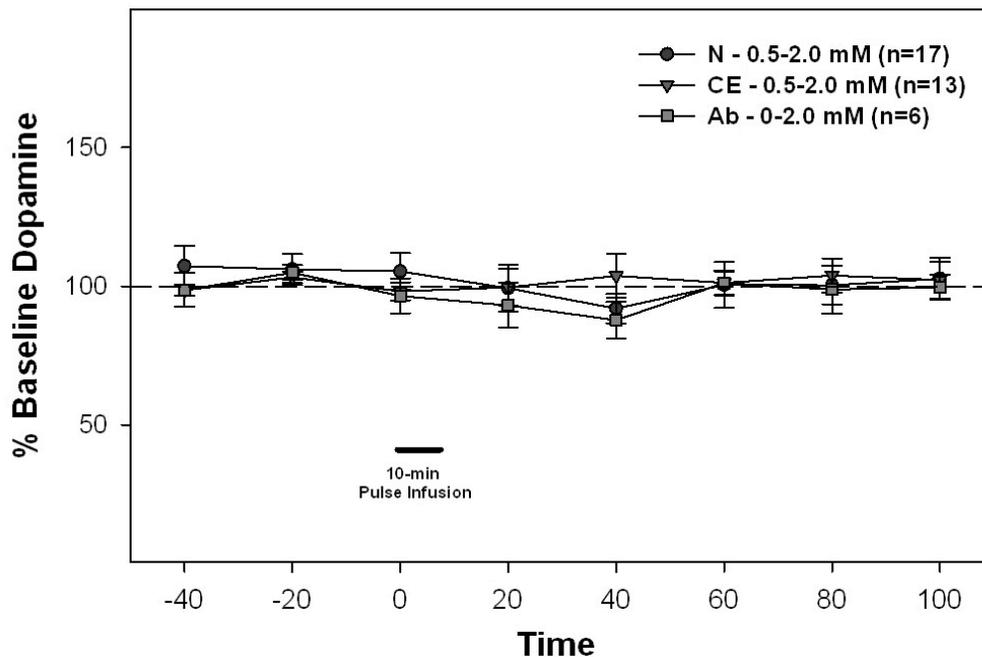


Figure 22. Time course of extracellular DA levels in the AcbSh of P rats before and after the microinjection of 0.5, 1.0, or 2.0 mM cocaine or aCSF into areas adjacent to the VTA. N: no ethanol-drinking experience; CE: continuous ethanol-drinking experience; Ab: chronic ethanol-drinking experience followed by a period of protracted abstinence. Data are the means  $\pm$  SEM.

## 4. DISCUSSION

### Sensitization of Cocaine Reinforcement within the Nucleus Accumbens Shell Following Chronic Ethanol Drinking

The results of the experiment indicate: (1) adult P rats initiated and maintained the self-infusion of cocaine into the AcbSh, but not the AcbC (Figures 3 and 12); and (2) voluntarily consumption of significant amounts of ethanol under continuous conditions, compared to water access only, reduced the threshold dose for self-infusion of cocaine and produced greater increases in responding for cocaine after a drug-free period (Figures 3, 6, 10, and 11). The results confirm the AcbSh as a critical brain site mediating the initiation of cocaine reinforcement and support the hypothesis that chronic continuous ethanol-drinking experience increases the sensitivity of the AcbSh to the reinforcing effects of cocaine.

### Cocaine Reinforcement within the Nucleus Accumbens Shell

The present study is in agreement with previous research finding both outbred Wistar and selectively-bred P rats, as well as mice, will self-administer physiologically relevant concentrations of cocaine into the AcbSh, but not the AcbC (David et al., 2001; Katner et al., 2011; McKinzie et al., 1999; Rodd-Henricks et al., 2002a). Ethanol-naïve P rats reliably self-infused concentrations of 2.0, 4.0, and 8.0 mM cocaine (200-800 pmol / infusion) into the AcbSh, but did not self-infuse cocaine at any concentration into the AcbC (Figures 3, 6, 12). P rats in the current study reliably self-infused cocaine into the AcbSh at the same

range of concentrations (2.0-8.0 mM) as previous experiments with P rats self-infusing cocaine into the AcbSh (Katner et al., 2011). Neither the current study nor Katner et al. (2011) examined the descending limb of the dose-response curve (> 8.0 mM cocaine) for the self-infusion of cocaine into the AcbSh of P rats. However, the self-infusion of cocaine into the AcbSh or pVTA of Wistar rats (Rodd-Henricks et al., 2002a; Rodd et al., 2005a) exhibited an inverted-U-shaped dose-response curve.

Doses supporting self-infusion in the current study correspond well with the amount of locally delivered cocaine required to increase DA and serotonin levels within the Acb, elicit locomotor activation, or produce a CPP (Andrews and Lucki, 2001; Delfs et al., 1990; Filip and Siwanowicz, 2001; Ikemoto, 2002; Teneud et al., 1996). The discrimination of the active from the inactive lever during Sessions 1-4 and Session 8 suggests the self-infusion of 2.0-8.0 mM cocaine into the AcbSh is not the result of an increase in general locomotor activity (Figure 11; Ikemoto and Wise, 2004). Lever discrimination is relevant because microinjection of cocaine or mixtures of D<sub>1</sub> and D<sub>2</sub> receptor agonists into the AcbSh, but not AcbC, elicited significant locomotion and rearing (Choi et al., 2000; Filip and Siwanowicz, 2001; Ikemoto, 2002). Additionally, sodium channel inhibition did not play a significant role in cocaine's neurochemical or reinforcing effects within the AcbSh (Hernandez et al., 1991; Ikemoto, 2003).

Lack of reliable cocaine self-infusion into the AcbC or areas ventral to the AcbSh (see Figures 12 and 13) confirms the findings of Rodd-Henricks et al. (2002a). Anatomical controls are critical for mapping the boundaries of a drug's local site of action. Concerns of anatomical specificity arise due to the unknown extent of drug diffusion away from the injection site and the tendency for drug efflux up the cannula shaft due to differential pressure (Ikemoto and Wise, 2004; Wise and Hoffman, 1992). Previous studies using the same or a similar drug-delivery system as the current experiments show a similar neuroanatomical specificity (AcbSh but not AcbC) for other drugs of abuse (Engleman et al., 2009, Hoebel et al., 1983; Ikemoto et al., 2005; Olds, 1982; Rodd et al., 2003b).

Diffusion away from injection site did not appear to contribute to the observed self-infusion behaviors in the current experiment, given the use of small injection volumes and the range of cocaine concentrations tested. The functional properties of DAT differ between the AcbSh and AcbC, corresponding to the increased sensitivity to the effects of psychostimulants of the AcbSh compared to the AcbC (David et al., 1998). The AcbSh, but not the AcbC, contained potential sites on the DAT for functional interactions between DA and norepinephrine (Nirenberg et al., 1997). The AcbSh also exhibited more transient DA release relative to the AcbC, associated with less efficient DA uptake in this subregion (Jones et al., 1996).

The direct effects of cocaine within the Acb are likely mediated via its actions as a non-specific monoamine reuptake inhibitor (Reith et al., 1986; Ritz et al., 1987), as local application produced significant increases in DA, serotonin, and norepinephrine (Andrews and Lucki, 2001; Hernandez et al., 1991; Li et al., 1996; Teneud et al., 1996). Cocaine-evoked increases serotonin and norepinephrine also potentially modulate the effects of DA within the Acb (Filip et al., 2005; Li et al., 1996; Smith and Aston-Jones, 2008), and serotonin can act locally to increase DA release (Campbell and McBride, 1995; Chen et al., 1991; Parsons and Justice, 1993; White, 1990). Cocaine's ability to increase DA levels in the Acb results in the activation of local  $D_1$  and  $D_2$  receptors. The cooperative activation of  $D_1$  and  $D_2$  receptors in the Acb (Clark and White, 1987) appears to be essential in the reinforcing actions of stimulants within the Acb (Ikemoto et al., 1997a), as well as cocaine sensitization (Henry et al., 1998).

Another significant finding from the current study is the increased level of active lever responding and self-infusions of N rats at 'optimal concentrations' after cocaine access was restored in Session 8. N rats obtained more infusions of 2.0 or 4.0 mM cocaine during Session 8 compared to the final session before extinction (Session 4; Figure 7). Furthermore, rats self-infusing a marginally reinforcing concentration (1.0 mM) did not display lever discrimination during the initial four sessions, but produced clear lever discrimination and a significant

number of self-infusions during Session 8 (Figures 6 and 10). Previous ICSEA studies with ethanol-naïve P rats found robust increases in active lever responding and infusions after previous access to either ethanol or cocaine was restored (Katner et al., 2011; Rodd et al., 2004a, 2005b,c). Moreover, P rats receiving ethanol access for seven consecutive sessions did not show this effect by the final session (Rodd et al., 2005d), suggesting potentiated responding resulted from neuronal alterations within the AcbSh or afferent and efferent connections occurring during the drug-free period. A number of molecular, neurochemical, and behavioral adaptations appear to contribute to the sensitization of cocaine reinforcement and locomotor response (Nestler, 2001; Self, 2004; Wolf, 2010). For example, rats exhibited increased breakpoints for cocaine IVSA only after 10 d of discrete trial self-administration followed by 7 d of deprivation, but not with continuous access to cocaine or shorter periods of deprivation (Morgan and Roberts, 2004). On the other hand, the observed effects could reflect tolerance to cocaine's neurochemical effects within the Acb. Rats were potentially compensating for lower basal levels of DA in the Acb by self-infusing greater amounts of cocaine, as 1-2 wk of contingent or non-contingent cocaine injections produced transient decreases in basal Acb DA levels 3-10 d after the final exposure (Meil et al., 1995; Rossetti et al., 1992).

Genetic factors potentially mediated the increased responding for cocaine during Session 8, as outbred Wistar rats generally do not show increased active lever responding and self-infusions after ethanol or cocaine access is restored (Rodd et al., 2004a,b; Rodd-Henricks et al., 2002a, 2003). Supporting this idea, animals with high novelty seeking and sensitivity to drugs of abuse (HR) showed an 'up-ward' shift of the dose-response curve of IVSA cocaine compared to low novelty-seeking (LR) animals which was associated with an increased maximal response to cocaine (Piazza et al., 2000). Rats selectively bred for alcohol preference, compared to alcohol-non-preferring and outbred rat strains, displayed an increased sensitivity to the reinforcing and neurochemical effects of cocaine (Honkanen et al., 1999; Katner et al., 2011; Le et al., 2006; Leggio et al.,

2003; Marttila et al., 2007; Mikkola et al., 2001; 2002). Thus, selective breeding for alcohol preference potentially produced a phenotype associated with a more general preference for drugs of abuse. Alcohol-preferring rats are innately different from non-preferring and outbred rat strains when examining basal neurotransmitter levels, receptor expression, and pharmacological responsiveness in limbic brain regions (Bell et al., 2006a; McBride and Li, 1998; Murphy et al., 2002).

### Effects of Continuous Ethanol Experience

Following continuous, ethanol-drinking experience, P rats displayed an increased sensitivity to the reinforcing effects of cocaine within the AcbSh relative to their ethanol-naïve counterparts. The observation of leftward shift in the dose-response curve for active lever responding and infusions during the acquisition of cocaine ICSA under a FR1 schedule supports this concept. The results of this study are congruent with previous work finding P rats with the same protocol of chronic continuous ethanol drinking displayed an increased sensitivity to the reinforcing effects of ethanol within the pVTA (Rodd et al., 2005b,c). CE animals reliably self-infusing a concentration of cocaine (0.5 mM) not self-infused by N animals (Figures 3 and 10), also supports the interpretation of an increased sensitivity to cocaine's reinforcing effects. Lastly, CE reliably self-infused 1.0 mM cocaine, whereas this concentration was only marginally reinforcing for N animals (Figure 3).

The enhanced sensitivity of the AcbSh to the reinforcing effects of cocaine in CE rats compared to N rats was not likely a result of the presence of appreciable ethanol levels within the brain or plasma, as access to the 15% ethanol solution was removed 4 h before the beginning of each ICSA session. Adult P rats receiving unlimited free-choice access to 10% ethanol drank 5-8 g/kg/d and showed peak blood ethanol levels of 60-120 mg% during the dark cycle, with an average content of 55 mg% at the end of the dark cycle (Bell et al.,

2006b; Murphy et al., 1986). Four h after the removal of ethanol access, increased peripheral and central ethanol levels resulting from consumption should have diminished to negligible levels (Engleman et al., 2008; Robinson et al., 2000; Yim et al., 2000). Indeed, removal of ethanol bottles 3 h before the beginning of microdialysis produced no detectable levels of ethanol within the VTA extracellular milieu during the first baseline sample (Engleman et al., 2011). On the other hand, P rats potentially underwent acute withdrawal and were responding for lower concentrations of cocaine and self-administering more infusions to partially counteract the local withdrawal effects. This is unlikely though, as CE rats in the current study displayed higher overall levels of infusion only during reinstatement and not during acquisition. Nevertheless, P rats given 6 wk of unlimited access to 10% ethanol voluntarily consumed more than 5 g/kg/d and displayed signs of physical dependence (Kampov-Polevoy et al., 2000), but more robust signs of withdrawal was not observed until after 20 wk of ethanol drinking (Waller et al., 1982). Additionally, animals were habituated to the removal of ethanol access 4 h before the ICSEA experimental session, preventing or attenuating withdrawal symptoms by eliminating the unexpected removal of ethanol access.

Similar to the current study, outbred Wistar rats with high ethanol intakes were more sensitive to cocaine-induced locomotion and CPP than rats with low ethanol intake (Stromberg and Mackler, 2005). Mice receiving 7 d of repeated ethanol injections displayed a CPP for cocaine associated with enhanced synaptic plasticity of NMDA receptors on VTA DA neurons (Bernier et al., 2011). Furthermore, high levels of operant lever responding for oral ethanol predicted a more rapid acquisition of lever responding for IVSA cocaine compared to lower levels of ethanol responding (Mierzejewski et al., 2003). Repeated systemic ethanol treatments produced an enhanced locomotor response to a subsequent injection of cocaine, which was associated with a long-term enhancement of evoked DA and ACh release in the Acb (Nestby et al., 1997) and an increased number of striatal DAT binding sites (Itzhak and Martin, 1999). Additionally, local

application of cocaine produced a greater increase in Acb DA levels for animals with ethanol-drinking experience compared to naïve controls (Yoshimoto et al., 2000). Chronic ethanol exposure also abolished the subsequent conditioned taste aversion to cocaine seen in ethanol-naïve animals, suggesting ethanol exposure may reduce the aversive effects of subsequent cocaine exposure (Kunin et al., 1999; Grakalic and Riley, 2002).

Another important finding is the observation of more pronounced differences in active lever responding and self-infusions of CE rats compared to N rats after cocaine access was restored in Session 8. In the current study, CE rats responded more on the active lever for and obtained more infusions of 0.5, 1.0, 2.0, or 4.0 mM cocaine during Session 8 compared to N rats and compared to Session 4 (Figures 7, 10, 11). Previous studies observed similar effects of ethanol-drinking experience on subsequent ethanol self-infusion into the pVTA of P rats (Rodd et al., 2005b,c). The increased rates of responding and infusion during Session 8 in CE rats may be due to a tolerance and not a sensitization to the reinforcing effects of cocaine, requiring animals to infuse more cocaine to obtain the same effect. This is unlikely, as a decrease in the threshold concentration for self-infusion during acquisition and reinstatement accompanied the 'up-ward' shift seen during Session 8. Similar to (Rodd et al., 2005c), self-administration of the highest drug concentration did not produce higher levels of active lever responding or infusions during Session 8 for CE rats relative to the ethanol-naïve counterparts. This finding suggests against a 'right-shift' in the curve associated with rate tolerance (Zernig et al., 2004). CE rats displayed larger increases in active lever responding and infusions during Session 8 compared to N controls. Thus, chronic ethanol drinking exacerbated the effects a drug-free period on cocaine reinforcement, potentially related to a further dysregulation of neurotransmitter release, uptake, and postsynaptic action.

During sessions where aCSF was substituted for cocaine (Sessions 5-7), the 2.0 mM group of N rats and the 2.0 and 4.0 mM groups of CE rats displayed a diminished extinction of responding for cocaine relative to the other groups

(Figures 10 and 11). During Sessions 5 and 7, N rats originally receiving 2.0 mM cocaine increased responding on the inactive lever (Figure 11). Rats initially self-infusing an 'optimal' concentration of ethanol (125-150 mg%) also increased inactive lever responding during the first extinction session (Rodd et al., 2004a,b; 2005b,c). The higher inactive lever responding during extinction may reflect enhanced cocaine-seeking behavior resulting from a concentration of 2.0 mM producing the highest level of conditioned cue reactivity. In the current study, as with a number of other ICSEA experiments, a cue light above the active lever was illuminated during all of the sessions, regardless of drug availability. Indeed, discriminative stimuli predicting IVSA cocaine access are particularly resistant to extinction and can induce lever responding even after extended periods of abstinence (Weiss et al., 2001).

Putative mechanisms mediating the increased sensitivity of the AcbSh to the reinforcing effects of cocaine produced by long-term ethanol exposure are unknown, but research suggests a role for alterations in the function of monoamine transporters and a number of neurotransmitter receptors. For example, long-term (e.g. at least 8 wk) continuous free-access ethanol drinking by animals selectively bred for high ethanol consumption produced a general up-regulation in basal and evoked dopaminergic neurotransmission within the Acb. This was indicated by: increased basal DA levels (Thielen et al., 2004), decreased D<sub>2</sub> autoreceptor function (Engleman et al., 2003), accelerated basal DA uptake (Carroll et al., 2006), and increased responsiveness to DAT-inhibited DA increases (Engleman et al., 2000), without alterations in the number of DA terminals (Zhou et al., 2006). The most evident substrate for changes in cocaine reinforcement within the AcbSh is the DAT. While cocaine binds to NET, SERT, and DAT (Ravna et al., 2009; Reith et al., 1986; Ritz et al., 1990), the potency for cocaine reinforcement is most strongly correlated with the affinity at the DAT (Ritz et al., 1987). On the other hand, studies with genetic knockout animals suggest both SERT and DAT play an important role in cocaine reinforcement (Filip et al., 2005; Hall et al., 2004; Sora et al., 2001; but see Chen et al., 2006).

Because kinetic and binding experiments were not performed in the current study, it is unclear if the potential up-regulation of cocaine-mediated DAT inhibition after ethanol exposure would result from an increased number of DAT binding sites for cocaine, an increased efficacy of cocaine to inhibit DAT, or an increased affinity of DAT for cocaine. For example, the up-regulation of Acb DA uptake inhibition by a selective DAT inhibitor after ethanol drinking (Engleman et al., 2000) is associated with a down-regulation of Acb D<sub>2</sub> autoreceptors, but not an increased level of DA clearance under basal conditions (Engleman et al., 2003; Thielen et al., 2004). Changes in DA uptake are likely a compensatory response to increased dopaminergic signaling as repeated ethanol exposure produces the accumulation of DA in the extracellular area (Grace, 2000; Thielen et al., 2004), and acute ethanol did not alter DA uptake in the Acb (Budygin et al., 2001; Jones et al., 2006). On the other hand, physiologically relevant concentrations of ethanol directly affected DAT function (Mayfield et al., 2001; Zahniser et al., 1999). Changes in DA uptake also potentially resulted from alterations in presynaptic D<sub>2</sub> autoreceptor function, however the specific mechanisms remain unclear (Mayfield and Zahniser, 2001; Schmitz et al., 2002).

Increases in cocaine reinforcement could also be promoted by an ethanol-induced enhanced sensitivity of postsynaptic D<sub>1</sub> receptors and subsensitivity of presynaptic D<sub>2</sub> autoreceptors within the AcbSh. Chronic ethanol drinking (free access or liquid diet) by outbred rats produced an up-regulation of Acb D<sub>1</sub> receptors (Bailey et al., 2001; May, 1992). However, both studies showed no alterations in the number of D<sub>1</sub> receptor binding sites, analogous to findings with chronic stimulant treatment (White and Kalivas, 1998). In addition to the *in vivo* microdialysis experiments using alcohol-preferring rats (Engleman et al., 2003; Thielen et al., 2004), binding studies using outbred rats also found evidence of D<sub>2</sub> receptor down-regulation within the Acb (Rommelspacher et al., 1992; Syvalahti et al., 1988). Moreover, human alcoholics displayed reduced D<sub>2</sub> binding in a number of brain regions (Volkow et al., 1996). Chronic ethanol drinking by inbred P rats produced intakes of 10 g/kg/d resulting in increased D<sub>1</sub> and D<sub>2</sub>

receptor density within rostral regions of the AcbSh and AcbC (Sari et al., 2006). Because D<sub>2</sub> receptors are located both pre- and postsynaptically within the Acb (Pickel et al., 2006; Sesack et al., 1994), *in vitro* changes are difficult to interpret. Lastly, ethanol-induced changes in AcbSh cocaine reinforcement may have resulted from an up-regulation of local D<sub>3</sub> receptors. Long-term (1 y) continuous ethanol drinking by both P and HAD rats produced increased striatal expression of D<sub>3</sub> receptors (Vengeliene et al., 2006), while D<sub>3</sub> receptor blockade within the AcbSh attenuated cocaine sensitization (Ramiro-Fuentes and Fernandez-Espejo, 2010).

The observed changes in cocaine reinforcement within the AcbSh are potentially partially mediated by an up-regulation of SERT function and down-regulation of 5-HT<sub>3</sub> receptor function within the AcbSh. Cocaine shows a higher affinity for SERT than DAT (Matecka et al., 1996; Ritz and Kuhar, 1989; Ritz et al., 1990) and is more potent at SERT- compared to DAT- and NET-inhibition within the Acb (Uchimura and North, 1990). Serotonin acts within the Acb at 5-HT<sub>3</sub> receptors to release DA locally (Campbell and McBride, 1995; Chen et al., 1991; Parsons and Justice, 1993; but see Campbell et al., 1995; Li et al., 1996). Ethanol positively modulates 5-HT<sub>3</sub> receptor function (Lovinger, 1999; Lovinger and Zhou, 1998; Sung et al., 2000), while cocaine binds to the 5-HT<sub>3</sub> receptor to inhibit ion conductance (Breitinger et al., 2001; Carta et al., 2003). The induction of cocaine sensitization resulted in a down-regulation of 5-HT<sub>3</sub> receptor immunoreactivity within the AcbSh, but not the AcbC (Ricci et al., 2004). Systemic injections of a 5-HT<sub>3</sub> receptor antagonist attenuated cocaine-induced CPP, locomotor activity, and Acb DA release (Kankaanpaa et al., 2002). Chronic ethanol treatment increased SERT mRNA within the Acb (Shibasaki et al., 2010), while P rats with chronic ethanol-drinking experience attenuated increases in Acb DA levels evoked by local 5-HT<sub>3</sub> receptor activation (Thielen et al., 2004; but see Yoshimoto et al., 1996).

Overall, the present results confirm the AcbSh as an important brain region mediating the acquisition of cocaine reinforcement and support the

hypothesis that a prolonged period of ethanol drinking increases the sensitivity to the reinforcing effects of cocaine within the AcbSh. Chronic ethanol drinking putatively produces neuronal alterations within the terminal region of mesoaccumbens brain circuitry, rendering an individual more sensitive to the reinforcing effects of cocaine and thus more susceptible to acquiring cocaine self-administration.

#### Persistent Alteration of Posterior Ventral Tegmental Area Dopamine Neurons to the Stimulating Effects of Cocaine Following Chronic Ethanol Drinking

The results of the experiment indicate: (1) administration of cocaine into the pVTA, but not the aVTA, increased extracellular DA levels in the AcbSh of adult P rats (Figures 16-19 and 21); (2) voluntarily consumption of ethanol under continuous conditions, compared to water access only, reduced the threshold dose of cocaine needed to increase DA levels, produced greater DA release at a moderate dose of cocaine, and resulted in decreased DA levels at high doses of cocaine (Figures 16-20); and (3) the altered response of pVTA DA neurons to cocaine was further augmented 30 d after termination of ethanol drinking (Figures 16-20). These results demonstrate a local activating effect of cocaine on pVTA DA neurons and support the hypothesis that chronic continuous ethanol-drinking experience induces persistent alterations in the sensitivity of pVTA DA neurons to the stimulating actions of cocaine.

#### Stimulating Effects of Cocaine on Posterior Ventral Tegmental Area Dopamine Neurons

The present study appears to be the first demonstrating transient increases in extracellular levels of DA within the AcbSh following pulsed microinjections of cocaine directly into the pVTA, at doses supporting local self-

administration (Rodd et al., 2005a). Injections of 0.5 or 1.0 mM (1.5 nmol or 3.0 nmol total) cocaine into the pVTA produced robust increases in AcbSh DA levels (160-170% baseline), while injections of 0.25 or 2.0 mM (0.75 nmol or 6.0 nmol) cocaine produced smaller and more transient increases in DA levels (130-160% baseline). The current results confirm previous research finding outbred Wistar rats reliably self-administered 0.5-2.0 mM, but not 0.25 or 4.0 mM, cocaine into the pVTA (Rodd et al., 2005a). Animals received 30 microinjections of cocaine over a 10-min period, however DA levels remained elevated for 20-60 minutes. A peripheral injection of a high dose of cocaine (15 mg/kg) has been shown to produce a similar time course and maximal effect in Acb DA levels (Kalivas and Duffy, 1991, 1993). The IC<sub>50</sub> of cocaine into the pVTA of P rats has not yet been examined, but the lack of significant DA-stimulating effect at 2.0 mM potentially resulted from P rats having a relatively greater sensitivity to cocaine within the pVTA compared to Wistar rats (e.g. leftward-shift in the dose-response curve). Supporting this, P rats were more sensitive to the reinforcing effects of cocaine or ethanol within the AcbSh and ethanol within the pVTA (Engleman et al., 2009; Katner et al., 2011; Rodd et al., 2004a). Concentrations greater than 2.0 mM were not examined in the current study, and it remains unclear if higher doses would produce decreases in Acb DA. This effect is plausible, given the putative mechanisms of cocaine within the VTA and the effects of pVTA cocaine following chronic continuous ethanol drinking.

Other experiments with intra-VTA infusion of cocaine also support the current findings regarding the stimulating effect of cocaine on pVTA neurons. For example, mice learned to self-infuse 0.6 or 3.0 mM cocaine into the pVTA (David et al., 2004). Moreover, daily bilateral intra-pVTA injections of 5-nmol cocaine for 4 d produced behavioral sensitization to a systemic cocaine challenge (Cornish and Kalivas, 2001). On the other hand, Initial *in vivo* studies suggested local effects of cocaine within the VTA partially and transiently inhibited most, but not all, DA neurons (Einhorn et al., 1988). However, these results were likely

complicated by chloral hydrate anesthesia inhibiting GABA neuronal activity (Lee et al., 2001; Steffensen et al., 2008).

In contrast to the pVTA, microinjections of cocaine into the aVTA did not alter DA levels in the AcbSh at any dose tested (Figure 21). Moreover, injections of cocaine into areas adjacent to the VTA also failed to alter DA levels in the AcbSh (Figure 22). Thus, the observed effects after microinjection of cocaine into pVTA were not due to diffusion of cocaine into the aVTA or areas surrounding the VTA. Also supporting this finding, cocaine is not self-administered into the aVTA (Rodd et al., 2005a). Regional differences within the VTA with regard to afferent innervation, synaptic connectivity, and relative receptor location and function appear to produce the functionally heterogeneous effects. Activation of GABA<sub>A</sub> receptors in the aVTA, but not the pVTA, increased the reinforcing effects of IVSA cocaine on a progressive ratio (PR) schedule (Lee et al., 2007). DA neurons within the pVTA, relative to the aVTA, are under greater D<sub>2</sub>-mediated tonic inhibition while DA neurons within the aVTA appeared to be under greater GABA<sub>A</sub>-mediated tonic inhibition (Ding et al., 2009b). Lastly, receptors for ORX exist on DA neurons in the pVTA projecting to the medial PFC and AcbSh, but not on neurons in the aVTA innervating the AcbC (Vittoz et al., 2008).

The mechanisms through which microinjections of cocaine into the pVTA produce increases in extracellular DA within the AcbSh are not completely clear, but a number of recent studies elucidated putative substrates. Cocaine's actions as a non-specific monoamine reuptake inhibitor (Reith et al., 1986) likely mediate the direct effects of cocaine within the pVTA. Local application of cocaine produced significant increases in VTA DA, serotonin, and norepinephrine (Chen and Reith, 1994a,b). On the other hand, increased VTA DA neuronal activity due to direct interaction between cocaine and 5-HT<sub>3</sub> or nicotinic ACh receptors on DA neurons is unlikely, as cocaine binding inhibited conductance of these ion channels (Breitinger et al., 2001; Carta et al., 2003; Hess et al., 2000; Ulrich et al., 1998). At concentrations equivalent to the current study, cocaine showed a higher affinity for SERT than DAT and was more potent inhibiting serotonin

versus DA uptake (Matecka et al., 1996; Ritz et al., 1990; Uchimura and North, 1990). Cocaine-induced increases in VTA serotonin levels activated 5-HT<sub>2A</sub> receptors (McMahon and Cunningham, 2001) located on VTA DA neurons (Doherty and Pickel, 2000) to increase DA neuronal firing (Pessia et al., 1994; Prisco et al., 1994). Cocaine acted indirectly through serotonin to: activate 5-HT<sub>1B</sub> receptors (O'Dell and Parsons, 2004) located presynaptically on GABA terminals (Sari et al., 1999), decrease GABA release (Johnson and North, 1992), reduce GABA-B-receptor-evoked inhibitory postsynaptic potentials in DA neurons (Cameron and Williams, 1994), and ultimately increase DA neuronal firing (Guan and McBride, 1989; Yan and Yan, 2001).

The indirect mechanisms by which acutely administered cocaine into the pVTA produced transient increases in AcbSh DA may also include glutamatergic and noradrenergic influences. Cocaine-induced increases in VTA DA levels activate presynaptic D<sub>1</sub> receptors to increase local glutamate levels (Kalivas and Duffy, 1995). Furthermore, the application of cocaine to VTA DA neurons increased the magnitude of NMDA excitatory postsynaptic currents (Schilstrom et al., 2006), which leads to increase VTA DA neuron burst firing (Canavier and Landry, 2006). Supporting this idea, selective NMDA blockade within the VTA eliminated both the tonic and phasic cocaine-induced DA increases within the AcbSh (Aragona et al., 2008; Sombers et al., 2009). Cocaine-induced increases in VTA norepinephrine increased DA neuronal excitability (Grenhoff et al., 1995) via actions at alpha-1 adrenergic receptors located on a subset of VTA DA neurons (Bayer and Picker, 1990). At higher doses of cocaine, significant binding to DAT produces increased levels of DA in the VTA, leading to DA neuronal inhibition. In this case, DA neurons are inhibited by activation of D<sub>2</sub> autoreceptors located on DA perikarya and dendrites to directly inhibit DA firing (White and Wang, 1984). At much higher concentrations, cocaine reduced tonic inhibition of pVTA DA neurons by acting presynaptically to block voltage-sensitive sodium ion channels on local GABA neurons (Steffensen et al., 2008). However, the doses of cocaine used in the current experiment correspond to concentrations much

lower than concentrations used in Steffensen et al. (2008). The relative contribution of afferent inputs, including the magnitude and temporal pattern of direct and indirect feedback/feedforward loops from the Acb to the VTA (Sesack and Grace, 2010; Xia et al., 2011), also affected the profile of AcbSh DA after pVTA cocaine injection.

### Effects of Continuous Ethanol Experience

Following continuous, free-choice ethanol-drinking experience, P rats displayed an augmentation in the sensitivity of pVTA DA neurons to the stimulating actions of cocaine relative to their ethanol-naïve counterparts. Chronic ethanol-drinking experience reduced the threshold dose of cocaine needed to increase DA levels, produced greater DA release at a moderate dose of cocaine, and resulted in decreased DA levels at high doses of cocaine. Generally, the data represent a leftward shift in the dose-response curve (Figure 20), although DA neuronal inhibition at concentrations higher than 2.0 mM cocaine was not confirmed in ethanol-naïve animals. Injections of 0.25 or 0.5 mM cocaine into the pVTA produced robust increases in AcbSh DA levels (200 and 320% baseline, respectively), while injections of 1.0 or 2.0 mM cocaine produced robust decreases in DA levels (70-75% baseline). The observed increase in maximal response to 0.5 mM cocaine suggests a sensitization to the DA-neuronal-stimulating effects because higher cocaine concentrations produced a decreased maximal response. Unfortunately, corroborating data from an ICSCA study examining the effects of chronic continuous ethanol drinking on the self-administration of cocaine into the pVTA is not available, as the experiments are ongoing. However, preliminary results suggest a similar finding as the current microinjection-microdialysis study (unpublished observations).

Moreover, the results are in agreement with a number of previous findings. First, chronic continuous ethanol drinking produced functional alterations in P rats, resulting in an increased sensitivity to the reinforcing effects of intra-AcbSh

cocaine (current study). Second, ethanol-experienced (3 g/kg/d intake), but not ethanol-naïve, Wistar rats displayed increased levels of DA in AcbSh after pVTA injections of 100 mg% ethanol (Rodd et al., 2006a). Lastly, P rats receiving the same protocol of chronic ethanol drinking as the current experiment displayed an increased sensitivity to the reinforcing effects of ethanol within the pVTA (Rodd et al., 2005b), as evidenced by an upward and leftward shift in the dose-response curve (Zernig et al., 2004). The enhanced sensitivity of the pVTA to the stimulating and inhibitory effects of cocaine in CE rats compared to N rats was not likely a result of the presence of appreciable ethanol levels within the brain or plasma, as access to the 15% ethanol solution was removed 4 h before the beginning of the microdialysis experiment.

Similar to experiments examining DA functioning in the Acb, P rats with long-term, voluntary ethanol-drinking experience exhibited an increased number of spontaneously active VTA DA neurons (Morzorati et al., 2010). Moreover, chronic ethanol drinking produced decreased pVTA DA levels along with increased DA clearance (Engleman et al., 2011). Divergent findings from other research underscore the importance of factors such as genetic background and voluntary drinking. Repeated ethanol injections (which often produce dependency) in outbred animals produced a hypodopaminergic state, including fewer spontaneous active VTA DA neurons (Shen et al., 2007), decreased firing rates (Diana et al., 1992, 1995, 1996), and decreased Acb DA levels (Diana et al., 2003; Rossetti et al., 1992; Weiss et al., 1996).

At low to moderate doses, cocaine injections into the pVTA produced increased levels of DA neuronal activity in ethanol-drinking animals compared to ethanol-naïve animals. Increased cocaine-evoked neuronal activity potentially resulted from an increased excitatory tone, decreased inhibitory tone, or alterations in serotonergic function. Chronic ethanol drinking or a single ethanol injection (Saal et al., 2003; Stuber et al., 2008) induced LTP of AMPA functioning in the VTA by enhancement of glutamatergic afferent input through the stimulation of the D<sub>1</sub> receptor (Deng et al., 2009; Xiao et al., 2009). AMPA LTP

enhanced tonic DA levels within terminal regions (Grace et al., 2007), and strengthened activity-dependent synaptic plasticity (Wolf et al., 2004). In a complementary manner, repeated ethanol injections induced NMDA LTP (Bernier et al., 2011), increasing the probability of DA neuronal burst firing.

Ethanol induction of NMDA and AMPA LTP is consistent with the increased expression of the NMDAR<sub>1</sub> and GluR<sub>1</sub> ionotropic glutamate receptor subunits within the VTA after chronic ethanol drinking (Ortiz et al., 1995). Moreover, pVTA DA neurons were more sensitive to *in vivo* NMDA-elicited burst firing in P rats with 8 wk of continuous ethanol drinking than in ethanol-naïve controls (Fitzgerald and Morzorati, 2010). Acute ethanol treatment impaired LTP induction at GABA synapses onto VTA DA neurons (Guan and Ye, 2010; but see Melis et al., 2002), and GABA inhibition can potently suppress NMDA-induced burst firing (Lobb et al., 2010). Overall, ethanol drinking potentially produced an enhancement of NMDA LTP and an impairment of GABA LTP promoting robust neuronal excitability of VTA DA neurons during cocaine treatment. Chronic ethanol drinking could also increase the stimulating effects of lower concentrations of cocaine via an up-regulation of cocaine-evoked serotonin uptake inhibition. Ethanol-induced adaptations in VTA SERT function are tenable because repeated ethanol treatment increased transporter expression in the Acb, cortex, and hippocampus (Shibasaki et al., 2010).

At high doses, cocaine injections into the pVTA produced decreased levels of DA neuronal activity in ethanol-drinking animals compared to both baseline levels and ethanol-naïve animals. Decreased cocaine-evoked neuronal activity potentially resulted from an up-regulation of: cocaine-evoked DAT inhibition, D<sub>2</sub> autoreceptor function, or GABAergic inhibition. For example, ethanol-induced alterations in DAT functioning within the VTA could increase the effectiveness of cocaine to inhibit the somatodendritic uptake of DA in a manner similar to the observed effect in the Acb (Yoshimoto et al., 2000). Supporting this concept, voluntary ethanol drinking by Wistar rats resulted in increased DAT binding within the VTA (Jiao et al., 2006). Repeated ethanol injections produced

a supersensitivity of D<sub>2</sub> autoreceptors on VTA DA neurons to inhibit neuronal firing (Perra et al., 2011). This was observed as an enhancement in both the magnitude and duration of neuronal inhibition, which resulted from a down-regulation of the calcium-dependent 'desensitization-cascade' of inositol 1,4,5-triphosphate and calcium/calmodulin-dependent protein kinase II. While acute ethanol activates the cAMP-protein kinase A (PKA) cascade via actions at D<sub>1</sub> receptors and/or AC, chronic ethanol exposure produces a down-regulation of cAMP-PKA activity and alters CREB functioning in a number of mesolimbic brain regions (Pandey, 2004; Ron and Jurd, 2005).

Genetic factors may affect ethanol-induced adaptations in D<sub>2</sub> receptor functioning within the VTA, as P rats displayed 25% less D<sub>2</sub> receptor binding in this region compared to NP rats (McBride et al., 1993). Increased inhibitory tone could also contribute to the observed decrease in cocaine-evoked neuronal activity, as chronic ethanol treatment increased VTA GABA neuronal firing and GABA release onto VTA DA neurons (Gallegos et al., 1999; Melis et al., 2002). Decreased GABA<sub>A</sub>-inhibition potentially contributed to increased VTA GABA neuronal activity, as chronic ethanol drinking produced decreased expression of the VTA GABA<sub>A</sub> alpha-1 receptor subunit (Charlton et al., 1997; Ortiz et al., 1995; Papadeas et al., 2001) located only on GABA, and not DA, VTA neurons (Tan et al., 2010).

Ethanol-induced alterations in the neuronal stimulating profile of cocaine within the pVTA may also result in part from changes in local neuropeptide functioning. When released within the VTA, CRF and ORX peptides impart modulatory effects on drug-related behaviors through plasticity at NMDA receptors (Bonci and Borgland, 2009; Shalev et al., 2010; Wise and Morales, 2010). Both CRF and ORX act within the VTA to increase DA neuronal firing and potentiate NMDA-excitation of DA neurons (Borgland et al., 2010). These actions ultimately increase DA release in terminal regions, such as the Acb. Intra-VTA injections of either ORX or CRF produce increases in local DA and glutamate levels and concomitantly trigger cocaine seeking (Wang et al., 2005, 2009). An

ethanol-induced up-regulation in type 1 and/or type 2 CRF receptor (CRF<sub>1</sub>, CRF<sub>2</sub>) functioning within the VTA could facilitate CRF potentiation of LTP, as chronic ethanol exposure produced increased expression of both receptor types and the CRF peptide in limbic brain regions (Heilig and Koob, 2007). Cocaine-induced alterations in ORX transmission within VTA were associated with local synaptic plasticity, behavioral sensitization, and reinforcement (Aston-Jones et al., 2009, 2010; Borgland et al., 2006; Espana et al., 2010, 2011). Thus, ethanol-induced increases in ORX afferent input to VTA DA neurons could reduce the threshold for cocaine activation of VTA DA neurons and/or promote cocaine-induced plasticity. Injections of an ORX antagonist into the VTA attenuated cue- and stress-induced reinstatement of ethanol seeking (Richards et al. 2008). Chronic ethanol drinking by alcohol preferring rats increased the area of hypothalamic ORX mRNA expression (Lawrence et al., 2006).

#### Effects of Ethanol Experience Followed by Forced Abstinence

Somewhat unexpectedly, P rats displayed an additional augmentation in the sensitivity of pVTA DA neurons to the stimulating actions of cocaine after 30 d of abstinence from chronic ethanol drinking, relative to their ethanol-naïve and continuous-ethanol-access counterparts. Compared to the continuous drinking condition, an additional period of protracted abstinence produced: an increased maximal response of DA release at a low dose of cocaine; no significant change (instead of a robust increase) in DA levels at moderate dose; and similar pattern of dopaminergic inhibition at the highest dose of cocaine (Figures 16, 17, 19). Generally, the data represent a leftward shift in the dose-response curve (Figure 20). Injections of 0.25 mM cocaine into the pVTA produced a robust increase in AcbSh DA levels (235% baseline), while injections of 0.5 or 2.0 mM cocaine produced no change or robust decreases in DA levels, respectively (60-80% baseline). The observation of a more robust increase in AcbSh DA after pVTA injections at the lowest concentration of cocaine examined (0.25 mM) in CE

versus N animals supports the interpretation of an increased sensitivity to cocaine's DA-neuronal-stimulating effects after chronic ethanol drinking.

Unfortunately, a reduction of threshold dose of cocaine needed to produce increased AcbSh DA levels was not confirmed, as 0.25 mM was the lowest concentration of cocaine examined. Additional experiments are warranted to examine the effects of protracted abstinence on the activation pVTA DA neurons by very low concentrations (0.05-0.10 mM) of locally applied cocaine. The concentration of 1.0 mM cocaine was not tested in the abstinent group of rats. However, given the pattern of dopaminergic inhibition at 1.0 mM in CE animals and at 2.0 mM in CE and Ab animals, a similar inhibitory response is likely. To this effect, concentrations of 1.0 and 2.0 mM cocaine potentially produced a maximal physiological level of pVTA DA neuronal inhibition (i.e. floor-effect).

As with the previous experiment, accompanying data from an ICSA study examining the effects of protracted abstinence from chronic ethanol drinking on the self-administration of cocaine into the pVTA is not currently available. P rats trained for 6 wk to respond for 15% ethanol or water displayed increased lever responding for ethanol in either a FR or PR schedule after a 2- or 5-wk period of abstinence, suggesting an enhancement of reinforcer value during protracted abstinence (Rodd et al., 2003a). Furthermore, P rats receiving repeated cycles of ethanol (15% v/v) access and deprivation followed by a 7-wk period of abstinence displayed an increased sensitivity to the reinforcing effects of ethanol within the pVTA compared to both ethanol-naïve and continuous-ethanol-drinking animals (Rodd et al., 2005c). Along with the current experiments, this suggests P rats given 12 wk of access to 15% v/v ethanol followed by a 30-d period of abstinence would display an increased sensitivity to the reinforcing effects of cocaine within the AcbSh compared to both ethanol-naïve and continuous-ethanol-drinking animals. Moreover, ethanol-abstinent P rats should also display higher levels of responding, compared to both ethanol-naïve and continuous-ethanol-drinking animals, for the infusion of optimal concentrations of cocaine into the AcbSh during the reinstatement session. Future studies examining the

effects of abstinence duration and ethanol re-exposure cycles on the reinforcing and DA-neuronal-stimulating effects of cocaine within the AcbSh and pVTA could significantly contribute to understanding the how the observed neurochemical effects of cocaine in the mesolimbic DA system are associated with the acquisition of reinforced behavior.

The enhancement of sensitivity of the pVTA to the DA neuronal effects of cocaine observed after abstinence from ethanol drinking likely reflects a further progression of neuroadaptive processes occurring during and after withdrawal from ethanol (Koob and Volkow, 2010). These progressive adaptations are manifest as an enhanced dysregulation of basal neurotransmission, receptors, neuromodulator function, and intracellular processes. Increased basal DA levels in the Acb of P rats persisted for at least a 2-wk period after termination of ethanol drinking (Thielen et al., 2004). Ethanol-induced changes in D<sub>1</sub> binding in the Acb was observed as far as seven months after the conclusion of ethanol treatment (May, 1992), while alterations in VTA DA neuronal firing persisted through at least a 6-wk period of abstinence (Shen et al., 2007).

Potential mechanisms for the effects observed in the current experiment involve the enhancement of CRF and ORX functioning during protracted abstinence. A significant role for CRF's actions within the VTA to mediate drug seeking and relapse at time points far into protracted abstinence has been well characterized (see Sarnyai et al., 2001; Shalev et al., 2010). Wistar rats with 2 wk of voluntary ethanol drinking followed by 20 d of abstinence showed a relatively selective increase in hypothalamic expression of CRF<sub>1</sub>, CRF<sub>2</sub>, and ORX receptor type 1 genes (Pickering et al., 2007). Chronic ethanol drinking produced a transient decrease in CRF levels within the amygdala of Wistar rats, with levels returning to normal after 3 wk (Zorrilla et al., 2001). Interestingly, CRF levels were significantly higher in ethanol-exposed animals after a 6-wk period of protracted abstinence, compared to ethanol-naïve controls. Thus, gradual increases in VTA CRF functioning occurring after acute ethanol withdrawal and during protracted abstinence could provide the enhanced ability of cocaine to

activate VTA DA neurons. Abstinence could also produce the observed effects via an enhancement in ORX excitatory tone to DA neurons in the pVTA. Inbred P rats displayed an increased Fos expression in a number of brain regions during the immediate cue-induced reinstatement from oral ethanol reinforcement, while pretreatment with a ORX antagonist eliminated reinstatement responding and blunted Fos expression (Jupp et al., 2011). Moreover, delaying reinstatement for five months potentiated the Fos response compared to immediate reinstatement, and ORX antagonism retained the ability to eliminate reinstatement and attenuate Fos expression.

Overall, the results confirm the pVTA as an important brain region mediating the neurochemical effects of cocaine-evoked increases in DA levels in mesocorticolimbic terminal regions, such as the AcbSh. The results also support the hypothesis that a prolonged period of ethanol drinking increases the sensitivity of the pVTA to the DA-neuronal-stimulating effects of cocaine. The inhibition of pVTA neurons in ethanol-naïve animals after injections of concentrations greater than 2.0 mM cocaine would suggest ethanol-drinking experience produced a global shift in the local actions of cocaine within the pVTA (i.e. increased sensitivity to the stimulating and inhibitory effects of pVTA cocaine). Furthermore, a period of protracted abstinence enhanced the effects of ethanol drinking, in contrast to the hypothesis that the effects of continuous ethanol drinking on the DA-neuronal-stimulating actions of intra-pVTA cocaine would endure for 30 d. For abstinent animals, the confirming pVTA injections of cocaine concentrations less than 0.25 mM elicit significant increases in AcbSh DA not seen in continuous ethanol drinking rats (i.e. observation of a leftward shift of the dose-response curve) will increase support for the current findings. In summary, the results point to the occurrence of ongoing changes in neuronal functioning beyond acute withdrawal from chronic ethanol drinking and into periods of protracted abstinence.

### Integrated Discussion

Adult P rats will voluntarily consume significant amounts of ethanol if given continuous free-choice access, and this ethanol-drinking experience increased the sensitivity to the local reinforcing and stimulatory effects of cocaine within the mesoaccumbens DA circuit. The consequences of continuous ethanol-drinking experience were manifest as alterations to the behavioral and neurochemical profile of the central actions of cocaine within brain regions containing mesocorticolimbic DA cell bodies (pVTA) or terminals (AcbSh).

Confirmation of similar ethanol-induced alterations in the complementary behavioral or neurochemical dose-response to cocaine within the same brain loci will increase support for the current findings. In the AcbSh, data was obtained for the behavioral response to local self-infusion to cocaine, but an assessment of the associated neurochemical changes is warranted. This is accomplished by the concurrent local application of cocaine via perfusion into the AcbSh (i.e. reverse-microdialysis) and collection of brain dialysates for DA measurement through a single in vivo microdialysis probe (for description, see Andrews and Lucki, 2001; Engleman et al., 2003). Additional experiments using quantitative methods can obtain information correlated with DA uptake (for description, see Chefer and Shippenberg, 2002; Yim and Gonzales, 2000). In the pVTA, data was obtained for the 'down-stream' neurochemical response to experimenter-administered cocaine, but an assessment of the associated behavioral changes is not complete. Dose-response experiments examining self-infusion of cocaine into the pVTA are crucial for a comprehensive interpretation of the effects of chronic continuous ethanol drinking and subsequent ethanol abstinence on the reinforcing and DA-neuronal-stimulating effects within the pVTA.

In agreement with other ICSA, intracranial place conditioning, and microinjection-microdialysis studies examining the neurocircuitry of drugs of abuse, the effects of cocaine were localized within the pVTA, as opposed to the aVTA or adjacent midbrain regions. Also of interest is the site-specific nature and

administration contingency of ethanol-induced alterations in the central reinforcing and DA-neuronal-stimulating effects of cocaine. For example, repeated daily ethanol or nicotine pretreatment injections directly into the pVTA were sufficient to induce a neurochemical sensitization to the DA-neuronal-stimulating effects of ethanol (Ding et al., 2009a, 2011). In this case, the drug pre-exposure was both site-specific and non-contingent. Experiments examining the relative magnitude and time course of such effects will be useful to characterize the specific influence of these variables. For example, does local pretreatment of the pVTA with repeated microinjections of ethanol enhance the subsequent ICSCA of cocaine into the pVTA, and do these effects persist to the same degree as an effect induced by voluntary ethanol drinking? Lastly, experiments examining the effects of the pattern and time course of drinking behavior and abstinence periods will also provide additional insight into the development of ethanol-induced neuronal adaptations associated with the enhancement of the reinforcing effects of cocaine within the MVM DA circuit. For example, limited access schedules of alcohol availability, repeated cycles of ethanol access and abstinence, and periods of protracted abstinence beyond 30 d would mimic the human behaviors associated with binge drinking and relapse.

### Conclusions

In summary, chronic continuous voluntary ethanol drinking by female P rats enhanced both the reinforcing effects of cocaine within the AcbSh and the stimulatory and inhibitory effects of cocaine on pVTA DA neurons. Alterations in the stimulatory and inhibitory effects of cocaine on pVTA DA neurons were not only enduring, but also enhanced, following a period of protracted abstinence from ethanol exposure. Thus, voluntary ethanol-drinking experience, by animals with a genetic predisposition for high ethanol intake, altered the site-specific actions of cocaine within the mesolimbic regions of the CNS. In human populations, moderate to high levels of alcohol drinking potentially produces

neuroplastic alterations within the brain mediating an increased susceptibility to the positive reinforcing effects of cocaine often observed during early drug experiences. Functional alterations in MVM DA circuits during the acquisition of cocaine self-administration suggest a potential increased vulnerability may be associated with the earlier stages of compulsive drug taking behaviors. Therefore, prevention of chronic and excessive alcohol intake in populations with a genetic predisposition for substance abuse could reduce the proclivity to develop maladaptive patterns of cocaine use. It is unknown, however, if the observed effects are specific to animals with a genetic preference for ethanol. Also uncertain is whether other putative 'gateway' drugs, such as nicotine and marijuana, share ethanol's mechanisms of action or produce neuronal alterations through distinct substrates. For example, repeated nicotine exposure enhanced cocaine-evoked synaptic plasticity, behavioral sensitization, and reinforcement via increased histone acetylation (Levine et al., 2011), a post-translational adaptation also observed after chronic ethanol exposure (McBride et al., 2009; Pascual et al., 2009). Thus, treatments targeting common putative substrates and addressing both direct drug effects and the sequential progression of polydrug use may be more effective at preventing drug and alcohol misuse than individual treatments.

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- Zweifel LS, Argilli E, Bonci A, Palmiter RD (2008) Role of NMDA receptors in dopamine neurons for plasticity and addictive behaviors. *Neuron* 59:486-496.

VITA

## VITA

SCOTT M. OSTER

## EDUCATION

Doctor of Philosophy. Psychobiology of Addictions, Psychology, Purdue University. May 2012.

Dissertation: Chronic ethanol drinking by alcohol-preferring rats increases the sensitivity of the mesolimbic dopamine system to the reinforcing and stimulating effects of cocaine.

Master of Science. Psychobiology of Addictions, Psychology, Purdue University. May 2007.

Thesis: Examining the role of GABA-A receptors within the ventral tegmental area of Wistar rats on reward mechanisms and the intracranial self-administration of ethanol.

Bachelor of Science with Distinction. Chemical Engineering. Minor in Psychology, Purdue University. May 2003.

## RESEARCH SKILLS

Intracranial self-administration methods.

Microdialysis methods.

Microinjection methods.

Operant self-administration methods.

Intravenous self-administration methods.

Rodent stereotaxic surgery methods: intracranial cannula implantation.

High performance liquid chromatography (HPLC): equipment use, calibration, and maintenance.

Electrolytic microinfusion transducer (EMIT): equipment construction, validation, methodology development, calibration, and maintenance.

Coulbourn Instruments Habitest operant systems and Graphic State Notation interface: equipment construction, system validation, and software program protocol development.

## RESEARCH EXPERIENCE

Graduate Research Assistant, Indiana University Purdue University Indianapolis. August 2003 to August 2011.

Experimental design, data collection and analysis, and methodology development using operant, microdialysis, microinjection, intravenous self-administration, and intracranial self-administration methods to examine the neural circuitry of alcohol and drug reinforcement.

Advisors: Dr. James M. Murphy, Department of Psychology. Dr. William J. McBride, Department of Psychiatry. Dr. Zachary Rodd, Department of Psychiatry.

Research Assistant, Purdue University. January 2003 to May 2003.

Collected and analyzed data using ellipsometry to examine thin-film dynamics associated with lung surfactant replacement drugs.

Advisor: Dr. Elias Frances, Department of Chemical Engineering

Research Assistant, Purdue University. January 2002 to May 2002.

Compiled and analyzed data from the Minnesota Multiphasic Personality Inventory (MMPI) to validate psychometric systems used for the assessment of personality disorders.

Advisor: Dr. Rebecca Merritt, Department of Psychology

## ACADEMIC EXPERIENCE

Instructor, Department of Psychology, IUPUI. Fall 2007 and Spring 2008.

PSY B105: Introduction to Psychology as a Biological Science

Lab Instructor, Department of Psychology, IUPUI. Spring 2006.

PSY B307: Tests and Measurement

Teaching Assistant, Department of Psychology, IUPUI. Fall 2005.

PSY B305: Statistics

Tutor, Department of Chemistry, Purdue University. Spring and Fall 2002.

Chemistry Resource Room, focus on general and organic chemistry.

## MEMBERSHIP IN PROFESSIONAL SOCIETIES

Student Member of the Society for Neuroscience.

Student Member of Research Society on Alcoholism.

## PUBLICATIONS

Ding ZM, Oster SM, Hauser SR, Toalston JE, Bell RL, McBride WJ, Rodd ZA (2012) Synergistic self-administration of ethanol and cocaine directly into the posterior ventral tegmental area: involvement of serotonin-3 receptors. *J Pharmacol Exp Ther* 340:202-209.

Hauser SR, Getachew B, Oster SM, Dhaher R, Ding ZM, Bell RL, McBride WJ, Rodd ZA (2012) Nicotine modulates alcohol-seeking and relapse by alcohol-preferring (P) rats in a time-dependent manner. *Alcohol Clin Exp Res* 36:43-54.

- Katner SN, Oster SM, Ding ZM, Deehan GA Jr, Toalston JE, Hauser SR, McBride WJ, Rodd ZA (2011) Alcohol-preferring (P) rats are more sensitive than Wistar rats to the reinforcing effects of cocaine self-administered directly into the nucleus accumbens shell. *Pharmacol Biochem Behav* 99:688-695.
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- Rodd ZA, Gryszowka VE, Toalston JE, Oster SM, Ji D, Bell RL, McBride WJ. (2007) The reinforcing actions of a serotonin-3 receptor agonist within the ventral tegmental area: evidence for subregional and genetic differences, and involvement of dopamine neurons. *J Pharmacol Exp Ther* 321:1003-1012.

Oster SM, Toalston JE, Kuc KA, Pommer TJ, Murphy JM, Lumeng L, Bell RL, McBride WJ, Rodd ZA. (2006) Effects of multiple alcohol deprivations on operant ethanol self-administration by high-alcohol-drinking replicate rat lines. *Alcohol* 38:155-164.

#### INVITED TALKS

Oster SM, Toalston JE, Ding ZM, Deehan GA, McBride WJ, Rodd ZA. Salsolinol self-infusion into the posterior ventral tegmental area (VTA) is dependent upon dopamine activity. Invited talk for the 2007 Society for Neuroscience meeting, San Diego, CA.

#### SELECTED POSTERS AND PRESENTATIONS

Oster SM, Ding, ZM, Toalston JE, Engleman EA, Murphy JM, McBride WJ, Rodd ZA. Chronic ethanol drinking induces persistent alterations to the stimulatory effects of cocaine on posterior ventral tegmental neurons. Poster presentation at the 2011 Research Society on Alcoholism meeting. Atlanta, GA.

Oster SM, Pommer TJ, Toalston JE, Bell RL, McBride WJ, Rodd ZA. Operant oral EtOH self-administration with concurrent access to multiple EtOH concentrations in Alcohol-Preferring (P) rats: Evidence for drug escalation and excessive blood EtOH concentrations. Poster presentation at the 2010 Research Society on Alcoholism meeting. San Antonio, TX.

Oster SM, Ding, ZM, Toalston JE, Engleman EA, Murphy JM, McBride WJ, Rodd ZA. Alcohol drinking experience increases the sensitivity of posterior ventral tegmental area neurons to the stimulating effects of cocaine. Poster presentation at the 2009 Society for Neuroscience meeting, Chicago, IL.

Bracken AL, Oster SM, Toalston JE, McQueen VK, McBride WJ, Rodd ZA. Positive contrast and sustained ethanol self-administration in alcohol-preferring (P) rats given concurrent operant access to ethanol and sucrose. Poster presentation at the 2009 Research Society on Alcoholism meeting. San Diego, CA.

Toalston JE, Rodd ZA, Oster SM, Bell RL, Murphy JM, McBride WJ. Effects of ethanol drinking by alcohol-preferring (P) rats during peri-adolescence on subsequent ethanol intracranial self-administration in the ventral tegmental area during adulthood. Poster presentation at the 2009 Research Society on Alcoholism meeting. San Diego, CA.

Oster SM, Ding ZM, Toalston JE, Rodd ZA, Bell RL, Pommer TJ, Lumeng L, Murphy JM, McBride WJ. Alcohol drinking increases the sensitivity of the nucleus accumbens shell to the reinforcing effects of cocaine in alcohol-preferring (P) rats. Poster presentation at the 2008 Research Society on Alcoholism meeting, Washington, D.C.

- Oster SM, Ding ZM, Hauser SR, Pommer TJ, Bell RL, McBride WJ, Rodd ZA. Microinjection of atrial natriuretic peptide into the shell, but not core, of the nucleus accumbens reduces ethanol self-administration in alcohol-preferring (P) rats. Poster presentation at the 2008 Research Society on Alcoholism meeting, Washington, D.C.
- Hauser SR, Ding Z-M, Getachew B, Dhaher R, Toalston JE, Oster SM, McQueen VK, McBride WJ, Rodd ZA. Involvement of the posterior ventral tegmental area (p-VTA) in mediating alcohol-seeking behavior in alcohol-preferring (P) rats. Poster presentation at the 2008 Research Society on Alcoholism meeting, Washington, D.C.
- Oster SM, Toalston JE, Ding Z-M, Deehan GA, McBride WJ, Rodd ZA. Salsolinol self-infusion into the posterior ventral tegmental area (VTA) is dependent upon dopamine activity. Invited talk for the 2007 Society for Neuroscience meeting, San Diego, CA.
- Rodd ZA, Toalston JE, Ding ZM, Oster SM, Davids MR, Bell RL, Engleman, EA, McBride WJ. Ethanol-activation of ventral tegmental area (VTA) dopamine neurons is mediated by local serotonin-3 receptors. Poster presentation at the 2007 Society for Neuroscience meeting, San Diego, CA.
- Ding Z-M, Toalston JE, Oster SM, McBride WJ, Rodd ZA. Reinforcing effects of ethanol in the posterior ventral tegmental area (VTA) are not mediated by serotonin-1B receptors. Poster presentation at the 2007 Society for Neuroscience meeting, San Diego, CA.
- Oster SM, Pommer TJ, Toalston JE, Murphy JM, Bell RL, McBride WJ, Rodd ZA. Exposure to multiple concentrations of ethanol under operant oral self-administration conditions: Effects of repeated ethanol deprivations. Poster presentation at the 2007 Research Society on Alcoholism meeting, Chicago, IL.
- Toalston JE, Rodd ZA, Oster SM, Murphy JM, Bell RL, McBride WJ. Effects of saccharin or ethanol drinking by adult alcohol-preferring (P) rats during peri-adolescence on subsequent ethanol self-administration during adulthood. Poster presentation at the 2007 Research Society on Alcoholism meeting, Chicago, IL.
- Pommer TJ, Oster SM, Toalston JE, Murphy JM, Bell RL, McBride WJ, Rodd ZA. Introduction of a 'lock-out' during concurrent access to multiple ethanol concentrations: effects of repeated deprivations. Poster presentation at the 2007 Research Society on Alcoholism meeting, Chicago, IL.
- Bell RL, Rodd ZA, Schultz JA, Toalston JE, Oster SM, Murphy JM, Lumeng L, McBride WJ. An evaluation of the consummatory behavior of genetically selected alcohol-preferring rats during adolescence and adulthood. Poster presentation at the 2007 Research Society on Alcoholism meeting, Chicago, IL.

- Oster SM, Toalston JE, Murphy JM, Bell RL, McBride WJ, Rodd ZA. Concurrent self-infusion of cocaine and ethanol into the posterior ventral tegmental area of Wistar rats is blocked by co-infusion of 5-HT3 antagonists. Poster presentation at the 2006 Society for Neuroscience meeting, Atlanta, GA.
- Oster SM, Rodd ZA, Toalston JE, Pommer TJ, Li T-K, Bell RL, McBride WJ. Salsolinol is self-administered into the posterior ventral tegmental area (VTA) by Wistar rats. Poster presentation at the 2006 Research Society on Alcoholism meeting, Baltimore, MD.
- Rodd ZA, Toalston JE, Oster SM, Davids MR, Bell RL, Engleman EA, Murphy JM, McBride WJ. Dopamine release in the nucleus accumbens (Acb) by microinjections of ethanol into the posterior ventral tegmental area (VTA) is increased by chronic ethanol consumption in Wistar rats. Poster presentation at the 2006 Research Society on Alcoholism meeting, Baltimore, MD.
- Rodd ZA, Toalston JE, Oster SM, Davids MR, Bell RL, Engleman EA, Murphy JM, McBride WJ. Microinjections of acetaldehyde into the posterior ventral tegmental area (VTA) increase dopamine release in the nucleus accumbens, but not dorsal striatum. Poster presentation at the 2006 Research Society on Alcoholism meeting, Baltimore, MD.
- Toalston JE, Rodd ZA, Oster SM, Murphy JM, Bell RL, McBride WJ. Effects of peri-adolescent ethanol drinking on oral saccharin self-administration by adult Alcohol Preferring (P) rats. Poster presentation at the 2006 Research Society on Alcoholism meeting, Baltimore, MD.
- Oster SM, Rodd ZA, Toalston JE, Murphy JM, Bell RL, McBride WJ. Ethanol self-administration into the posterior ventral tegmental area (VTA) is not mediated by GABA-A receptors. Poster presentation at the 2005 Society for Neuroscience meeting, Washington, D.C.

## GRANTS

- F31 AA164292, Kirschstein National Research Service Award.  
Chronic drinking effects on cocaine self-administration.  
PI: 09/01/06 to 08/31/09.

## HONORS AND AWARDS

- Indianapolis Chapter of the Society for Neuroscience Poster Award, 2009.  
Paul J. McKinley Award for outstanding student in Psychobiology of Addictions Ph.D. program at IUPUI Department of Psychology, 2008.  
IUPUI School of Science Graduate Student Council Travel Award for attendance of Research Society on Alcoholism meeting 2006 and Society for Neuroscience meeting 2008.  
IUPUI Graduate Student Organization Educational Enhancement Grant for attendance of Research Society on Alcoholism meeting 2007 and 2008  
Research Society on Alcoholism Student Merit Award, 2006, 2007, and 2008.