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Mesocorticolimbic Adaptations in Synaptic Plasticity Underlie the Development of Alcohol Dependence

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MESOCORTICOLIMBIC ADAPTATIONS IN SYNAPTIC PLASTICITY UNDERLIE THE DEVELOPMENT OF ALCOHOL DEPENDENCE

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August, 2012

Dedication

This dissertation is dedicated to my amazing wife, Jacquelyn, and her smile.

Acknowledgements

I would like to acknowledge my dissertation committee members for their time, effort, and support of my doctoral candidacy:

Richard A. Morrisett, Ph.D.

Rueben A. Gonzales, Ph.D.

Christine L. Duvauchelle, Ph.D.

Hitoshi Morikawa, Ph.D.

R. Dayne Mayfield, Ph.D.

I would like to acknowledge my supervisor Dr. Richard A. Morrisett for his unwavering support and belief in my abilities as a graduate student despite occasional evidence to the contrary, his knowledge, and his patience throughout my graduate career.

I would like to acknowledge the current and former members of the Morrisett Lab for their extensive support and general agreeable dispositions that made my graduate career inordinately fun and memorable:

Armando Salinas, Esther Maier, Ph.D., Tavanna Porter M.S., Jonathan Theile, Ph.D., Rafael Renteria, Min Yang Ph.D., Douglas Lee, special mention to Tao Zhang Ph.D. who first introduced me to the world of electrophysiology.

I would like to acknowledge: the Staff of the College of Pharmacy for their immense help and support; the Waggoner Center for Alcohol and Addiction Research for supplemental financial support, and the NIAAA for funding me for the past two years

through my Ruth L. Kirschstein National Research Service Award for Individual Predoctoral Fellows, F31AA018941 (2010-2012).

I would also like to acknowledge my friends who have always provided much needed laughs and good conversation.

Last, but certainly not least, I would like to acknowledge my family who have always encouraged me to explore the depths of what I am capable of achieving (especially you, Mom) and continued to support and love me along the way.

Mesocorticolimbic Adaptations in Synaptic Plasticity Underlie the Development of Alcohol Dependence

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The University of Texas at Austin, 2012

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Synaptic alterations in the nucleus accumbens (NAc) are crucial for the aberrant reward-associated learning that forms the foundation of drug dependence. Glutamatergic synaptic plasticity in the NAc has been implicated in several behavioral responses to psychomotor stimulating agents, such as cocaine and amphetamine, yet no studies, at present, have investigated its modulation by ethanol. We demonstrated that both *in vitro* and *in vivo* ethanol treatment significantly disrupts normal synaptic functioning in medium spiny neurons (MSNs) of the NAc shell. Utilizing whole-cell voltage clamp recording techniques, synaptic conditioning (low frequency stimulation with concurrent postsynaptic depolarization) reliably depressed (NAc-LTD) AMPA-mediated excitatory postsynaptic currents (EPSCs). Acute ethanol exposure inhibited the depression of AMPA EPSCs differentially with increasing concentrations, but this inhibitory action of ethanol was reversed by a D1-like dopamine receptor agonist. When examined 24 hours following a single bout of *in vivo* chronic intermittent ethanol (CIE) vapor exposure, NAc-LTD was absent and instead synaptic potentiation (LTP) was reliably observed. We further investigated CIE-induced modulation of NAc-LTD by distinguishing between the two subpopulations of MSNs in the NAc, D1 receptor-expressing (D1+) and D2 receptor-expressing (D1-). We determined that NAc-LTD is expressed solely in D1+ but not D1-

MSNs. In addition, 24 hours following a repeated regimen of *in vivo* CIE exposure NAc-LTD is completely occluded in D1+ MSNs, while D1- MSNs are able to express LTD. Complete recovery of normal synaptic plasticity expression in both D1+ and D1- MSNs does not occur until two weeks of withdrawal from CIE vapor exposure. To our knowledge, this is the first demonstration of a reversal in the cell type-specificity of synaptic plasticity in the NAc shell, as well as, the gradual recovery of the pre-drug exposure plasticity state following extended withdrawal. This study suggests that NAc-LTD is cell type-specific and highly sensitive to both acute and chronic ethanol exposure. We believe these observations also highlight the adaptability of NAc MSNs to the effects of long-term ethanol exposure. A change in these synaptic processes may constitute a neural adaptation that contributes to the induction and/or expression of alcohol dependence.

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CHAPTER 1:

BACKGROUND

History of Alcohol Use

Originally proposed by Professor Robert Dudley of UC-Berkeley, the "drunken monkey hypothesis" proposes that the human attraction to alcohol may have a genetic basis due to the high reliance of early primates on fruit as a food source. He suggests that yeasts on fruit skin would convert fruit sugars to ethanol (alcohol), which was absorbed by the fruit producing an alcoholic smell identifying the fruit as being at peak ripeness and ready to consume (Dudley, 2004). Thus, natural selection would favor those of the closest ancestors of humans who could more precisely detect the smell and taste of alcohol, thereby helping them locate nutritious, perfectly ripe fruit more easily. The validity of this theory is not entirely as important as the history it attempts to explain. Alcohol has been one of the most commonly used intoxicating chemical substances by humans in history. The first historical evidence of alcoholic beverages came from an archeological discovery of Stone Age beer jugs from approximately 10,000 BC. References and descriptions regarding the use of alcoholic beverages are prevalent from almost all civilizations and time periods since 3000-2000 BC. Alcohol has traditionally been used for medicinal, antiseptic, analgesic, social, religious, or recreational purposes across different cultures throughout history. Even Hippocrates (ca. 460-370 BC) identified numerous medicinal properties of wine, including safe consumption in lieu of contaminated water, but was critical of drunkenness (Adams, 1894).

Yet, the dichotomous nature of alcohol contributed to inconsistent societal attitudes towards its consumption. Alcohol use or non-use was one of the central features of division within Hindu society. Throughout Asia, probably attributable to the tenets of Islam and Buddhism that required abstinence from alcohol, varying trends of alcohol use spread at the same time of increased prevalence of these religions (600 BC-700 AD). European culture, on the other hand, had well-established, broad-based social patterns of consumption of fermented alcoholic beverages (Hanson, 1995). Distillation of spirits began in the 15th century, and in conjunction with European colonization, a significant cultural component of alcohol use started to permeate throughout the world. However, the importance of moderation, either from secular or religious origins, is typically stressed during the same time periods where alcohol use is prevalent. In 12th-centruy Russia, a moving sermon by St. Basil the Great is titled, "On how it is seemly to refrain from drunkenness" (Keller, 1979). Egyptians warned against taverns and excessive drinking, and the New Testament recommended abstinence for those who could not control their drinking (Hanson, 1995). One reason alcohol use has persisted through our history is that it can serve an important role in enhancing the enjoyment and quality of life. Alcohol can act as a social lubricant, provide entertainment, facilitate relaxation, provide pharmacological pleasure, and enhance the flavors of food. The famous pharmacologist Louis Lewin described his views on why humans consume alcohol in his work, 'Phantastica' (Lewin, 1924), almost a century ago:

> …to tear the soul away from everyday life and direct it into another sphere where it is not confined within the walls of plain and customary monotony, or burdened with disagreeable and sad impressions but, on the contrary, where it attains gaiety, temporary happiness and even forgetfulness. This has always impelled man to the use of alcoholic beverages (Glatt, 1977).

Yet, excessive alcohol consumption has been and continues to be a major problem in our society. Thus, the dichotomous nature of alcohol and its use and misuse provides ample questions for scientific researchers in many disciplines to investigate.

Alcohol Dependence

DEFINITION

Drug addiction (defined as Substance Dependence by the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV: APA, 1994) of the American Psychiatric Association) is a disease recognized by the clinical community as a maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by a person exhibiting three (or more) of a specific set of symptoms within any 12-month period. These criteria help differentiate between substance abuse and dependence, where dependence requires at least three of the following: (1) tolerance- a need for increase drug amounts to achieve desired effect, (2) withdrawal- characteristic syndrome of the substance, (3) loss of control in limiting intake- substance taken in larger amounts and longer than intended, (4) compulsion to take the drug- persistent desire or unsuccessful efforts to control use, (5) compulsion to seek the drug- great amount of time devoted to obtain the substance (6) important social, occupational, or recreational activities reduced or given up because of substance use, (7) substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem likely to have been caused or exacerbated by the substance- continued use despite negative consequences.

Drug dependence is a chronically relapsing disorder that can result in the emergence of a negative emotional state (e.g., dysphoria, anxiety, irritability) reflecting a motivational withdrawal syndrome when access to the drug is prevented (Koob and Le Moal, 1997). The loss of control over limiting drug intake and the chronic, compulsive drug-seeking that characterize dependence is clinically distinct from occasional, but limited use of an abusable drug. Approximately 15.6% (29 million) of the U.S. adult population will go on to engage in nonmedical or illicit drug use at some time in their lives, with approximately 2.9% (5.4 million) going on to substance dependence on illicit drugs (Grant and Dawson, 1998; Grant et al., 2004). As of 2008, 51% (120 million) of people over the age of 12 were current alcohol users, and of these, 7.7% (18 million) met the DSM criteria for Substance Abuse or Dependence on Alcohol (Substance Abuse and Mental Health Services Administration, 2008). Although sometimes confusing, more common terms, such as addiction and alcoholism, are assumed to be identical to the clinical syndromes of substance dependence and substance dependence on alcohol. The broader term of addiction can be useful in describing how behaviors leading to alcohol abuse and dependence actually fall on a continuum rather than into specific categories (Saha et al., 2006). Perhaps simply having two diagnostic criteria can be misleading, but they do provide a more precise meaning to exceedingly complicated disorders.

Yet, it is important to mention that substance dependence is not synonymous with physical dependence–physical adaptations that result in largely somatic withdrawal symptoms when drugs such as alcohol, heroin, and benzodiazepines are abruptly discontinued. Adaptations associated with physical drug withdrawal are distinct from the motivational changes of acute withdrawal and protracted abstinence (Koob and Volkow, 2010). Excessive drug use does not absolutely lead to substance dependence. Drug dependence consists of two major components: (1) a genetic predisposition- whereby 40-

60% of the vulnerability to dependence can be attributed to genetic factors (Goldman et al., 2005; Hiroi and Agatsuma, 2005), and (2) the abuse component-whereby excessive drug use leads to dysfunctions in the structure and function of brain signaling systems that manifest themselves into the addicted behavioral state.

ECONOMICS AND SOCIETAL IMPACT

According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), over 550 million gallons of ethanol in the form of roughly 7.5 billion total gallons of beer, wine, and spirits were consumed in the U.S. in 2006. The Center for Disease Control (CDC) reported that excessive alcohol consumption is responsible for an average of 79,000 deaths and 2.3 million years of potential life lost in the U.S. each year (CDC, 2004), making it the third-leading cause of preventable death in the U.S. (Mokdad et al., 2004). Alcohol dependence is associated with multiple adverse health and social consequences, including liver cirrhosis, certain cancers, unintentional injuries, violence, and fetal alcohol spectrum disorder. In addition, excessive alcohol consumption causes premature death, increased healthcare costs, property damage from fire and motor vehicle crashes, increased crime and criminal justice system costs, and lost worker productivity in the form of missed work, diminished output, and reduced earnings potential. Comprehensive analysis conservatively estimated the total economic cost of excessive drinking to be $223.5 billion in 2006, which on a per capita basis is approximately $746 per person (Bouchery et al., 2011). That overall economic cost figure is an increase from $148 billion in 1992, and $184.6 billion in 1998 (Harwood et al., 1999). Certainly, the overall impact of excessive alcohol use on health and social well-being in our nation is only increasing, and evidence-based strategies for reducing excessive drinking should be widely implemented. Given the significant societal cost, research aimed at understanding

and treating alcohol dependence is essential, and the necessary resources must be made available to discover more effective therapies.

CURRENT TREATMENT

A cure for alcoholism has yet to be discovered; however, several medications have been developed that display at least some efficacy in promoting abstinence from ethanol consumption in humans. These pharmacological therapies fall into two main types of categories: aversive medications, which deter the patient from drinking, and anti-craving medications, which reduce the patient's desire to drink. Medications that cause an unpleasant (aversive) reaction when taken with alcohol have long been a mainstay of alcoholism treatment (Gunzerath et al., 2011). The most widely used of these, disulfiram, was approved for use by the FDA in 1949 and is a medication that disrupts the normal metabolism of alcohol in the body through inhibition of the enzyme, aldehyde dehydrogenase. Thus, with disulfiram in a person's blood, subsequent consumption of alcohol will lead to a build-up of acetaldehyde causing the person to become quite ill. The effectiveness of such a drinking deterrent is limited to highly motivated, fully compliant patients, or for short-term use when facing temporary high-risk situations (Chick et al., 1992). The anti-craving medications are more amenable to tailoring to typologies and comorbities. Nearly 50 years after the approval of disulfiram, NIAAA supported human studies of the anti-craving drug, naltrexone (Revia®, Depade®), formed the basis for the Food and Drug Administration's (FDA's) approval of the drug for the treatment of alcoholism in 1994 (O'Malley et al., 1992; Volpicelli et al., 1992). Naltrexone is believed to suppress the rewarding properties of alcohol through inhibition of mu opioid receptors, resulting in a dampening of the excitatory effects of alcohol on the mesolimbic system. Yet, the exact mechanism of naltrexone's efficacy is still under

investigation. In 2006, a more therapeutically useful long-acting depot preparation of naltrexone was approved by the FDA. In 2004, the FDA approved acamprosate, another drug aimed at reducing alcohol craving. Acamprosate is believed to be a partial agonist of *N*-methyl-D-aspartate (NMDA) glutamate receptors, yet its exact mechanism of therapeutic action is unclear and clinical results have been contradictory; with efficacy repeatedly found in European trials, but not in the U.S. trials (Anton et al., 2006; Mason and Crean, 2007). Currently, NIAAA is also funding exploration of new molecular targets, and testing of about 20 new compounds–including topiramate, an anticonvulsant that inhibits glutamate function, and ondansetron, a 5-HT3 receptor antagonist–is underway (Gunzerath et al., 2011). Topiramate has been shown to help severe alcoholism patients reach abstinence more quickly, while odansetron has been efficacious in the treatment of early-onset, but not late-onset, alcoholism (Johnson, 2008). Other medications currently under investigation, but not yet FDA approved, include the neurokinin-1 receptor antagonist, LY686017, which blunted or suppressed craving in recently detoxified inpatients (George et al., 2008), and varenicline, a partial nicotinic acetylcholine receptor agonist, which has been shown to reduce ethanol seeking and voluntary ethanol consumption in animal models (Steensland et al., 2007). Hopefully, with more directed research efforts and continuing financial support, more effective treatment options for alcoholism will be developed and made available to the public.

Mesocorticolimbic System and Reward

The neurotransmitter dopamine (DA) is released from neurons in the midbrain ventral tegmental area (VTA) that have widespread projections to regions known to be involved in reward processes and in guiding goal-directed behavior (Wise, 2004; Grace et

al., 2007; Ikemoto, 2007). One area of the brain that is particularly important as a region where many of these systems converge is the nucleus accumbens (NAc). The NAc has a central role in the integration of cortical afferent systems under the modulatory influence of DA. In turn, the NAc and many of its inputs are also involved in directly or indirectly regulating DA neuron activity states. By examining the afferent synaptic input to the NAc, its modulation by DA, and the regulation of VTA DA neurons, a functional circuit can be envisaged that illustrates the function of these two major structures in modulating behavioral responses that mediate reward acquisition (Sesack and Grace, 2010).

NUCLEUS ACCUMBENS

Anatomy and Connectivity

The NAc is part of the ventral striatal complex and serves as a critical region where motivations derived from limbic regions interface with motor control circuitry to regulate appropriate goal-directed behavior (Mogenson et al., 1980; Groenewegen et al., 1999; Nicola et al., 2000; Wise, 2004). The NAc shares similarities with other parts of the striatal complex as it receives extensive excitatory afferents from the cerebral cortex and thalamus, as well as, input from the hippocampus and basolateral amygdala. The completion of cortico-striato-pallidal-thalamocortical loops is achieved through the connection of the output from the NAc to the ventral pallidum (VP), which innervates the mediodorsal and other thalamic divisions which are known to participate in the initiation of voluntary movements (Swerdlow and Koob, 1987; Zahm and Brog, 1992; O'Donnell et al., 1997). The NAc also receives modulatory synaptic input from the brainstem, including DA and GABA projections from the medial substantia nigra zona compacta (SNc) and VTA (Ikemoto, 2007). The DA innervation is especially noteworthy as it

forms an essential component of reward circuitry and is recruited by both natural rewards and drugs of abuse (Koob, 1992; Wise, 2004). Serotonin and non-serotonin inputs from the dorsal raphe nucleus, as well as, a small norepinephrine projection from the locus coeruleus and nucleus of the solitary tract converge in the NAc (Swanson and Hartman, 1975; Brog et al., 1993). The major GABAergic projections from the NAc MSNs are to the VP, substantia nigra, VTA, hypothalamus, and brainstem (Haber et al., 1990; Usuda et al., 1998).

The NAc is divided into two primary subdivisions: the core is the central aspect surrounding the anterior commissure and located directly beneath and contiguous with the dorsal striatum, and the shell occupies the most ventral and medial portions of the NAc (Zahm and Brog, 1992). The NAc core and shell differ in their precise cellular morphology, neurochemistry, projection patterns, and functions (Heimer et al., 1991; Zahm and Brog, 1992; Meredith, 1999). The NAc core projects primarily to the dorsolateral portion of the VP, the entopeduncular nucleus, and the substatia nigra zona reticulata (SNr). The shell mainly innervates the ventromedial VP, lateral hypothalamic area, lateral preoptic area, SNc, VTA, and periaqueductal gray. Efferent projections from the NAc shell are thought to the VTA influence DA cells that in turn project to the NAc core, creating a medial to lateral series of spiraling projections that allow limbic associated structures to influence neurotransmission in successively more motor-related parts of the basal ganglia circuitry. Furthermore, a subset of outputs from the NAc can be viewed as functionally analogous to the direct and indirect pathways that are involved in behavioral activation and response inhibition (Alexander et al., 1990).

Cell Types and Electrophysiological Properties

Striatal characteristics are shared between the NAc core and shell, given that 90-95% of the cells are γ-aminobutyric acid (GABA)-ergic medium spiny neurons (MSNs), which comprise the primary output of the region (Meredith, 1999). The projecting axons give off local collaterals that contact dendritic trees of neighboring MSNs (Wilson and Groves, 1980). The remaining 5-10% of the neuron population comprises at least three main interneuron classes, the giant aspiny cholinergic neurons, the medium aspiny GABAergic neurons, and the neurons co-expressing nitric oxide, somatostatin, and neuropeptide Y (Tepper and Bolam, 2004). In vivo experiments have shown that MSNs show a pattern of spontaneous activity consisting of brief episodes of firing interspersed with extended periods of silence and exhibit membrane potential bistability, characterized by robust shifts between a hyperpolarized "DOWN" state (around -80 mV) and a depolarized "UP" state (around -60 mV) (O'Donnell and Grace, 1995). Transitions to the UP state in NAc neurons are correlated with population activity in the ventral hippocampus (Goto and O'Donnell, 2001a). An ensemble of NAc neurons are thought to simultaneously go in to the depolarized state whenever a situation arises that demands the attention of the animal, which could result from activation of attention-related DA pathways (Goto and O'Donnell, 2001b). Thus, electrophysiological properties of MSNs directly support the involvement of the NAc as an integration point of several afferent inputs.

Principal NAc MSNs are commonly divided into two major subsets based on their expression of releasable peptides, dopamine receptor expression , and their axonal projection targets (Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1990). Five G-protein coupled receptors (GPCRs) mediate DA signaling, and these receptors are

grouped into two classes on the basis of the G-protein to which they couple: D1 and D5 (D1-like) receptors stimulate G_s and G_{olf} proteins, which stimulate adenylyl cyclase leading to increased PKA activity, whereas D2, D3, and D4 (D2-like) receptors stimulate $G_{i/o}$ proteins which inhibit adenylyl cyclase causing decreased PKA activity (Neve et al., 2004). D1 and D2 receptors are, by far, the most abundant in the NAc, and MSNs are segregated based on their respective expression of either D1 (direct pathway MSNs) or D2 receptors (indirect pathway MSNs) (Alexander et al., 1986). Based on the coupling of DA receptors to specific G-proteins, DA acting on D2 receptors potently inhibits NAc neurons (O'Donnell and Grace, 1996), whereas, D1 receptor stimulation potentiates glutamatergic drive onto MSNs (Cepeda et al., 1998). Accordingly, experiments conducted using locally applied antagonists *in vivo* demonstrate that D2 antagonists increase NAc neuron firing, while D1 antagonists decrease cell excitability (West and Grace, 2002).

Given their opposing roles in modulating intracellular signaling pathways and their different outputs, researchers have begun investigating the functional differences in D1 and D2 receptor-expressing MSNs. To facilitate these efforts, bacterial artificial chromosome (BAC) transgenic mice in which expression of enhanced green fluorescent protein (eGFP) is controlled by the D1 or D2 receptor promoters (Gong et al., 2003) have been created and subsequently provided insights into specific synaptic characteristics of both MSN subtypes (Cepeda et al., 2008; Grueter et al., 2010). Thus, an experimenter can record electrophysiological activity from D1-eGFP expressing MSNs simply by identifying the fluorescing cells, which carry a high probability that if the eGFP is under control of the D1 promoter, then a fluorescing neuron will express D1 receptors (Matamales et al., 2009). Likewise, the non-fluorescing neurons are highly likely to

express D2 receptors, so both subtypes of MSNs can be analyzed using a single BAC transgenic mouse line.

Drug Reward

It is well-accepted that most drugs of abuse, including ethanol, activate the mesocorticolimbic dopamine system and lead to an increase in extracellular dopamine concentrations in the NAc (Imperato and Di Chiara, 1986; Weiss et al., 1993; Doyon et al., 2003). DA signals released by a given stimulus are thought to exert their influence on responses to subsequent stimuli that follow the initial triggering one. Release of DA in the NAc shell by unfamiliar and unpredicted primary appetitive rewards might serve to associate the sensory properties of the rewarding stimulus with its biological outcome. (Di Chiara et al., 2004). All drugs of abuse elicit, to different extents depending on the pharmacological class they belong to, an incentive arousal state as a result of their ability to increase extracellular DA in the NAc shell. This incentive arousal state facilitates the rate of current instrumental behavior, the acquisition and expression of secondary reinforcement and the reinstatement of previously extinguished instrumental responding as well as the consolidation of mnemonic traces of salient stimuli to be associated with affective states (Di Chiara et al., 2004). In short, DA in the NAc alerts the animal to the significance of a certain stimulus so that the appropriate associations can be made between the surroundings and behaviors that preceded the subsequent biological result of that stimulus. Repeated, non-contingent exposure to drugs of abuse is thought to cause maladaptive activation of DA transmission which could result in the aberrant motivational behaviors typical of addiction–compulsive perseverance on drugs and drug-related stimuli at the expense of more conventional non-drug rewards. Thus, rather than directly mediating the rewarding effects of natural and drug reinforcers, more recent

hypotheses focus on the role of mesolimbic dopamine as a motivational learning signal (Spanagel and Weiss, 1999), a signal of pathological associative learning in addiction (Di Chiara, 2002), a neural substrate of incentive salience (Robinson and Berridge, 2003), or a signal that informs about the predictability of reward related to cues associate with drug availability (Fiorillo et al., 2003).

Cortical neurons are the likely promoters of goal-directed behaviors, with the ventral subiculum of the hippocampus providing spatial and contextual information, the prefrontal cortex supplying executive control, including task switching and response inhibition, and the basolateral amygdala communicating information regarding conditioned associations as well as affective drive (Wolf, 2002; Kalivas et al., 2005; Ambroggi et al., 2008; Ito et al., 2008; Gruber et al., 2009). By the direct route, cortical activation of NAc neurons leads ultimately to disinhibition of appropriate action plans that facilitate reward acquisition. In contrast, cortical activation of the indirect circuit is likely to inhibit motor plans that are maladaptive, either for obtaining reward or for avoiding punishment (Mink, 1996; Redgrave et al., 1999). The complex interaction between these distinct circuits may function to optimize the behavioral response to rewards and conditioned associations.

The NAc shell subregion, in particular, has been associated with aspects of drug reward (Carlezon et al., 1995; Rodd-Henricks et al., 2002; Sellings and Clarke, 2003; Ikemoto, 2007). Evidence suggests that the transition to an addicted state follows adaptations first in the NAc shell, then NAc core, and finally the dorsal striatum. In non-human primates, the NAc shell and core subregions are organized in a series of parallel circuits linked in an ascending spiral to the dorsal striatum in a manner that could account for the transition from goal-directed to habitual behaviors during the development of addiction (Haber et al., 2000). Thus, investigations into the synaptic modifications that

underlie these neural circuit adaptations could significant advance our understanding of how these complex behaviors originate and are eventually expressed.

Synaptic Plasticity and Drug Dependence

Synaptic plasticity of neural circuits allows for reorganization of synapses in response to different stimuli. Long-term depression (LTD) and long-term potentiation (LTP) are the best characterized mechanisms for modulating synaptic strength in an experience-dependent manner– a long-lasting decrease or increase in synaptic strength, respectively. Both processes are thought to be involved in information storage and therefore in learning and memory and other physiological processes. The occurrence of long-lasting synaptic changes can reinforce some inputs, through LTP, or reduce others, when LTD is induced, while other inputs may maintain their original synaptic signal unaltered.

HISTORICAL BACKGROUND OF SYNAPTIC PLASTICITY

Synaptic plasticity has primarily been studied in the hippocampus ever since the first demonstration *in vivo* of a long-lasting enhancement of the excitatory transmission in the perforant pathway-dentate gyrus synapse, in response to high frequency afferent stimulation (Bliss and Lomo, 1973). This groundbreaking observation cemented the foundation for all future discoveries on synaptic plasticity in the vertebrate central nervous system. Yet, this discovery would not have proved as valuable had not an almost simultaneous methodological breakthrough occurred in the late 1960s. Yamamoto and McIlwain first pioneered the use of thin brain slices, maintained *in vitro* in an appropriate extracellular medium (Yamamoto and McIlwain, 1966a, b); however, it wasn't until over

a decade later that the utility of this technique was realized, when it was shown that hippocampal LTP could be studied *in vitro* on brain slices (Schwartzkroin and Wester, 1975; Andersen et al., 1977). Thus, the discovery of LTP in the hippocampus, paralleled by the development of electrophysiology from hippocampal slices, constituted a fortunate and highly prolific marriage for all future findings on the neurobiology of synaptic plasticity. It also bears mentioning that the invention of the patch clamp method of electrophysiology (Neher and Sakmann, 1976) further extended the experimental possibilities in investigating ion channel-related biological phenomena.

SYNAPTIC PLASTICITY MECHANISMS

Most synapses the undergo LTP and LTD use the excitatory amino acid, L-glutamate, as their neurotransmitter. L-glutamate acts on NMDA receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) receptors (AMPARs), kainite receptors, and metabotropic glutamate recptors (mGluRs) (Collingridge et al., 2009). AMPARs are the principal transducers of fast excitatory neurotransmission in the mammalian brain, and are targets for multiple signaling pathways that regulate the strength of glutamatergic excitatory synapses. NMDARs are widespread triggers for synaptic plasticity, but do not determine the direction of change in synaptic efficacy; thus activation of NMDARs can lead to LTP or LTD (Luscher and Frerking, 2001). Since their initial discovery, many forms of LTP and LTD have been described, which can be classified by their specific mechanisms of induction (i.e. which type of activity is required to evoke plasticity) and expression (i.e. which molecular changes mediate the change in synaptic efficacy). Some of the major forms of synaptic plasticity with a known relationship to drug exposure will now be discussed.

Long-term Potentiation

NMDAR-dependent LTP is induced by releasing glutamate (traditionally via brief (1-3 seconds) high frequency (100 Hz) afferent electrical tetanus stimulation) onto depolarized NMDARs (active after magnesium (Mg^{2+}) block has been relieved). This allows calcium (Ca^{2+}) to enter the postsynaptic terminal, which triggers an intracellular cascade of events (involving activation of Ca^{2+}-calmodulin kinase type II (CAMKII), eventually leading to an increase of AMPARs at the synapse, thus strengthening the excitatory transmission (Luscher and Frerking, 2001).

Long-term Depression

Mechanisms of Induction

NMDAR-dependent LTD is induced by a weak activation of NMDARs (traditionally longer (5-15 minutes) low frequency (1-3 Hz) afferent electrical stimulation) resulting in a small rise of Ca^{2+} in the postsynaptic terminal. Ca^{2+} that enters through NMDARs binds to calmodulin to activate protein phosphatase 2B (PP2B, also known as calcineurin), which dephosphorylates inhibitor-1 and this leads to the activation of protein phosphatase 1 (PP1) (Mulkey et al., 1993). PP1 then dephosphorylates its substrate(s), including serine845 on the AMPAR subunit GluA1, which results in the subsequent removal of these receptors from synapses via a clathrin- and dynamin-dependent process (Selig et al., 1995; Luscher et al., 1999). NMDARs are tetramers of various subunits (GluN subunits) (Collingridge et al., 2009), usually composed of two GluN1 subunits and two GluN2 subunits. The two GluN2 subunits can be identical (GluN2A, GluN2B, GluN2C, or GluN2D), forming a diheteromer, or they can be different from each other, forming a triheteromer with two identical GluN1 subunits (Ulbrich and Isacoff, 2008). Although some studies have suggested that NMDAR-LTD

involves the activation of specific NMDAR subtypes, it is increasingly likely that various NMDAR subtypes can trigger LTD and that the actual subtype(s) that are involved depend on various factors– induction protocol employed, expression levels in brain region, or environmental conditions.

A second major form of LTD is mGluR-dependent LTD. Any of the seven mGluR subtypes that are expressed in the brain could conceivably trigger LTD. The canonical signaling pathway of group I mGluRs involves G_q protein signaling, leading to the hydrolysis of phosphatidyl inositol to generate inositol triphosphate (IP3) and diacylglycerol (DAG), which in turn can activate PKC and release of Ca^{2+} from intracellular stores. This leads to a decrease in synaptic strength through a variety of distinct mechanisms.

Mechanisms of Expression

LTD is mediated by persistent pre- and postsynaptic changes, and the proportion of presynaptic compared with postsynaptic alterations probably depends on various factors, including the type of synapse and the developmental stage of the animal. There is evidence that both forms of LTD outlined here can involve a reduction in the probability of glutamate release (which would constitute presynaptic LTD). This decrease in neurotransmitter release could be triggered by changes in the presynaptic terminal or by postsynaptic changes that are communicated across the synapse via a retrograde messenger (Collingridge et al., 2010). Endocannabinoids, in particular, are known to function as retrograde messengers in the striatum (Gerdeman and Lovinger, 2003), neocortex (Nevian and Sakmann, 2006), and cerebellum (Qiu and Knopfel, 2009).

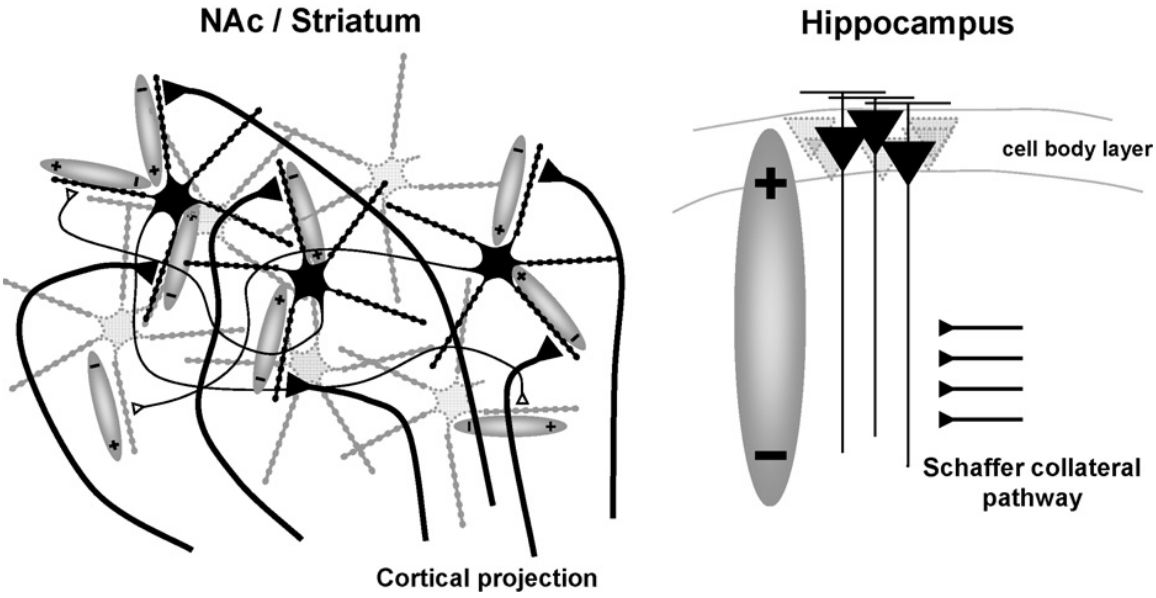
NMDAR-LTD is mainly a postsynaptic phenomenon, and most of the evidence points to the removal of AMPARs from the synapse as the primary mechanism through

which decreased sensitivity to glutamate in the postsynaptic neuron is achieved. AMPARs are stabilized on the cellular membrane by an interaction between *N*-ethylmaleimide-sensitive factor (NSF; an ATPase involved in membrane fusion events) and the GluA2 subunit; the initiation of AMPAR endocytosis occurs when the clathrin adaptor protein (AP2) replaces NSF during NMDAR-LTD (Collingridge et al., 2004). NMDAR-LTD is also associated with tyrosine phosphorylation of GluA2 and this suggests that protein tyrosine kinases (PTKs) are also involved. PTKs of the sarcoma (Src) family phosphorylate GluA2 at a tyrosine residue in a tyrosine-rich region of the Carboxy-terminal tail of GluA2 and this is thought to be required for AMPAR endocytosis. Consistent with this idea, a peptide that mimics this tyrosine-rich region (thus, acting as a competitive substrate for phosphorylation from Src PTKs) has been found to block NMDAR-LTD (Ahmadian et al., 2004). Postsynaptic receptor changes can also be involved in mGluR-LTD although the mechanisms of AMPAR trafficking differ from NMDAR-LTD.

SYNAPTIC PLASTICITY IN THE NUCLEUS ACCUMBENS

In general, using the knowledge obtained from hippocampal synaptic plasticity studies as a framework does not fully translate to the NAc because the intrinsic circuitry within the NAc contains a few more chemical, anatomical, and electrical peculiarities than the more organized hippocampus, which contains reliable sink-source electrical dipoles (Illustration 1). The GABAergic MSNs neurons of the NAc are not anatomically organized into distinct cell layers; furthermore, their dendritic arbor is not polarized and is fully covered by dendritic spines making excitatory and inhibitory synaptic contacts with extrinsic and intrinsic axons. Therefore, no clear electrical polarization exists at the level of a single MSN let alone of the whole neuronal population (Berretta et al., 2008).

Illustration 1. Different synaptic organization in the striatum/NAc and in the hippocampus.



MSNs of the NAc receive glutamatergic excitation to their dendritic arbor through cortical projection (thick, filled terminals). Their anatomical organization gives rise to several local electrical dipoles, which do not summate into a sing sink-source electrical field. Intrinsic GABAergic networks are also generated through synaptic contacts among MSNs (thin, hallowed terminals). In the hippocampus, glutamatergic afferents and cell bodies of target neurons are segregated into distinct layers, so that a single sing-source electrical dipole is generated between the two layers. Adapted with permission from *Berretta et al./Progress in Neurobiology 84 (2008) 343-362.*

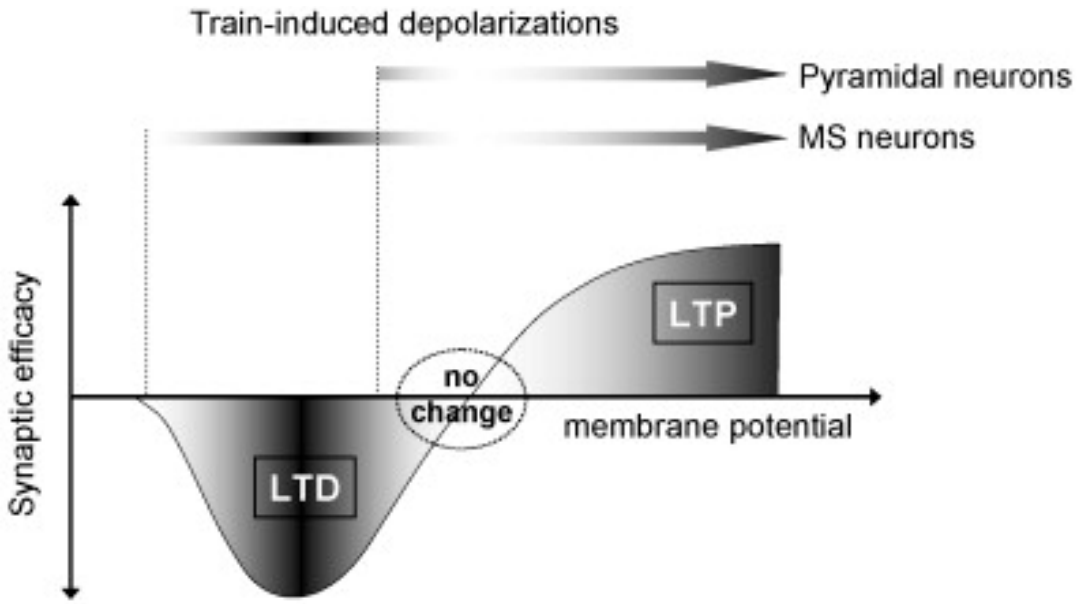
The form of synaptic plasticity that is more frequently reported in the NAc is LTD of AMPAR-mediated synaptic transmission, although LTP of glutamatergic synaptic transmission has also been induced using high frequency stimulation (Pennartz et al., 1993; Kombian and Malenka, 1994). When differing patterns of low frequency electrical stimulation are delivered to synapses on NAc MSNs, at least three forms of LTD,

triggered by different mechanisms of induction, have been demonstrated. The first form of LTD (referred to at 1 Hz-LTD) requires NMDAR activation, is induced via low frequency stimulation (500 stimuli at 1 Hz) paired with a postsynaptic depolarization to -50 mV, and is expressed through decreased postsynaptic AMPAR receptor expression, as described above for NMDAR-LTD (Thomas et al., 2000; Brebner et al., 2005; Jeanes et al., 2011). Two forms of presynaptic LTD, independent of NMDAR activation, have been demonstrated: one form requires endocannabinoid (eCB) signaling through postsynaptic group I mGluR activation and subsequent eCB release as a retrograde messenger (Robbe et al., 2002a); the other form is mediated by presynaptic mGlu2/3 receptors through inhibition of Ca^{2+} channels by the cyclic AMP/protein kinase A pathway (Robbe et al., 2002b). Each presynaptic form of LTD is expressed via decreased glutamate release and induced through the delivery of higher frequency (10-13 Hz) stimulation of afferent inputs than that used in 1 Hz-LTD.

Functional Implications

According to the Artola-Brocher-Singer rule (Illustration 2), induction of LTP or LTD depends on the ability of the postsynaptic membrane to reach different sequential thresholds for LTP and LTP induction (Artola and Singer, 1993). An NMDAR-mediated increase in postsynaptic Ca^{2+} levels subsequent to afferent stimulation can reach three potential concentrations: high (leading to LTP), intermediate (no plasticity or maintenance of current synaptic strength), or low (resulting in LTD) (Lisman, 1989; Cho et al., 2001). By using these three codes of synaptic communication, the dynamic shaping of the intrinsic synaptic connections in the NAc may control the final output signals to function in gating salient information, while ignoring unwanted impulses, through activation or depression of target neurons (Berretta et al., 2008).

Illustration 2. Different susceptibility to synaptic plasticity of MSNs, predicted by the Artola-Brocher-Singer (ABS) rule.



According to the ABS rule, synapses express LTD to LTP depending on the level of postsynaptic depolarization reached during the conditioning train stimulation and no change in synaptic efficacy may occur if intermediate levels of excitation are reached. Since MSNs have a very negative resting membrane potential, in *in vitro* slice preparations, they are much more susceptible to LTD than LTP. Adapted with permission from *Berretta et al./Progress in Neurobiology 84 (2008) 343-362.*

Any abnormal form of long-lasting adaptations at excitatory synapses within the NAc can affect the output of the target neurons, thereby altering the responsiveness of neuronal networks and mediating many of the behavioral changes that underlie pathological conditions. Bidirectional synaptic plasticity may be crucial to prevent saturation of the informational storage capacity in neural circuits. Pathological

behaviors, then, could originate from the modification of a pre-existing equilibrium of synaptic potentiation and depression, potentially resulting in the preservation of unessential information normally erased (Berretta et al., 2008). Thus, rather than the aberrant expression of one specific form of synaptic plasticity determining the output of the circuit, it may be the shifting of the overall synaptic plasticity equilibrium that contributes to abnormal behaviors.

Both LTP and LTD have been reported at glutamatergic synapses in the NAc (Pennartz et al., 1993; Thomas et al., 2000; Schotanus and Chergui, 2008a, b; Jeanes et al., 2011; Pascoli et al., 2012), and it is believed that these long-term modifications in the efficacy of the excitatory signals contribute to optimization, shaping, and maintenance of NAc functions. Plastic synaptic changes could be either bidirectional or less delineated in terms of polarity. Consequently, not only the plastic modification in excitatory postsynaptic current (EPSC) amplitude but also the long-term maintenance of a synaptic message resistance to change could represent key codes for controlling either pleasure, the therapeutic effects of medication, or addiction (Berretta et al., 2008).

NAc Synaptic Plasticity and Drug Dependence

Synaptic plasticity in the NAc appears to uniquely encode behavioral responses to repetitive drug experience. MSNs of the NAc are relatively quiescent neurons whose activity depends heavily on excitatory inputs from cortical and limbic regions; thus, alterations in glutamatergic synaptic plasticity onto MSNs in the NAc can profoundly influence the output of ventral striatal circuits.

Psychostimulants

Over the past decade, understanding of psychostimulant (primarily cocaine and amphetamine)-evoked synaptic plasticity has expanded greatly. The ability of low

frequency synaptic activity to elicit NMDAR-LTD is occluded following repeated cocaine injection (Thomas et al., 2001), while a single cocaine exposure alters the induction of mGluR-LTD in indirect pathway (D2+) MSNs of the NAc core (Grueter et al., 2010). Following a prolonged abstinence (21 days) from cocaine self-administration, NMDAR-LTD in the NAc core is suppressed (Martin et al., 2006). Repeated exposure to psychostimulants can also result in an increased density of dendritic spines in NAc MSNs (Russo et al., 2010), with the increase in spine density potentially being more robust in direct pathway (D1+) MSNs following cocaine exposure (Lee et al., 2006; Dobi et al., 2011; Kim et al., 2011). Furthermore, cocaine-induced increase in miniature EPSC frequency in direct pathways MSNs was observed in the NAc shell (Kim et al., 2011) but not the NAc core (Dobi et al., 2011), suggesting that the NAc shell may be the site of initial synaptic adaptations caused by repeated cocaine exposure.

Shortly following repeated cocaine exposure, an increased proportion of 'silent' synapses, containing NMDARs but no detectable functional AMPARs, was detected in the NAc shell (Huang et al., 2009), which could contribute to the decreased ratio of AMPAR-mediated to NMDAR-mediated currents observed at the same time point (Kourrich et al., 2007). The presence of silent synapses in the NAc peaks towards the end of chronic cocaine exposure and dissipates during one to two weeks of withdrawal (Huang et al., 2009), and evidence suggests these newly generated silent synapses are not lost during this withdrawal period but 'unsilenced' by insertion of functional AMPARs (Conrad et al., 2008; Dobi et al., 2011). During withdrawal, increased synaptic drive onto NAc MSNs may promote drug seeking behaviors as indicated by the finding that intra-NAc infusion of drugs that selectively block GluA2-lacking AMPARs attenuates drug seeking in response to cocaine-associate cues (Conrad et al., 2008). Intra-NAc injection of a peptide that prevents internalization of AMPARs from synapses (thus

inhibiting LTD) has been demonstrated to block the expression of behavioral sensitization to amphetamine (Brebner et al., 2005). A recent report demonstrated that activation of D1+ MSNs in the NAc enhanced cocaine sensitization and conditioned place preference, whereas activating D2+ MSNs diminished these behaviors (Lobo et al., 2010). Additionally, permanent disruption of NMDAR-LTD in the NAc facilitates the transition of rats to an 'addicted' behavioral state while LTD was recovered in 'unaddicted' rats that displayed controlled cocaine intake (Kasanetz et al., 2010). Recently, a group reported that *in vivo* optogenetic depotentiation of cocaine-induced potentiation of excitatory transmission in D1+ MSNs abolished cocaine-induced locomotor sensitization (Pascoli et al., 2012). Overall, many noteworthy advances have been made in pursuit of defining the specific neuroadaptations responsible for psychostimulant-induced behaviors.

Ethanol

While we may learn from and consider the significant advances in understanding the neuroadaptive responses induced by direct reinforcers such as cocaine and amphetamine, it is important to note that alterations induced by chronic ethanol exposure are likely due to both shared and distinct mechanisms. Our lab recently published the first report demonstrating a disruption in NAc shell NMDAR-dependent LTD following *in vivo* ethanol exposure. In that study, we observed that chronic intermittent ethanol (CIE) exposure reverses the polarity of synaptic plasticity in the NAc shell, converting LTD to synaptic potentiation of EPSCs (Jeanes et al., 2011). Due to the unique NMDAR blocking actions of ethanol (Lovinger et al., 1989, 1990), it is likely that neuroadaptive changes in NAc glutamatergic function due to CIE exposure are fundamentally distinct from those of other drugs of abuse. Additional studies will be necessary to identify the

precise neuroadaptations governing these changes in NAc long-term synaptic plasticity, especially with respect to the two subtypes (D1 and D2 receptor-expressing) of MSNs.

Animals Models of Alcohol Dependence

HUMAN ALCOHOL USE PHENOTYPES

Given the significant societal cost, research aimed at treating alcohol dependence and alcohol use disorders are essential. To that end, numerous models of alcohol dependence have been developed for basic research scientists to better model human patterns of alcohol consumption in laboratory animals. More recently, there has been a return to emphasis on chronic alcohol use and the role of dependence in vulnerability to relapse and in sustaining substance use. Three primary factors have led to this shift:

(1) Recognition that affective (psychological) components of withdrawal and protracted abstinence influence alcohol/drug use rather than simply avoidance of physiological symptoms of withdrawal.

(2) Advances in research approaches that enable detecting changes in affective components of withdrawal (i.e. human functional brain imaging and laboratory procedures; and animal behavioral and neurochemical/molecular procedures (Koob, 2009).

(3) Refinement of animal models that better reflect the course of alcohol/drug use from initiation and sustained use to the development of dependence.

A neuroadaptive view of alcoholism focuses on long-term plasticity that leads to a persistent negative affective state and altered function of key motivation systems as the proximal cause of relapse and excessive alcohol drinking. In this scenario, phenomena

encountered during acute withdrawal and early abstinence are of interest primarily if they reflect events that are critical to the induction of long-term plasticity (Heilig et al., 2010).

Acute withdrawal from alcohol use in humans reflects generalized nervous system hyperexcitability and is dominated by tremors, autonomic nervous system hyperactivity, risk for delirium tremens (peak around 72 hours) and seizures (for 48 hours with a peak around 24 hours). Early abstinence refers to an intermediate period (about 3-6 weeks) that follows discontinued alcohol use during which anxiety, low mood, and disturbed sleep continue but in the absence of acute physical symptoms (Schuckit and Hesselbrock, 1994). Protracted abstinence is a final phase where elevated anxiety and dysphoria for not necessarily detectable, but patients continue to report what could be called a shift in affective processing. During this phase, small, normally insignificant challenges provoke negative affect, craving, and relapse (Sinha and Li, 2007).

LABORATORY ANIMAL PHENOTYPES

A major limitation in modeling clinical withdrawal phenomena had been for a long time that most laboratory animals will not voluntarily consume sufficient amounts of alcohol to produce dependence. However, over the years, certain strains of animals have been selected for their willingness to drink ethanol, and one mouse strain in particular, C57BL/6J, are well known to voluntarily consume high quantities of ethanol while other strains drink comparatively little (Belknap et al., 1993). Most animal models of physical alcohol dependence involve experimenter-administered alcohol, with three approaches being the most common: repeated gastric intubations (Majchrowicz, 1975), forced liquid diet containing alcohol as sole source of fluid and calories (Wise, 1975), and chronic inhalation of alcohol vapor (Goldstein and Pal, 1971; Rogers et al., 1979). In both animal and clinical studies, the amount and duration of alcohol exposure play a significant role

for the development of dependence and expression of withdrawal (Finn and Crabbe, 1997). Additionally, greater severity of withdrawal symptoms have consistently been found when chronic alcohol exposure is delivered in an intermittent rather than continuous manner (chronic intermittent ethanol or CIE) (Becker, 1998).

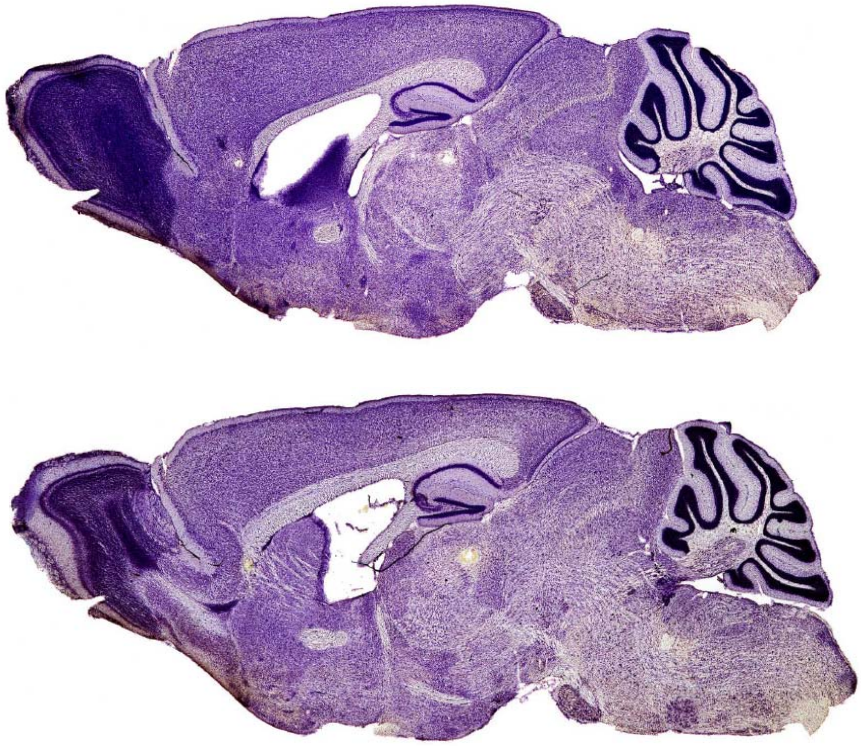
The phenotypes most directly relevant for linking withdrawal processes with maintenance of alcoholic drinking are excessive voluntary alcohol intake/self-administration and propensity for relapse. Several studies in mice (Becker and Lopez, 2004; Lopez and Becker, 2005; Chu et al., 2007; Finn et al., 2007; Dhaher et al., 2008) and rats (Roberts et al., 2000; Rimondini et al., 2002; O'Dell et al., 2004; Sommer et al., 2008) have demonstrated increased alcohol responding and/or drinking in dependent compared with non-dependent animals. In fact, the enhanced alcohol consumption during withdrawal in dependent animals was shown to produce blood and brain alcohol levels that nearly reached the levels attained during the forced chronic alcohol exposure that preceded the dependent state (Roberts et al., 2000; Griffin et al., 2009b). Similar to findings in clinical studies, animals with a history of alcohol dependence exhibit exaggerated sensitivity to the effect of alcohol-related cues and stressors to enhance alcohol-seeking behavior (Liu and Weiss, 2002; Sommer et al., 2008). These effects have been observed up to several months after termination of the alcohol exposure (Valdez et al., 2002; Lopez and Becker, 2005; Sommer et al., 2008), supporting the notion that during protracted abstinence, there is an enhanced vulnerability to relapse that perpetuates the addicted state. Animal models that involve a history of physical dependence on alcohol with repeated cycles of withdrawal appear to be accompanied by an increased motivation to self-administer alcohol and a propensity to relapse (Heilig et al., 2010). These types of animal models may more closely mimic the motivational

adaptations relevant for maintaining drug-seeking and taking behaviors in human alcoholism.

Representative Brain Slices for Electrophysiological Recordings

Two sagittal brain slices containing the NAc were typically prepared from each hemisphere for each experimental animal. Much care was taken to ensure that similar orientation and thickness (220-250µm) were maintained for the slices each experimental day. These brain slices contain the NAc core and shell subregions, as well as, prefrontal cortex and some dorsal striatum. Recordings were only performed in the most medial portions of the NAc, and in the most rostral and ventral areas away from the anterior commissure. Thus, we believe that our recordings fully represent MSNs from the NAc shell subregion in all experimental findings.

Illustration 3. Sagittal mouse brain sections representative of areas of NAc used for electrophysiological experiments.



Hypothesis and Aims

OVERALL PROJECT RATIONALE

It is our assertion that changes in glutamatergic synaptic plasticity in NAc shell MSNs contribute to the expression of ethanol dependence. Several lines of evidence to that end include: 1) LTD in the NAc has been implicated in the expression of behavioral sensitization to other drugs of abuse, cocaine and amphetamine (Thomas et al., 2001; Brebner et al., 2005), 2) chronic ethanol exposure leads to the conversion of synaptic depression to potentiation at excitatory synapses in MSNs of the dorsal striatum (Xia et al., 2006; Yamamoto et al., 1999), 3) our preliminary data demonstrate a shift from synaptic depression to potentiation at excitatory synapses in the NAc shell following a CIE procedure known to induce ethanol dependence in mice (Becker and Lopez, 2004, Lopez and Becker, 2005). We have chosen to characterize the plasticity at a time point (72 hours post-CIE exposure) known to coincide with the initiation of increased ethanol drinking in mice. We further hypothesize that D1 and D2 MSNs differentially express LTD in ethanol-naïve mice leading to additional variations in the expression of synaptic plasticity following CIE exposure. Our support for this hypothesis resides primarily in our preliminary results that demonstrate two distinct populations of MSNs neurons in the NAc shell based on their distinct expression of differing LTD magnitudes in response to identical conditioning stimuli. These data indicate that one population of MSNs exhibits a greater magnitude of LTD (55% of baseline) compared to another which displays a smaller LTD (88% of baseline) (see section C). Differences in the intrinsic properties of D1 and D2 MSNs have been shown in the dorsal striatum, which indicate D2 MSNs are more excitable and responsive to cortical glutamatergic synaptic transmission (Cepeda et al., 2008). We believe that these properties of D2 MSNs are more conducive to the induction of synaptic potentiation rather than synaptic depression. Induction of synaptic

plasticity from depression to potentiation reflects progressively higher levels of postsynaptic free calcium entry (Cho et al., 2001; Lisman, 1989). Taken together, D1 MSNs likely comprise the population of neurons we have shown to exhibit the greater magnitude of LTD because a decreased responsiveness to excitatory input would result in a lower intracellular calcium level reached during conditioning stimuli. Additionally, we contend that the conversion of synaptic depression to potentiation observed following CIE exposure is expressed primarily in D2 MSNs because they are expected to achieve a greater postsynaptic calcium level given their greater responsiveness to cortical glutamatergic activity. Therefore, following CIE exposure, I believe that a shift in synaptic plasticity of D2 MSNs to synaptic potentiation leads to a dysfunction in the output of the NAc shell that plays a crucial role in ethanol dependence.

To test these hypotheses, experiments are designed to 1) elucidate the mechanisms and time course of the conversion from synaptic depression to potentiation following CIE exposure (Aim 1), and 2) test whether D1 MSNs more readily exhibit LTD in ethanol-naïve mice, and D2 MSNs express the conversion to potentiation in CIE treated mice (Aim 2) will be performed.

SPECIFIC AIMS

Specific Aim 1: To investigate the mechanisms underlying the conversion of synaptic depression to potentiation and its persistence following extended withdrawal periods.

Our preliminary data indicate that MSNs exhibit a switch from synaptic depression to potentiation 24 hours following withdrawal from CIE exposure. LTD induced by low frequency (1 Hz) stimulation (paired with postsynaptic depolarization to -50mV) in NAc MSNs requires NMDA receptor activation (Thomas et al., 2000; Brebner

et al., 2005). It is not known, however, whether this conversion to potentiation also requires NMDA receptor activation or persists at extended withdrawal periods.

Aim 1.1: Characterization of synaptic plasticity observed 72 hours after CIE exposure.

Aim 1.2: Persistence of synaptic potentiation at extended withdrawal time points and after a second series of CIE exposure.

Specific Aim 2: To determine the differential expression of synaptic plasticity in D1 and D2-expressing MSNs.

Our preliminary data suggest that two populations of MSNs exist in the NAc shell, which respond to LTD-inducing stimuli with different magnitudes of synaptic depression. Our goal in this aim is to determine if D1 or D2-expressing MSNs comprise one or both of these two populations. We will examine synaptic plasticity in BAC transgenic mice expressing either eGFP-labeled D1 or D2 receptors. Using these mice, we will be able to distinguish between the two major types of MSNs and determine whether synaptic plasticity is preferentially expressed in one neuronal population or the other.

Aim 2.1: To investigate glutamatergic synaptic depression in D1 and D2-expressing MSNs of ethanol-naïve mice.

Aim 2.2: Analysis of synaptic plasticity of D1 and D2 MSNs following CIE exposure.

CHAPTER 2:

IN VIVO CHRONIC INTERMITTENT ETHANOL EXPOSURE REVERSES THE POLARITY OF SYNAPTIC PLASTICITY IN THE NUCLUES ACCUMBENS

 Jeanes ZM, Buske TR, Morrisett RA. In Vivo Chronic Intermittent Ethanol Exposure Reverses the Polarity of Synaptic Plasticity in the Nucleus Accumbens Shell. JPET 336:155-164, 2011.

Abstract

Glutamatergic synaptic plasticity in the nucleus accumbens (NAc) is implicated in response to sensitization to psychomotor stimulating agents yet ethanol effects here are undefined. We studied the acute in vitro and in vivo effects of ethanol in medium spiny neurons from the shell NAc subregion of slices of C57BL/6 mice using whole-cell voltage clamp recordings of AMPA EPSCs. Synaptic conditioning (low frequency stimulation with concurrent postsynaptic depolarization) reliably depressed AMPA EPSCs by nearly 30%; this accumbal long-term depression (NAc LTD) was blocked by a non-selective NMDA receptor antagonist (DL -APV) and a selective NMDAR2B antagonist (Ro 25-6981). Acute ethanol exposure inhibited the depression of AMPA EPSCs differentially with increasing concentrations, but this inhibitory action of ethanol was occluded by a D1-selective dopamine receptor agonist. Ethanol dependence was

elicited in C57BL/6 mice by two separate four day bouts of chronic intermittent ethanol (CIE) vapor exposure. When assessed 24 hrs following a single bout of in vivo CIE vapor exposure, NAc LTD was absent and instead NMDAR receptor-dependent synaptic potentiation (LTP) was reliably observed. Interestingly, both LTP and LTD were completely absent following an extended withdrawal (72 hours) after a single 3 day CIE vapor bout. These observations demonstrate that accumbal synaptic depression 1) is mediated by NR2B receptors, 2) is highly sensitive to both acute and chronic ethanol exposure, and 3) alterations in this synaptic process may constitute a neural adaptation that contributes to the induction and/or expression of ethanol dependence.

Introduction

GABAergic medium spiny neurons (MSNs) of the nucleus accumbens (NAc) in the ventral striatum are principal neurons in the mesocorticolimbic system that process information concerning reward behavior (Nestler, 2001). These neurons receive a dopaminergic projection from the ventral tegmental area (VTA) and glutamatergic projections from prefrontal cortex and other limbic structures. It is generally thought that neuroadaptations in response to chronic drug abuse underlie development of craving and other drug-seeking behaviors associated with dependence. Much evidence indicates that NAc MSNs are very likely involved in such aberrant neuroadaptive responses. While neuroadaptations underlying chronic ethanol abuse remain undefined, interactions between dopaminergic, glutamatergic and GABAergic systems are likely crucial in this regard (for reviews see (Gonzales et al., 2004; Zhang et al., 2006).

Indeed, a large literature indicates that ethanol reinforcement involves the activation of the VTA-accumbal dopamine system (Gonzales et al., 2004). Ethanol has

unique pharmacological actions to excite VTA dopamine neurons, and withdrawal from chronic ethanol exposure reduces their firing (Brodie et al., 1990; Shen and Chiodo, 1993; Brodie et al., 1999; Shen, 2003). Dopamine release increases in the NAc during operant self-administration of ethanol (Weiss et al., 1993; Gonzales and Weiss, 1998; Yim et al., 1998) and D1-dopamine receptor antagonists reduce operant ethanol responding (Rassnick et al., 1992; Hodge et al., 1993; Samson et al., 1993). Additionally, ethanol self-administration is reduced in animals lacking D1 receptors (El-Ghundi et al., 1998) or one of its intracellular partners, DARPP-32 (Risinger et al., 2001).

Neuroadaptations which contribute to ethanol abuse likely share common mechanisms with those seen in other abused reinforcers – especially the psychomotor stimulating agents such as cocaine and amphetamine. Recent evidence indicates that adaptations in accumbal glutamatergic plasticity constitute a mechanism encoding repetitive drug experience to psychomotor stimulants. In control NAc MSNs, low frequency conditioning stimulation (LFS) paired with postsynaptic depolarization, which mimics the upstate of about -50 mV common in bistable MSNs, produces long-term depression (LTD) of AMPA EPSCs (Thomas et al., 2000; Thomas et al., 2001). Like hippocampal NMDA LTD (Dudek and Bear, 1992; Man et al., 2000), NAc LTD is induced by a moderate increase in intracellular Ca2+ through NMDA receptor activation (Thomas et al., 2000).

Thomas and colleagues first reported marked differences in basal AMPA EPSCs in NAc MSNs from cocaine sensitized animals (Thomas et al., 2001). Furthermore, NAc LTD expression was completely occluded in these sensitized animals thus suggesting that repetitive cocaine experience directly induced LTD. In a very elegant series of experiments, Wang and colleagues also reported LTD occlusion in NAc MSNs following amphetamine sensitization and dissected the mechanisms underlying neuroadaptation to

psychomotor stimulants (Brebner et al., 2005). Since expression of hippocampal LTD had previously been demonstrated to be dependent upon GluR2 subunit internalization (Luscher et al., 1999), these investigators also proposed that endocytotic process as a prime mechanism whereby amphetamine experience modulated glutamatergic plasticity. The role of GluR2 internalization in expression of NAc LTD and sensitization to amphetamine was directly tested using a peptide which disrupted internalization of GluR2-containing AMPA receptors. Active, but not inactive, peptides completely occluded NAc LTD, and the most critical observations further came from in vivo studies. Intravenous or intra-accumbal, but not intra-VTA, injection of active, but not inactive, peptides completely occluded expression of amphetamine sensitization in previously sensitized rats (Brebner et al., 2005).

No studies investigating neuroadaptive changes in glutamatergic synaptic plasticity in the nucleus accumbens following ethanol exposure exist. This is particularly significant since, in contrast with other drugs, ethanol has a unique action to inhibit NMDA receptors and to disrupt NMDA receptor-dependent plasticity in hippocampal and other structures (Sinclair and Lo, 1986; Lovinger et al., 1989, 1990; Morrisett and Swartzwelder, 1993; Nie et al., 1993; Nie et al., 1994; Maldve et al., 2002; Zhang et al., 2005). Reports of plasticity changes in MSNs of the dorsal striatum in response to ethanol exposure do exist (Yamamoto et al., 1999; Xia et al., 2006; Wang et al., 2007); however, those involve different forms of plasticity from that involved herein. Additionally, the MSNs of the dorsal striatum are involved in habit formation and not thought to be involved in ethanol reinforcement and reward such as processed in the NAc (Everitt and Robbins, 2005). Taken together, these findings prompted us to investigate neuroadaptive changes in glutamatergic transmission in NAc medium spiny neurons following in vivo ethanol exposure. Since induction of NAc LTD is NMDA receptor-

dependent, we analyzed the direct effects of in vitro ethanol exposure on LTD in the NAc as well.

Materials and Methods

BRAIN SLICE PREPARATION

Parasagittal slices (210-250 μm thick) containing the NAc were prepared from the brains of 4-8 week-old male C57BL/6J mice (Jackson Labs, Bar Harbor, ME). Mice were lightly anesthetized by inhalation of halothane, and the brains were rapidly removed and placed in ice-cold (4ºC) oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 110 choline, 25 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 7 MgCl2, 0.5 CaCl2, 25 dextrose, 11.6 Na+-ascorbate, and 3.1 Na+-pyruvate, bubbled with 95% O2/5% CO2. Slices were then transferred to an incubation ACSF for a minimum of 45-60 minutes prior to recording that contained the following (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 2.4 MgCl2, 1.8 CaCl2, 10 dextrose, bubbled with 95% O2/5% CO2; pH 7.4, 32oC. Unless otherwise noted, all drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

PATCH CLAMP ELECTROPHYSIOLOGY

We conducted all recordings at 31-33oC in ACSF containing (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 0.9 MgCl2, 2 CaCl2, 10 dextrose, bubbled with 95% O2/5% CO2. The GABAA receptor antagonist, picrotoxin (50 μM), was added to the external recording solution throughout all recordings to inhibit GABAA receptor-mediated synaptic currents; this improves the reliability of synaptic plasticity in the dorsal and ventral striatum by favoring postsynaptic depolarization during conditioning

stimuli (Berretta et al., 2008). Whole-cell voltage clamp recordings were obtained from NAc shell MSNs visually identified using the MRK200 Modular Imaging system (Siskiyou Corporation, Grants Pass, OR) mounted on a vibration isolation table under IR-Dodt optics. MSNs represent ~95% of the neurons in the NAc and have distinctly smaller cell bodies (about 10 µm in diameter). MSNs were also identified by their highly negative resting membrane potential (less than -75 mV). MSNs from the most rostral and ventral areas of the NAc were chosen to make sure all recordings arose from the NAc shell subregion. Only one neuron per slice was used for recording. ACSF continuously perfused the recording chamber at 2.0-2.5 mL/min. Recording electrodes (thin-wall glass, WPI Instruments) were made using a Brown-Flaming model P-88 electrode puller (Sutter Instruments, San Rafael, CA) to yield resistances between 3-5 MΩ and contained (in mM): 135 KMeSO4, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Tris-GTP, (pH 7.3 with KOH). Input and access resistances were monitored throughout all experiments, and the recording was terminated if either resistance varied by more than 20%. These parameters were measured by application of a -10 mV, 100 ms voltage step at 5-10 min intervals. Synaptic currents were monitored at a holding potential of -80 mV. Changes in the holding current were observed to detect any resealing or other instability of the patch.

DATA ACQUISITION AND ANALYSIS

Excitatory afferents, the majority of which arise from the prefrontal cortex, were stimulated with a stainless steel bipolar stimulating electrode (FHC, Inc., Bowdoin, ME) placed between the recorded MSN and prefrontal cortex, typically 150-300 µm from the MSN cell body. EPSCs were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, and digitized at 10-20 kHz via a Digidata

1440A interface board using pClamp 10.2 (Axon Instruments, Foster City, CA). Standard evoked EPSCs elicited by local stimulation were established in NAc shell MSNs for at least 10 minutes (at 0.1 Hz) to ensure stable recordings. LTD induction was assessed by delivering conditioning stimuli (500 pulses at 1 Hz at baseline stimulation intensity) while continuously and simultaneously depolarizing the postsynaptic cell to -50 mV (referred to below as conditioning stimulation). EPSCs were then monitored for 30-45 minutes post-pairing (at 0.1 Hz).

Peak EPSC values were determined using Clampfit 10.2 software (Axon Instruments). For each recording, peak EPSC amplitude values were normalized to the average EPSC amplitude of the final 10 minutes of baseline (60 sweeps) for that single recording. The mean normalized EPSC amplitudes for 12 consecutive sweeps were condensed into 2 minute bins and represented as a single data point in scatter plots for each treatment group. Each data point represents the average of 12 consecutive EPSC amplitudes at that time point from each neuron within its respective treatment group. We used two parameters to determine whether plasticity of EPSC amplitudes (either depression or potentiation) occurred. An unpaired Student's t test (p value < 0.5) was used to compare the five normalized EPSC values from 20 to 30 minutes (minutes 40-50 on figures) after the pairing protocol to the five normalized EPSC values during the last 10 minutes of baseline. In addition, the change in average EPSC amplitude after conditioning needed to be greater than 2 standard deviations from baseline. If both of these criteria were met, that treatment group was determined to exhibit plasticity.

For each experiment, the 40-50 minute time period was used to compare the magnitude of plasticity after different drug exposures. The five normalized EPSC values between min 40-50 were compared between groups using a single factor ANOVA with Bonferroni post-hoc analyses. Statistical significance for between treatment group

comparisons was defined as p value < 0.05. Thus, LTD was considered the control outcome to which all drug (either in vitro or in vivo) exposures were compared. LTD was determined to be reduced and not completely blocked in situations where the post-pairing average EPSC amplitude (min 40-50) was significantly increased from control LTD (ANOVA) and significantly decreased from its respective baseline (Student's t test). Experiments testing different antagonists were interleaved with control experiments using slices prepared from the same animals where possible.

CHRONIC INTERMITTENT ETHANOL EXPOSURE

Ethanol dependence was induced by exposing mice to chronic intermittent ethanol vapor (Becker and Hale, 1993; Becker and Lopez, 2004; Lopez and Becker, 2005). Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 0.2-0.3 L/min. The resulting ethanol vapor then combined with a separate air stream to give a total flow rate of around 4 L/min which was delivered to mice in special mouse chamber units (Allentown Inc., Allentown, NJ). These chambers resembled normal acrylic cages but contain an additional air-tight seal top, a vapor inlet, and an exhaust outlet. Food and water was available ad libitum on the wire cage tops. The ethanol flow rate was determined empirically to yield target blood ethanol concentrations (35-45 mM, or 150-200 mg/dL) measured from a 10 µL tail blood sample using an Analox AM1 alcohol analyzer (Analox, Lunenberg, MA). Two identical cages of mice were always run simultaneously; one cage for exposure to ethanol vapor and the second cage for an air only control. The ethanol group received a single, daily intraperitoneal injection containing both ethanol (20% v/v, 1.5 g/kg) and pyrazole (68 mg/kg) in sterile PBS. Mice were then immediately chambered and exposed to ethanol vapor or air (from 1700 to 0900 hrs daily under a reverse light/dark cycle- lights off at 1200 hrs) for three

consecutive days. Air control mice received only the pyrazole injection but were otherwise handled exactly as the ethanol group. On the fourth day, animals were returned to home cages for 24 or 72 hours (depending on experiment). On the fifth or seventh day, electrophysiological experiments were performed as described above.

Two bottle choice drinking. Eight-week old C57BL/6J mice (Jackson Labs) were acclimated to a 12:12 hour reverse light environment (lights off at 1200 hrs) for two weeks. The animals had free access to food and water throughout the experiment. Subjects were tested for baseline ethanol consumption (g/kg) using a two-hour, two bottle choice limited access drinking paradigm (15% ethanol/water or water choice) for 21 days, administered 30 minutes prior to the beginning of the dark cycle. Ethanol consumption was recorded for the last five days of the baseline period and used to divide subjects equally into control and experimental groups. Mice were subjected to ethanol vapor or control air chamber exposure as described above for four consecutive days followed by 74 hours of rest. Post chamber ethanol consumption was recorded for five days as before. A second round of chamber exposure was administered for four consecutive days followed by 74 hours of rest. Post chamber ethanol consumption was again recorded for five days. Statistical significance for average ethanol consumption over each five day period was defined as $p < 0.05$ using a student's t test with Bonferroni post-hoc analyses.

Results

NMDAR-MEDIATED LTD IN THE NAC SHELL OF ETHANOL-NAÏVE MICE

All data in this study were gathered using whole-cell voltage clamp of MSNs solely in the mouse NAc shell region in the presence of the GABAA receptor antagonist, picrotoxin (50 µM). Low frequency stimulation (500 pulses @ 1Hz) paired with postsynaptic depolarization to -50 mV has been reported to induce NMDA receptor-dependent LTD in the NAc core and shell (Thomas et al., 2000). Following a similar protocol involving coincident depolarization and low frequency afferent stimulation (referred to below as conditioning stimulation), we consistently observed reliable LTD of EPSCs from all ethanol-naïve MSNs examined (71.4 ± 0.7% of baseline, n=21 neurons from 15 animals, $p<0.001$, post-conditioning vs baseline, Fig. 1). Baseline and post-conditioning EPSCs recorded at -80 mV were solely AMPA receptor-mediated since they were completely abolished by the AMPA/kainate receptor antagonist, DNQX (6,7-dinitroquinoxaline-2,3-dione , 10 µM, supplemental data).

We measured the paired-pulse ratio (PPR) of EPSCs (2 pulses, 50ms apart) before and after conditioning stimulation to determine whether LTD expression was related to presynaptic and/or postsynaptic changes in glutamatergic transmission. In six neurons from different mice, we observed LTD using paired test stimuli identical to that of LTD using single stimuli (72.9 ± 1.4% of baseline, n=6 neurons from 6 animals, $p>0.05$, paired stimuli LTD vs single stimuli LTD, Fig. 1). Additionally, we observed no significant difference ($p>0.05$, student's t test) in paired-pulse facilitation (PPF) between baseline EPSCs (1.73 ± 0.18) and following induction of LTD (1.85 ± 0.18) indicating that changes in neurotransmitter release do not contribute to 1Hz-LTD in the NAc (Fig. 1C).

NON-SPECIFIC OR SUBUNIT-SPECIFIC NMDA RECEPTOR INHIBITION BLOCKS NAC LTD

Thomas and colleagues first described low frequency NAc LTD and showed its induction depended upon NMDA receptor activation (Thomas et al., 2000); therefore, we determined whether the plasticity we observed in response to conditioning was likewise dependent upon NMDA receptors. In the presence of the non-selective NMDA receptor antagonist, DL-APV (100 μM), LTD was completely blocked (96.6 ± 1.0% of baseline, n=4 neurons from 4 animals, $p>0.05$, vs baseline; $p<0.05$, vs control LTD, Fig. 2).

We also tested whether NMDA receptors containing the NR2B subunit are required for induction of NAc LTD. In presence of the specific NR2B antagonist, Ro 25-6981(0.5 μM), LTD in ethanol-naïve mice was completely abolished (105.6 ± 1.2% of baseline, n=5 neurons from 4 animals, $p<0.05$, vs baseline; $p<0.05$, vs control LTD, vs DL-APV, Fig. 2). The small potentiation observed in the presence of Ro 25-6981 was significant from baseline; however, the magnitude of potentiation was not greater than two standard deviations from baseline, which does not meet our criteria for plasticity expression.

ACUTE IN VITRO ETHANOL EXPOSURE OF INCREASING CONCENTRATIONS DIFFERENTIALLY INHIBITS NAC LTD

Ethanol is well known to inhibit NMDA receptors; therefore, we next tested whether ethanol inhibits NAc LTD expression. Bath application of a low, intoxicating concentration of ethanol (20 mM) partially but significantly reduced NAc LTD (79.7 ± 2.0% of baseline, n=6 neurons from 5 animals, $p<0.05$, vs baseline; $p<0.05$, vs control LTD, Fig. 3). LTD expression was completely inhibited by a moderately to strongly intoxicating concentration of ethanol (40 mM) equivalent to the target concentration used in the in vivo vapor model described below (104.3 ± 1.3% of baseline, n=7 neurons from 6 animals, $p>0.05$, vs baseline; $p<0.05$, vs 20 mM EtOH, vs control LTD, Fig. 3). Interestingly, when conditioning was performed in a highly intoxicating concentration of

ethanol (60 mM), NAc LTD appeared similar in magnitude to that observed in the presence of the lowest concentration of ethanol (20 mM) tested (81.5 ± 0.7% of baseline, n=7 neurons from 6 animals, $p<0.05$, vs baseline, vs 40 mM EtOH, vs control LTD; $p>0.05$, vs 20 mM EtOH, Fig. 3).

ACTIVATION OF DOPAMINE D1 RECEPTORS RESTORES LTD EXPRESSION IN THE PRESENCE OF ETHANOL

Previous work in our lab demonstrated that activation of D1-dopamine receptors decreased the ethanol sensitivity of NMDA receptors on MSNs in the NAc (Maldve et al., 2002; Zhang et al., 2005). Therefore, we next tested whether D1-dopamine receptor activation might antagonize the inhibitory effects of ethanol on NAc LTD. Bath application of the selective D1 receptor agonist (±SKF38393, 50 μM) did not affect the magnitude of LTD expression in control slices (65.9 ± 1.7% of baseline, n=7 neurons from 6 animals, $p<0.001$, vs baseline, Fig. 4). However, pre-treatment of slices with SKF38393 for 30 minutes prior to ethanol (40 mM) application rescued LTD from acute ethanol inhibition (66.8 ± 1.5% of baseline, n=6 neurons from 5 animals, $p<0.001$, vs baseline, Fig. 4). When LTD magnitude was compared between the SKF only, SKF+ EtOH, and control NAc LTD groups, there were no significant differences apparent ($p>0.05$, one-way ANOVA, Bonferroni post-hoc). Likewise, a specific D1 receptor antagonist, SCH23390 (10 μM), had no effect on LTD expression when applied alone (63.9 ± 1.8% of baseline, n=4 neurons from 3 animals, $p<0.001$, vs baseline, Fig. 5). Co-application of both the D1 receptor agonist and antagonist 30 minutes prior to ethanol reversed the ability of the D1 agonist to occlude ethanol inhibition of NAc LTD. Thus, in the presence of both D1 agonist and antagonist and ethanol (40 mM), we observed NAc

LTD similar to that from the ethanol alone treatment group (97.8 ± 1.7% of baseline, n=5 neurons from 4 animals, p>0.05, vs baseline, Fig. 5).

NAc LTD is completely occluded in the presence of ethanol (60mM) when D1 receptors are inhibited

We next investigated the possibility that the highest concentration of ethanol (60 mM) did not fully inhibit NAc LTD because of a subsequent release of dopamine upon ethanol application. When pre-treated for 20 minutes with the specific D1 receptor antagonist, SCH23390 (10 μM), the application of ethanol (60 mM) completely blocked NAc LTD (99.1 ± 2.6% of baseline, n=6 from 4 animals, p>0.05 vs baseline; p<0.05 vs control LTD, vs 60mM EtOH alone; Fig. 6).

Chronic intermittent ethanol exposure increases voluntary ethanol consumption in C57BL/6J mice

Repeated cycles of chronic intermittent ethanol (CIE) exposure to C57BL/6 mice will significantly increase voluntary ethanol consumption (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009b). In order to determine whether NAc LTD may represent an important synaptic process that may be altered in ethanol dependence, we first developed this mouse model in our lab. We examined ethanol intake in two hour drinking bouts initiated 30 minutes prior to the dark cycle before and after two successive periods of four days of intermittent 16 hour ethanol exposure. The average baseline ethanol consumption for air vapor control and ethanol vapor groups prior to CIE exposure was not significantly different (1.51 ± 0.08 g/kg/day vs. 1.51 ± 0.05 g/kg/day, n=8 animals per group, p>0.05, student's t test, Bonferroni post-hoc, Fig. 7). Following the first bout of CIE exposure (four days, 16 hour vapor exposure), the average ethanol

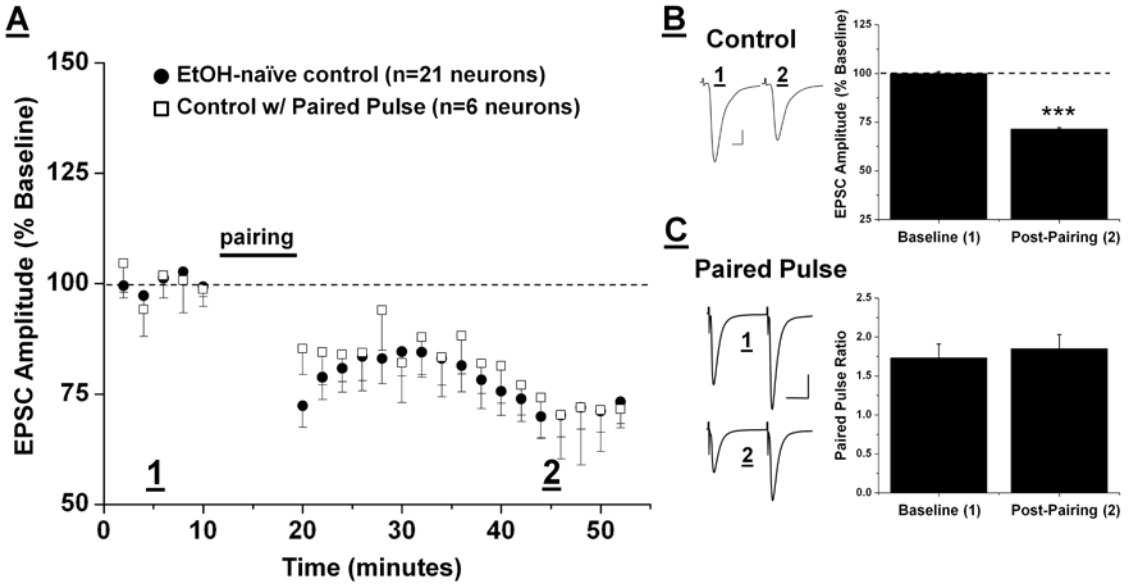
intake of the ethanol vapor group was not significantly increased compared to the air vapor control group (2.90 ± 0.33 g/kg/day vs. 2.19 ± 0.23 g/kg/day, n=8 animals per group, $p>0.05$, student's t test, Bonferroni post-hoc, Fig. 7). However, only the ethanol vapor group showed a significantly increased ethanol intake compared to baseline ($p>0.05$ for air baseline vs. air post-CIE 1; $p<0.05$ for ethanol baseline vs. ethanol post-CIE 1). Following the second bout of CIE exposure, the average ethanol intake of the ethanol vapor group was significantly increased compared to the air vapor control group (2.77 ± 0.13 g/kg/day vs. 1.75 ± 0.23 g/kg/day, n=8 animals per group, $p<0.01$, student's t test, Bonferroni post-hoc, Fig. 7), and neither group differed significantly between their respective post-CIE 1 average ethanol consumptions ($p>0.05$).

SYNAPTIC CONDITIONING ELICITS LONG-TERM POTENTIATION FOLLOWING IN VIVO CHRONIC INTERMITTENT ETHANOL VAPOR EXPOSURE

Repetitive exposure to psychomotor stimulating agents occludes NAc LTD (Thomas et al., 2001; Brebner et al., 2005); therefore, we next investigated whether a similar neuroadaptation occurs following intermittent ethanol exposure. The standard CIE protocol involved two periods of ethanol exposure; however, since ethanol intake was significantly increased following the first ethanol exposure period, we chose to investigate the immediate effects following the first few 16 hour bouts of intermittent ethanol exposure for three consecutive days. NAc MSNs prepared from mice 24 hours following brief CIE exposure displayed marked differences in excitatory transmission in response to the standard conditioning stimulation (Fig. 8). Normal synaptic depression was present in NAc MSNs from the air control mouse group (65.3 ± 1.1% of baseline, n=4 from 3 animals, $p<0.001$ vs baseline; $p>0.05$ vs control LTD). Instead, synaptic conditioning elicited a striking and highly significant synaptic potentiation of EPSC

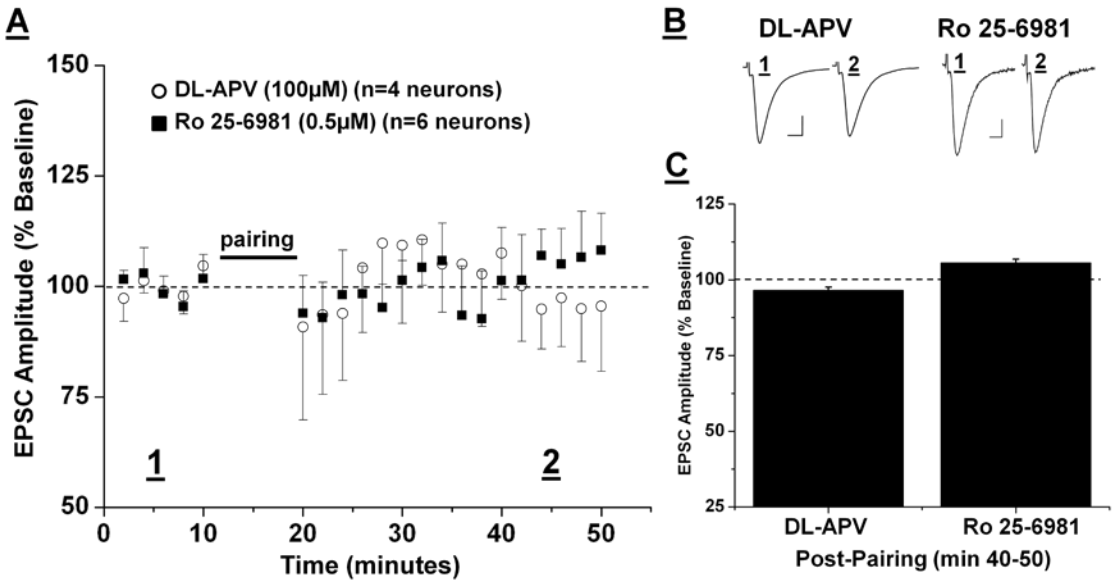
amplitudes in NAc MSNs prepared 24 hours following CIE treatment (124.9 ± 1.3% of baseline, n=8 neurons from 8 animals, $p<0.05$, vs baseline; $p<0.001$, vs air control). Furthermore, DL-APV (100 μM) completely abolished this synaptic potentiation observed in slices from CIE-exposed mice (91.2 ± 1.6% of baseline, n=7 neurons from 4 animals, $p<0.05$, vs baseline; $p<0.05$, vs air control, vs 24hrs post-CIE, Fig. 9). This small depression did satisfy our criteria for plasticity, yet it was still significantly different from air control LTD. Finally, we assessed NAc plasticity in slices from mice that had been allowed 72 hours of recovery after the conclusion of their CIE exposure to investigate the endurance of these synaptic changes (Fig. 9). In these mice, conditioning elicited a slight, yet significantly less depression of synaptic transmission than air control LTD (91.1 ± 0.9% of baseline, n=5 neurons from 3 animals, $p<0.05$, vs baseline, vs 24hr post CIE, vs air control; $p>0.05$ vs CIE-APV).

Figure 1. Low frequency stimulation induces LTD in the NAc shell.



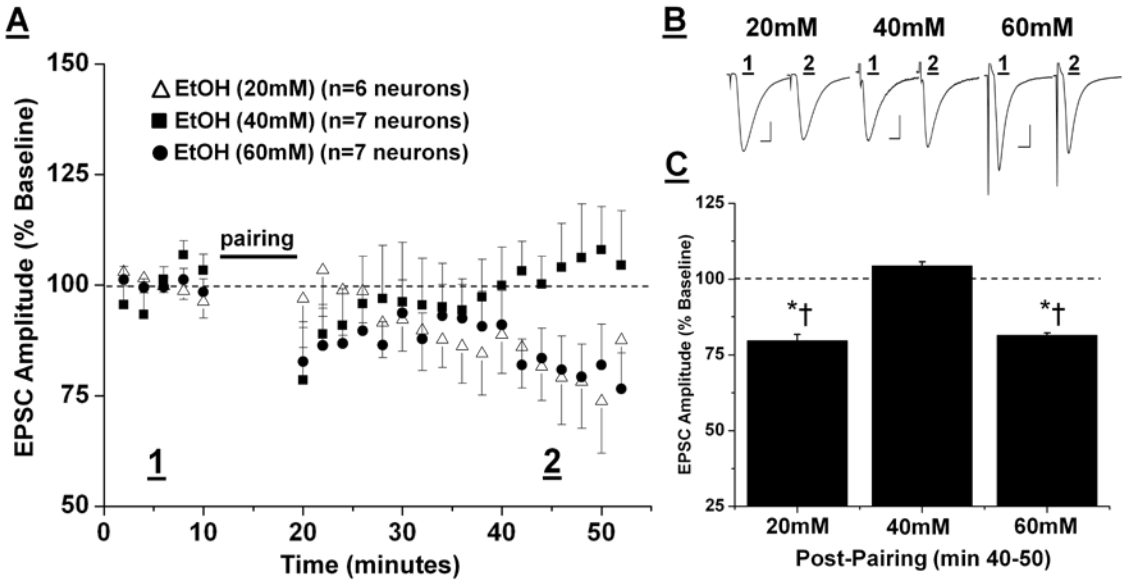
A, conditioning stimulation (500 pulses @ 1Hz with concurrent depolarization to -50 mV, denoted as "pairing") induces long-term depression of evoked AMPA receptor-mediated EPSCs in NAc shell MSNs of ethanol-naïve mice (71.4 ± 0.7% of baseline, n=21 neurons from 15 animals, ***$p<0.001$, post-pairing vs baseline). Each data point represents an average of 12 consecutive normalized EPSC amplitudes condensed into 2 minute bins (% Baseline ± SEM) from each neuron studied at that time point. Pairing stimulation also induced LTD when paired EPSCs (50 ms apart) were evoked during baseline and post-pairing (72.9 ± 1.4% of baseline, n=6 neurons from 6 animals, $p<0.001$, vs baseline; $p>0.05$, vs control LTD). B, sample EPSC traces from a single neuron. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). Bar graph representing the percentage change ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50). C, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from single and paired-pulse groups. Scale bars represent 20 ms (horizontal) and 50 pA (vertical). Bar graph represents the paired-pulse ratio (PPR) ± SEM between baseline and post-pairing. PPR determined by dividing the amplitude of EPSC 2 by EPSC1 for each sweep. Average PPR during baseline and post-pairing were not significantly different (1.73 ± 0.18 and 1.85 ± 0.18 respectively, $p>0.05$).

Figure 2. NR2B-containing NMDA receptors required for NAc LTD expression.



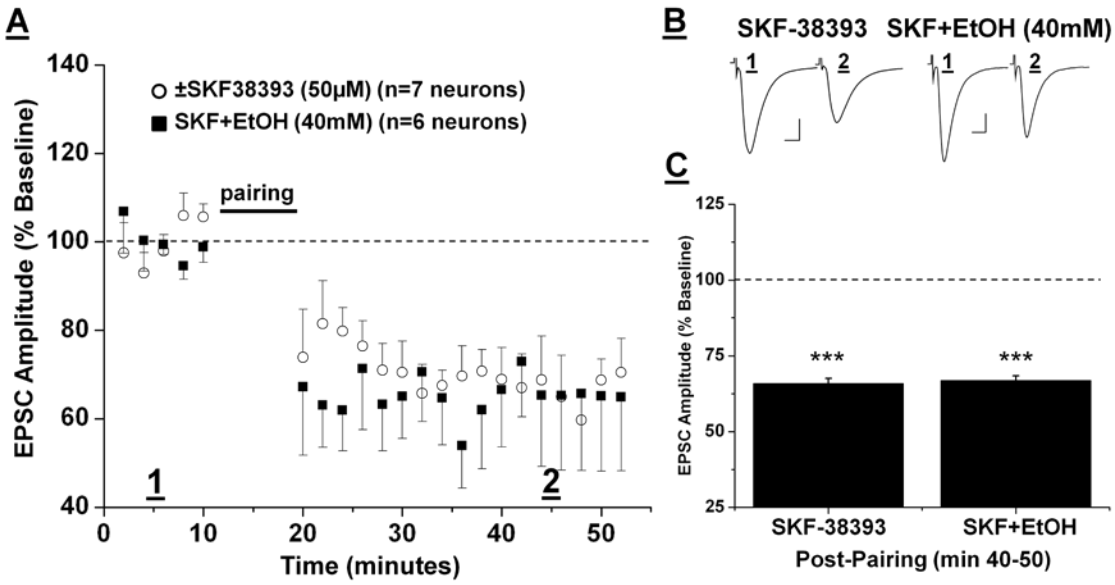
A, pairing stimulation does not induce LTD expression in the presence of the non-selective NMDA receptor antagonist, DL-APV (100 μM), (96.6 ± 1.0% of baseline, n=4 neurons from 4 animals, $p>0.05$, vs baseline) or the NR2B subunit-selective NMDA receptor antagonist, Ro 25-6981 (0.5 μM), (105.6 ± 1.2% of baseline, n=5 neurons from 4 animals, $p<0.05$ vs baseline, vs DL-APV). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug exposure group.

Figure 3. In vitro ethanol exposure, at increasing concentrations, differentially alters NAc LTD expression.



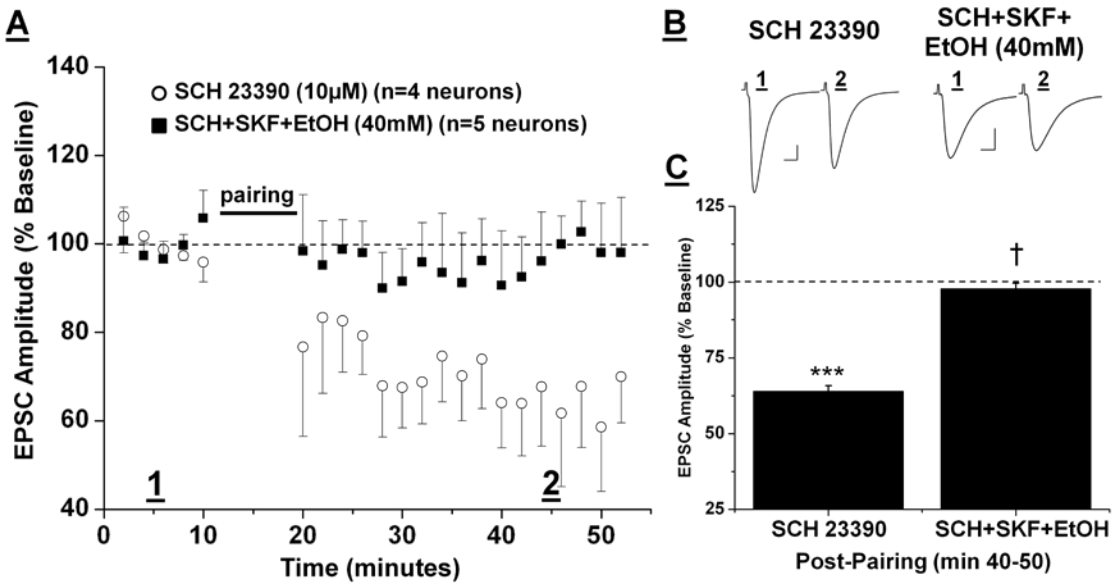
A, escalating concentrations of ethanol applied to the recording bath have varying effects on the magnitude of LTD. Maximum inhibition of LTD expression is achieved through application of ethanol (40 mM) (104.3 ± 1.3% of baseline, n=7 neurons from 6 animals, $p>0.05$, vs baseline). In the presence of ethanol (20 mM) and (60 mM), pairing stimulation induces an LTD magnitude that is decreased from control LTD but not completely occluded (79.7 ± 2.0% of baseline, n=6 neurons from 5 animals, *$p<0.05$, vs baseline, † $p<0.05$ vs 40mM) and (81.5 ± 0.7% of baseline, n=7 neurons from 6 animals, *$p<0.05$, vs baseline; † $p<0.05$ vs 40 mM; $p>0.05$, vs 20 mM) respectively. B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each ethanol exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each ethanol exposure group.

Figure 4. D1 receptor activation occludes ethanol (40 mM) inhibition of NAc LTD.



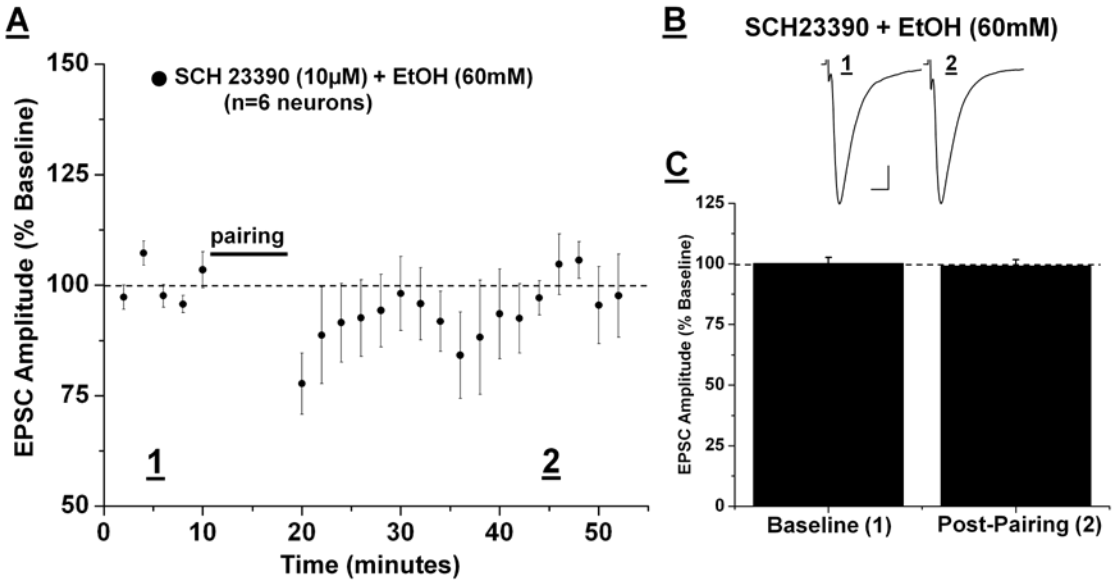
A, pre-treatment (30 min) of NAc slices with the D1 receptor agonist, (±)SKF38393 (50 μM), reverses ethanol inhibition of LTD expression (66.8 ± 1.5% of baseline, n=6 neurons from 5 animals, ***$p<0.001$, vs baseline; $p<0.05$, vs 40mM EtOH alone). Application of SKF38393 alone did not have any effect on LTD expression (65.9 ± 1.7% of baseline, n=7 neurons from 6 animals, ***$p<0.001$, vs baseline; $p>0.05$, vs SKF+EtOH). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug exposure group.

Figure 5. Ethanol recovers inhibition of NAc LTD when D1 activation is occluded.



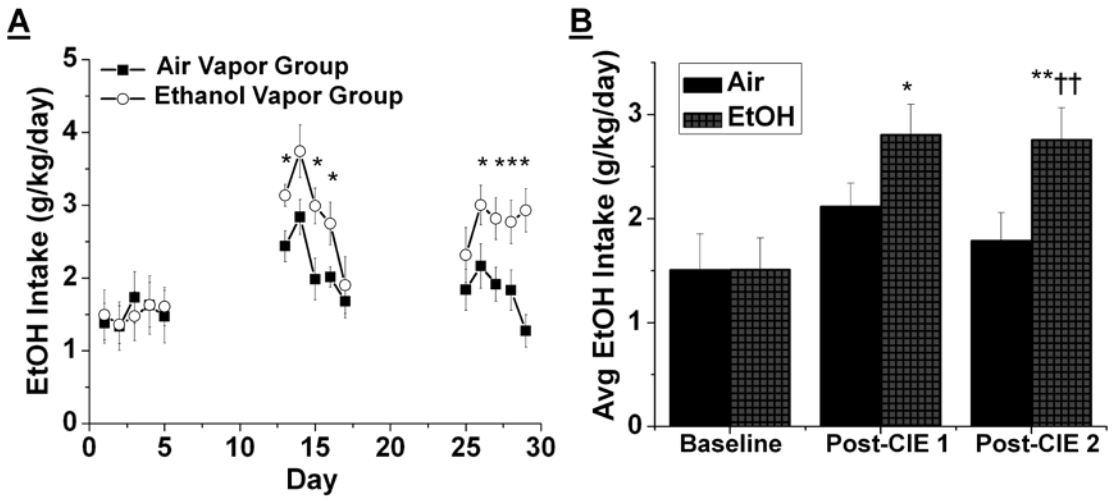
A, bath application of the D1 receptor antagonist, SCH23390 (10 μM), prior to SKF38393 pre-treatment, permits inhibition of LTD expression by ethanol (40 mM) similar to that observed with ethanol alone (97.8 ± 1.7% of baseline, n=5 neurons from 4 animals, $p>0.05$, vs baseline; † $p<0.05$ vs SCH23390 alone). SCH23390 alone does not alter the magnitude of LTD compared to control (63.9 ± 1.8% of baseline, n=4 neurons from 3 animals, ***$p<0.001$, vs baseline). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline, mean ± SEM, for average normalized EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug exposure group.

Figure 6. NAc LTD is completely inhibited by highest ethanol concentration (60mM) when D1 receptor activation is blocked.



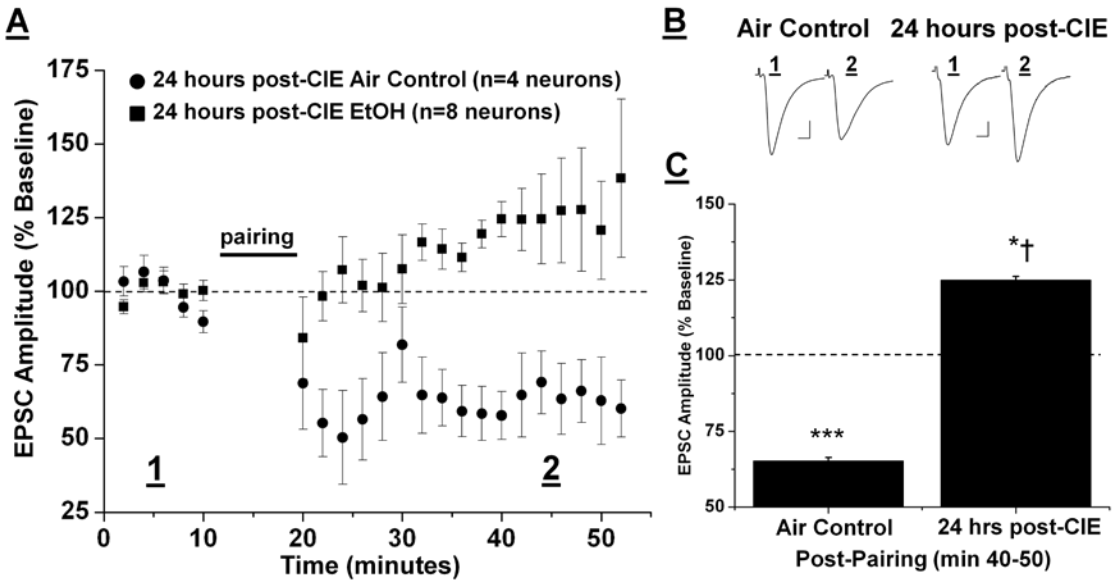
A, bath application of the D1 receptor antagonist, SCH23390 (10 μM), 20 min prior to ethanol (60 mM) completely occludes NAc LTD (99.1 ± 2.6% of baseline, n=6 neurons from 4 animals, $p>0.05$, vs baseline; $p<0.05$ vs 60mM EtOH alone). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative recording from all neurons recorded. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for this drug exposure group.

Figure 7. C57BL/6J mice increase voluntary ethanol consumption following two CIE exposures.



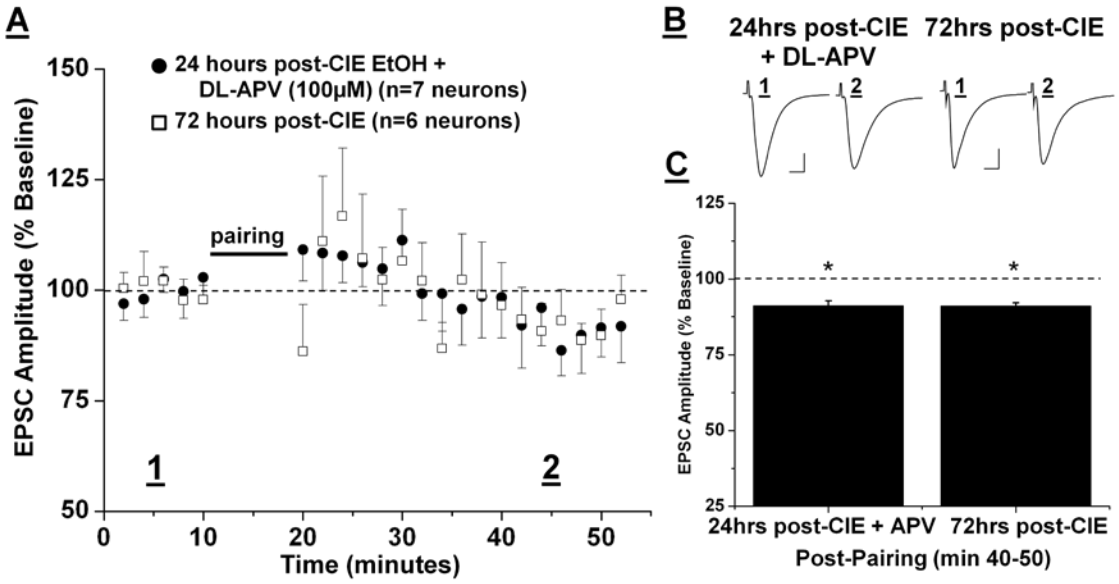
A, average daily EtOH intake (g/kg/day) in a 2 hour drinking bout begun 30 min prior to start of dark cycle over 5 days in a baseline (pre-vapor exposure) and following 2 successive periods of 4 days of 16 hr EtOH or air vapor exposure. Baseline EtOH intake was measured during the last five days of the 21-day baseline period. The CIE vapor exposure periods were delivered over days 6-9 and 18-21. Animals rested 74 hrs after the final vapor exposure before resuming drinking; *$p<0.05$ ethanol vs air group. B, bar graph representing average EtOH intake (g/kg/day), shown as mean ± SEM, for air and EtOH vapor treatment groups measured over each 5-day drinking period. Baseline ethanol intake did not differ between the air (1.51 ± 0.08) and EtOH vapor (1.51 ± 0.05) groups ($p>0.05$, student's t test, Bonferroni post-hoc). Only the ethanol vapor group significantly increased ethanol consumption after the first CIE vapor treatment compared to baseline (air, 2.19 ± 0.23, EtOH, 2.90 ± 0.33; *$p<0.05$). Following the second CIE treatment, the air group (1.75 ± 0.23) showed significantly lower ethanol intake than the EtOH group (2.77 ± 0.13) (†† $p<0.01$) (**$p<0.01$, ethanol post-CIE 2 vs baseline). N=8 animals per group.

Figure 8. CIE vapor exposure converts NAc LTD to synaptic potentiation.



A, 24 hours following three consecutive days in vivo CIE vapor exposure, pairing stimulation induces synaptic potentiation rather than LTD of EPSCs (124.9 ± 1.3% of baseline, n=8 neurons from 8 animals, *p<0.05, vs baseline; † p<0.05 vs air vapor control). In the air vapor control group, pairing stimulation induces a similar LTD to that observed in the ethanol-naïve control group (65.3 ± 1.1% of baseline, n=4 neurons from 3 animals, ***p<0.001 vs baseline). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each vapor (air or ethanol) exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each vapor (air or ethanol) exposure group.

Figure 9. Synaptic potentiation following CIE exposure is NMDAR-dependent and dissipates after 72 hours withdrawal.



A, 24 hours following three consecutive days in vivo CIE vapor exposure, synaptic potentiation is blocked by the NMDAR antagonist, DL-APV (100 μM), (91.2 ± 1.6% of baseline, n=7 neurons from 4 animals, *p<0.05, vs baseline, vs air vapor control, vs CIE EtOH 24hrs). Following 72 hours of withdrawal from the identical CIE vapor exposure, pairing stimulation does not induce either synaptic potentiation or LTD (91.1 ± 0.9% of baseline, n=5 neurons from 3 animals, *p<0.05, vs baseline, vs air vapor control, vs CIE EtOH 24hrs; p>0.05, vs CIE+APV). B, sample traces of averaged baseline and post-pairing EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug/vapor exposure group. Scale bars represent 5 ms (horizontal) and 50 pA (vertical). C, bar graph representing the percentage of baseline ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each drug/vapor exposure group.

Discussion

This report includes the following novel observations. First, that synaptic depression of glutamatergic excitatory transmission onto medium spiny neurons of the shell of the nucleus accumbens requires activation of NR2B-containing NMDA receptors. Second, acute ethanol exposure blocks NAc LTD in a differential manner depending on the concentration. Third, activation of D1-dopamine receptors completely occludes the ability of ethanol to inhibit NAc LTD. Fourth, a repeated regimen of intermittent ethanol exposure enhances voluntary ethanol intake in C57BL/6J mice, and such intermittent exposure induces NAc metaplasticity from LTD to LTP. Occlusion of both NAc LTD and LTP is retained for at least 72 hours following intermittent ethanol exposure.

NAc LTD, in vitro ethanol exposure and the role of NMDA and dopamine D1 receptors

Medium spiny neurons rest at more hyperpolarized membrane potentials (a "down-state" ~ -80 mV) and display transitions to a depolarized potential (the "up-state ~ -50 mV) coupled with an ensemble of action potentials. When differing patterns of conditioning stimulation are delivered to NAc MSNs at least three forms of synaptic depression may result (Berretta et al., 2008). The focus of this report is the only form expressed via NMDA receptor activation and decreased postsynaptic AMPA receptor function (Brebner et al., 2005). Two forms of presynaptic LTD, independent of NMDA receptor activation, have been demonstrated – these are induced by endocannabinoid or metabotropic glutamate receptor activation, are due to decreased glutamate release, and

occur following delivery of higher frequency synaptic stimuli than that used during NMDA-LTD (Robbe et al., 2002b; Robbe et al., 2002a).

In the present study, we also observed that the low frequency form of postsynaptic NAc LTD is NMDA receptor-dependent and additionally showed that this LTD is mediated by NR2B subunit-containing NMDA receptors. This is a novel result not previously documented concerning NAc LTD, yet it is not surprising since NR2B-containing receptors are highly expressed and targeted on NAc MSNs (Chapman et al., 2003; Dunah and Standaert, 2003). We did not investigate the involvement of NR2A-containing NMDARs in LTD expression given the lack of a specific pharmacological tool (Neyton and Paoletti, 2006). There is limited involvement of NR2C or NR2D-containing NMDA receptors since their NAc expression is nearly non-existent at this age (Standaert et al., 1994; Landwehrmeyer et al., 1995; Ghasemzadeh et al., 1996). The NR2A and NR2B subunits have been implicated to differentially contribute to LTP and LTD respectively in several brain regions (Liu et al., 2004; Massey et al., 2004; Zhao et al., 2005); however, these conclusions are controversial and specific contribution of one particular NMDA receptor subunit to exclusively LTD or LTP may not hold true in every instance (Berberich et al., 2005; Weitlauf et al., 2005; Morishita et al., 2007). Thus, we cannot derive any conclusions about the contributions of other subunits besides NR2B to LTD expression in the NAc and future studies will have to address the possibility.

Our observations demonstrate that pharmacologically-relevant concentrations (40 mM) of ethanol completely inhibit the expression of LTD when applied in vitro to a slice preparation from ethanol-naïve mice. Evidence exists for both promoting and inhibiting effects of ethanol on LTD expression in other brain regions including the hippocampus, dorsal striatum, and cerebellum (Hendricson et al., 2002; Izumi et al., 2005; Yin et al., 2007; Belmeguenai et al., 2008). These reports certainly support the likelihood that

ethanol modulates NAc LTD as we observe, but the different underlying mechanisms of plasticity expression interject a degree of complexity that tempers direct comparisons between these different reports.

Given the extensive literature that demonstrates ethanol inhibition of NMDA receptors, it is likely that a similar inhibition of NMDA receptors is the primary mechanism through which ethanol occludes the expression of NAc LTD. Excitatory transmission onto NMDA receptors is reduced by acute ethanol application in slice preparations of the hippocampus (Lovinger et al., 1989, 1990) and the nucleus accumbens (Nie et al., 1993; Nie et al., 1994; Maldve et al., 2002; Zhang et al., 2005; Wang et al., 2007). Our results indicate that both NR2B inhibition and application of ethanol prevent the expression of LTD, and it is tempting to speculate that ethanol blocks LTD expression by predominantly interacting with NR2B-containing NMDA receptors.

Our lab and others previously observed that activation of D1 receptors strongly suppressed the inhibitory effects of ethanol on NMDA receptor-mediated synaptic transmission dependent upon activation of DARPP-32 and phosphorylation of serine 897 of the NR1 subunit (Maldve et al., 2002; Zhang et al., 2005; Lin et al., 2006) but also see (Xu and Woodward, 2006). In the present report, we again observed that prior activation of D1-dopamine receptors rescues the expression of LTD in the NAc in the presence of ethanol. Thus, NAc LTD is ethanol-sensitive and displays characteristics consistent with NMDA receptor-mediated processes documented previously. One major goal is to determine how pharmacological manipulation of these processes can mitigate the long term effects of ethanol exposure and therefore may be therapeutic in ethanol dependence; hence, modulation of D1-dopamine signaling may give insights to novel therapeutic targets. Furthermore, the observation that a high concentration of ethanol (60 mM) did not occlude NAc LTD is consistent with the idea that such concentrations of ethanol can

induce dopamine release (Brodie et al., 1990; Brodie et al., 1999) and DARPP-32 phosphorylation directly (Maldve et al., 2002), Both of these effects therefore increase serine 897-NR1 phosphorylation and thereby decrease ethanol sensitivity of NMDA receptors (Maldve et al., 2002; Zhang et al., 2005; Lin et al., 2006). Therefore, the observation of NAc LTD in the presence of a high concentration of ethanol, which is occluded by D1 receptor inhibition, is consistent with prior observations.

IN VIVO CHRONIC ETHANOL EXPOSURE INCREASES INTAKE AND SIGNIFICANTLY ALTERS NAC LTD

One major hindrance for basic research into the neurobiological mechanisms of alcoholism has been the difficulties associated with development of a convenient animal model. Recently, a model based upon two 3-4 day regimens of passive ethanol administration via peripheral injection and subsequent 16 hour vapor inhalation (termed chronic intermittent exposure) to C57BL/6 mice has become widely adopted (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009b). In this study, we observed significant increases in voluntary ethanol consumption in our own cohort of animals following two bouts of CIE exposure. To test our hypothesis–as seen with chronic exposure to psychomotor stimulants–that occlusion of NAc LTD will result following CIE, we assessed the response of NAc MSNs to conditioning following a single bout of CIE as a baseline to determine the minimal level of ethanol exposure which might occlude LTD. Indeed, we were surprised that such a profound metaplasticity–a switch from LTD to LTP–occurred following such a brief ethanol exposure bout. We interpret such robust changes in NAc plasticity as an indicator of the potential importance of this process in ethanol neuroadaptation and therefore chose to first characterize this initial change in response to short-term CIE. Indeed, after a single 3-day cycle of this CIE

model, we contend that significant alterations in glutamatergic synaptic plasticity in the NAc have already begun.

While we observed increases in drinking at these short exposure durations, Becker and colleagues demonstrated that the maximum increases in voluntary ethanol consumption in mice are not observed until three days after withdrawal from CIE exposure. Thus, considerable study will be required to determine the temporal characteristics of the consolidation of this neuroadaptive response. Our results indicate that synaptic potentiation has subsided, while LTD remains occluded in the NAc shell at this same time point. Just as multiple CIE exposures are necessary to observe a sustained increase in ethanol consumption, we believe synaptic potentiation also persists after multiple CIE exposures. The current study indicates that synaptic activity is disrupted in the NAc shell at an early time point in a model of ethanol dependence. Future studies will address whether alterations in synaptic plasticity in the NAc shell directly contribute to the behaviors that lead toward increased voluntary ethanol consumption. The matter is made more complex since psychostimulants and ethanol have fundamentally distinct effects on NAc LTD. Chronic exposure to either drug appears to artificially induce an LTD-like state via occlusion of the response to in vitro conditioning stimulation. However, the unique NMDA receptor blocking actions of ethanol result in an additional potentiating action; thus, we contend that alterations in NAc shell glutamatergic synaptic function due to CIE exposure are fundamentally distinct from those of psychostimulants. Nonetheless, disruption of GluR2 internalization suppressed the sensitized response to psychostimulants (Brebner et al., 2005), and therefore, the likelihood remains that LTD occlusion is critical to ethanol-seeking behavior. However, the ethanol-dependent state may conceivably be further and uniquely driven by the resultant LTP and subsequent enhancement of glutamatergic transmission. Could blockade of synaptic potentiation in

the NAc after CIE exposure prevent an increase in voluntary ethanol consumption? Such possibilities deserve investigation. It is hoped that these results provide novel insights into the relevant contributions of NAc synaptic plasticity in the complex array of neuroadaptations that re-wire the motivational circuitry in the ethanol-dependent animal.

CHAPTER 3:

REVERSAL OF CELL TYPE-SPECIFIC EXPRESSION OF LONG-TERM DEPRESSION IN THE NUCLEUS ACCUMBENS SHELL

Abstract

Synaptic alterations in the nucleus accumbens (NAc) are crucial for the aberrant reward-associated learning that forms the foundation of drug dependence. Changes in NAc glutamatergic synaptic plasticity, in particular, are thought to be a vital component of the neurological underpinnings of addictive behavior. Medium spiny neurons (MSNs), the primary cell type in the NAc, are commonly divided into two major subtypes according to their expression of D1 or D2 dopamine receptors. Recent studies have begun to utilize bacterial artificial chromosome transgenic mice to investigate the cell type-specific synaptic modifications that occur in the NAc in response to drugs of abuse. Yet, few experimental findings related to ethanol exposure and NAc synaptic plasticity have been demonstrated. We studied the basic electrophysiological and synaptic properties NAc shell D1+ and D1- MSNs using whole-cell voltage and current clamp recording techniques. We observed that D1+ MSNs intrinsically more excitable than those lacking D1 receptors. Direct pathway synapses onto D1+ MSNs do not exhibit and increase in spontaneous excitatory neurotransmitter release subsequent to a 10Hz burst stimulation, whereas D1- MSNs do. Long-term depression (1 Hz-LTD) of glutamatergic excitatory transmission in the NAc shell is induced solely in D1+ but not D1- MSNs. 24 hours following a repeated regimen of in vivo chronic intermittent ethanol (CIE) vapor exposure 1 Hz-LTD is completely occluded in D1+ MSNs but present in D1- MSNs. Complete recovery of 1 Hz-LTD expression, or lack thereof, back to controls levels in

both D1+ and D1- MSNs respectively does not occur until 2 weeks of withdrawal from CIE vapor exposure. To our knowledge, this is the first demonstration of a reversal in the cell type-specificity of synaptic plasticity in the NAc shell, as well as, the gradual recovery of the pre-drug exposure plasticity state following extended withdrawal. We believe these observations highlight the adaptability of NAc MSNs to the effects of long-term ethanol exposure may represent a critical neuroadaptation underlying the development of ethanol dependence.

Introduction

The nucleus accumbens (NAc) has been extensively documented as an important brain region of convergence for the widespread projections involved in reward processing and guidance of goal-directed behaviors (Ikemoto, 2007). In the NAc, γ-aminobutyric acid (GABA)-ergic medium spiny neurons (MSNs) receive dopaminergic innervations from the ventral tegmental area and glutamatergic inputs from the prefrontal cortex, hippocampus, and amygdala. Accordingly, limbic region afferents to the NAc interface with motor control circuitry to regulate goal-directed behavior (Mogenson et al., 1980; Nicola et al., 2000; Wise, 2004). Persistent adaptive changes within the NAc in response to drugs of abuse are posited to underlie drug dependence (Koob and Le Moal, 2001; Kauer and Malenka, 2007). These adaptations have been shown to involve neuronal signaling and synaptic mechanisms similar to those implicated in neural models of learning and memory, in particular long-term synaptic plasticity (Nestler, 2001; Hyman et al., 2006). Specifically, long-term depression (LTD) of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)-mediated excitatory postsynaptic currents (EPSCs) normally present in drug-naïve animals is absent in MSNs from animals sensitized to the

behavioral effects of psychostimulants (Thomas et al., 2001; Brebner et al., 2005). In addition, LTD in the NAc core remained occluded in rats that had self-administered cocaine 21 days earlier (Martin et al., 2006). Thus, it has become increasingly apparent that drug-induced synaptic plasticity modifications may share a common mechanism that underlies dependence to drugs of abuse.

NAc MSNs are commonly divided into two major categories based on their expression of releasable peptides, dopamine receptor subtype expression, and their axonal projection targets (Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1990). Dopamine D1 receptor (D1R)-expressing and dopamine D2 receptor (D2R)-expressing MSNs characterize the striatonigral (direct) and striatopallidal (indirect) pathways, respectively (Alexander et al., 1986; Groenewegen et al., 1999). Given their opposing roles in modulating intracellular signaling pathways and their different outputs, researchers have begun investigating the functional differences in D1R- and D2R-expressing MSNs. To facilitate these efforts, bacterial artificial chromosome (BAC) transgenic mice in which expression of enhanced green fluorescent protein (eGFP) is controlled by D1R or D2R promoters (Gong et al., 2003) have been created and subsequently provided insights into the specific synaptic characteristics of both MSN subtypes in the dorsal striatum and NAc core (Cepeda et al., 2008; Grueter et al., 2010). A recent report demonstrated that activation of D1+ MSNs in the NAc enhanced cocaine sensitization and conditioned place preference, whereas activating D2+ MSNs diminished these behaviors (Lobo et al.). Additionally, permanent disruption in N-methyl-D-aspartate receptor (NMDAR) LTD in the NAc facilitates the transition of rats to an "addicted" behavioral state (Kasanetz et al., 2010).

While there is extensive literature detailing how alterations in NAc signaling are important in behavioral responses to psychostimulants, reports investigating NAc

synaptic plasticity and ethanol are few. In fact, our lab recently published the first report demonstrating a disruption in NAc shell NMDAR-dependent LTD following in vivo ethanol exposure. In that study, we observed that chronic intermittent ethanol (CIE) exposure reverses the polarity of synaptic plasticity in the NAc shell, converting LTD to synaptic potentiation (Jeanes et al., 2011). Toward the conclusion of that study, we suspected that our observed control LTD magnitudes originated from two distinct populations of MSNs and confirmed a bimodal distribution upon post-hoc analysis (unpublished observations). The psychostimulant literature also strongly supports the notion of drug-induced cell-type specific modifications in the NAc. In addition, recent reports have shown NAc dopamine receptor specific modulation of ethanol-related behaviors. One study revealed that siRNA-mediated downregulation of D1R in the NAc decreased ethanol intake and preference, behavioral sensitization to ethanol, as well as acquisition, but not expression of ethanol-induced CPP (Bahi and Dreyer, 2012), while another demonstrated that reinstated ethanol seeking in rats was dose-dependently attenuated by blocking D1Rs in the NAc (Chaudhri et al., 2009). Taken together, these observations formed the impetus for our investigation of the cell type-specificity of basal NAc shell LTD and its conversion to synaptic potentiation subsequent to CIE exposure.

In the current study, we used BAC transgenic mice that express eGFP in direct pathway MSNs that express dopamine D1 receptors to determine basal electrophysiological and synaptic properties in both D1+ and D1- (putative D2 receptor-expressing) MSNs. We then investigated how D1+ and D1- MSNs in the NAc shell expressed 1 Hz-LTD in ethanol-naïve and CIE-exposed mice at several withdrawal time points. Collectively, we observed distinct basal synaptic properties between D1+ and D1- MSNs, as well as, significant alterations in synaptic plasticity in a cell-type specific manner before and after CIE exposure that return to pre-CIE levels after extended

withdrawal. These findings could provide crucial insight into the cell-type specific synaptic adaptations within the NAc shell, which are thought to contribute to the development of ethanol dependence.

Materials and Methods

SUBJECTS

Dopamine D1a receptor (drd1a) promoter-dependent eGFP BAC transgenic mice, generated by the GENSAT (Gene Expression Nervous System Atlas) project, were purchased from the Mutant Mouse Regional Resource Center and outcrossed onto the Swiss Webster background to create hemizygous progeny. Mice were housed under a 12-h light/dark cycle (lights on at 0700 hours) and cared for by the University of Texas at Austin Animal Resource Center. Food and water were available ad libitum, and all of the following experimental procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

BRAIN SLICE PREPARATION

Parasagittal slices (210-250 μm thick) containing the NAc were prepared from the brains of 4-8 week-old male hemizygous eGFP transgenic mice outcrossed onto the Swiss Webster background (Harlan Laboratories, Indianapolis, IN). Mice were lightly anesthetized with isofluorane, and the brains were rapidly removed and placed in ice-cold (4ºC) oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 87 NaCl, 25 NaHCO3, 1.25 NaH2PO4, 2.5 KCl, 7 MgCl2, 0.5 CaCl2, 25 dextrose, 75 sucrose, bubbled with 95% O2/5% CO2. Sagittal slices were cut and then transferred to an incubation ACSF for a minimum of 60 minutes prior to recording that contained the

following (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 2.4 MgCl2, 1.8 CaCl2, 10 dextrose, bubbled with 95% O2/5% CO2; pH 7.4, 32oC. Unless otherwise noted, all drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

PATCH CLAMP ELECTROPHYSIOLOGY

We conducted all recordings at 31-33oC in ACSF containing (in mM): 120 NaCl, 25 NaHCO3, 1.23 NaH2PO4, 3.3 KCl, 0.9 MgCl2, 2 CaCl2, 10 dextrose, bubbled with 95% O2/5% CO2. The GABAA receptor antagonist, picrotoxin (50 μM), was added to the external recording solution throughout all recordings to inhibit GABAA receptor-mediated synaptic currents and improve the reliability of synaptic plasticity in the dorsal and ventral striatum by favoring postsynaptic depolarization during conditioning stimuli (Berretta et al., 2008). Whole-cell voltage and current clamp recordings were obtained from NAc shell eGFP(+) (referred to as D1+) and eGFP(-) (referred to as D1-) MSNs visually identified using a mercury lamp with a GFP filter on an Olympus BX-50WI microscope (Leeds Instruments, Irving, TX) mounted on a vibration isolation table. MSNs represent ~90-95% of the neurons in the NAc and have distinctly smaller cell bodies (about 10 μm in diameter) than cholinergic or GABA interneurons. MSNs were also identified by their highly negative resting membrane potential (less than -75 mV), presence of inward rectification to hyperpolarizing current injection, and prolonged ramp to firing first action potential. MSNs from the most rostral and ventral areas of the NAc were chosen to make sure all recordings arose from the NAc shell. Only one neuron per slice was used for recording and once 1 Hz-LTD induction stimuli were initiated in a slice, that slice was discarded whether the recording lasted the full length or not. ACSF continuously perfused the recording chamber at 2.0-2.5 mL/min. Recording electrodes

(thin-wall glass, WPI Instruments) were made using a Brown-Flaming model P-88 electrode puller (Sutter Instruments, San Rafael, CA) to yield resistances between 3-5 MΩ and contained (in mM): 135 KMeSO4, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Tris-GTP, (pH 7.3 with KOH). Input and access resistances were monitored throughout all experiments, and the recording was terminated if either resistance varied by more than 20%. These parameters were measured by application of a -10 mV, 100 ms voltage step at 5-10 min intervals. Synaptic currents were monitored at a holding potential of -80 mV. Changes in the holding current were observed to detect any resealing or other instability of the patch.

DATA ACQUISITION AND ANALYSIS

Excitatory afferents, the majority of which arise from the prefrontal cortex, were stimulated with a stainless steel bipolar stimulating electrode (FHC, Inc., Bowdoin, ME) placed between the recorded MSN and prefrontal cortex, typically 150-300 μm from the MSN cell body. EPSCs were acquired using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 1 kHz, and digitized at 10-20 kHz via a Digidata 1440A interface board using pClamp 10.2 (Axon Instruments, Foster City, CA). Standard evoked excitatory postsynaptic currents (EPSCs) elicited by local stimulation were established in NAc shell MSNs for at least 10 minutes (at 0.1 Hz) to ensure stable recordings. Paired pulse ratios (PPRs) were measured at 50-200-msec interstimulus intervals and calculated as the ratio of the second EPSC to the first EPSC amplitude. LTD induction was assessed by delivering conditioning stimuli (500 pulses at 1 Hz at baseline stimulation intensity) while continuously and simultaneously depolarizing the postsynaptic cell to -50 mV (referred to below as conditioning stimulation). EPSCs were

then monitored for 30-45 minutes post-pairing (at 0.1 Hz). For spontaneous EPSC recordings, quantal events (2 recordings, 20 sweeps each, 9.375 sec/sweep) were detected using the template sEPSC detection protocol contained within Clampfit (pClamp v10.2, Axon Instruments, Molecular Devices). Quantal events with > 5 pA amplitude were selected for analysis and verified by eye, while events > 100 pA (though quite rare, ~ 1 event per 2-3 cells) were excluded from analysis.

Spike firing, intrinsic membrane properties, and peak EPSC amplitude values were determined using Clampfit 10.2 software (Axon Instruments). For each plasticity recording, peak EPSC amplitude values were normalized to the average EPSC amplitude of the final 10 minutes of baseline (60 sweeps) for that single recording. The mean normalized EPSC amplitudes for 12 consecutive sweeps were condensed into 2 minute bins and represented as a single data point in scatter plots for each treatment group. Each data point represents the average of 12 consecutive EPSC amplitudes at that time point from each neuron within its respective treatment group. We used two parameters to determine whether plasticity of EPSC amplitudes (either depression or potentiation) occurred. A paired Student's t-test (p value < 0.5) was used to compare the normalized EPSC amplitudes from 20 to 30 minutes (minutes 40-50 on figures) after the pairing protocol to the normalized EPSC values during the last 10 minutes of baseline. In addition, the change in average EPSC amplitude after conditioning needed to be greater than 2 standard deviations from baseline. If both of these criteria were met, that treatment group was determined to exhibit plasticity (either depression or potentiation).

For each experiment, the 40-50 minute time period was used to compare the magnitude of plasticity after different drug exposures. Each cell was given a single average normalized EPSC value from min 40-50, which were then compiled for each experimental group and compared between groups using a single factor ANOVA with

Bonferroni post-hoc analyses. Statistical significance for between treatment group comparisons was defined as p value < 0.05. Thus, LTD was considered the control outcome to which all drug (either in vitro or in vivo) exposures were compared. LTD was determined to be reduced and not completely blocked in situations where the post-pairing average EPSC amplitude (min 40-50) was significantly increased from control LTD (ANOVA) and significantly decreased from its respective baseline (Student's t test). Experiments testing different antagonists were interleaved with control experiments using slices prepared from the same animals where possible.

CHRONIC INTERMITTENT ETHANOL EXPOSURE

Ethanol dependence was induced by exposing mice to chronic intermittent ethanol vapor (Becker and Hale, 1993; Becker and Lopez, 2004; Lopez and Becker, 2005). Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 95-120 mL/min. The resulting ethanol vapor then combined with a separate air stream to give a total flow rate of 2.5-3 L/min which was delivered to mice in airtight mouse chamber units (Allentown Inc., Allentown, NJ). These chambers resembled normal acrylic cages but contain an additional air-tight seal top, a vapor inlet, and an exhaust outlet. Food and water was available ad libitum on the wire cage tops. The ethanol flow rate was determined empirically to yield target blood ethanol concentrations (35-45 mM, or 150-200 mg/dL) measured from a 10 µL tail blood sample using an Analox AM1 alcohol analyzer (Analox, Lunenberg, MA). Two identical cages of mice were always run simultaneously; one cage for exposure to ethanol vapor and the second cage for an air only control. The ethanol group received a single, daily intraperitoneal injection containing both ethanol (20% v/v, 1.5 g/kg) and pyrazole (68 mg/kg) in sterile PBS.

Mice were then immediately chambered and exposed to ethanol vapor or air (from 1700 to 0900 hrs daily under a reverse light/dark cycle- lights off at 1200 hrs) for four consecutive days. Air control mice received only the pyrazole injection but were otherwise handled exactly as the ethanol group. On the fifth day, animals were returned to home cages for 24-h, 72-h, 1 week, or 2 weeks (depending on experiment). On the day corresponding to each specific withdrawal time point, electrophysiological experiments were performed as described above.

Results

BASIC MEMBRANE PROPERTIES OF NAC SHELL MSNS IN SLICES

We prepared parasagittal slices containing the NAc shell from BAC transgenic mice that expressed eGFP in D1+ MSNs of the direct pathway. We observed different basal electrophysiological and synaptic properties between D1+ and D1- MSNs of the NAc shell, including a greater basal frequency of spontaneous excitatory postsynaptic currents (sEPSCs) in D1+ MSNs (5.50 ± 0.57 Hz; n=18, Fig. 10a) than in D1- MSNs (3.69 ± 0.45 Hz; n=16; $p<0.05$; Fig. 10b), yet the amplitude of sEPSCs was not significantly different between D1+ and D1- MSNs (14.24 ± 0.58 and 15.87 ± 0.93 pA, respectively).

Consistent with these results, the paired-pulse ratios of EPSCs, which inversely correlate with the probability of neurotransmitter release, were lower in D1+ MSNs (50ms, 1.31 ± 0.12; 100 ms, 1.13 ± 0.06; 150ms, 1.10 ± 0.04; 200ms, 1.05 ± 0.05; n=7; $p<0.05$) than in D1- MSNs (50ms, 1.69 ± 0.14; 100 ms, 1.46 ± 0.12; 150ms, 1.39 ± 0.12; 200ms, 1.33 ± 0.12; n=5; Fig. 11a). In whole-cell current clamp recordings, we detected a more hyperpolarized firing threshold in D1+ MSNs (-41.53 ± 0.69mV; n=12) than in

D1- MSNs (-38.01 ± 0.85mV; n=11; $p<0.05$; Fig. 11b) but found no difference between D1+ and D1- MSNs in resting membrane potential (-76.64 ± 0.95 and -76.78 ± 0.74 mV) action potential amplitude (77.90 ± 2.12 and 77.15 ± 2.21 mV), half-amplitude duration (0.86 ± 0.05 and 0.84 ± 0.03 ms), or afterhyperpolarization amplitude (9.20 ± 0.45 and 9.36 ± 0.36 mV) (Fig. 11b). In addition, we observed significantly higher frequencies of action potential firing in response to fixed current injections in D1+ MSNs (250pA, 14.93 ± 3.24; 300pA, 16.93 ± 2.56 Hz; n=10; Fig. 11c,d) than in D1- MSNs (250pA, 6.19 ± 2.52Hz; 300pA, 8.47 ± 1.43Hz; n=7; $p<0.05$; Fig. 11c,d). These results show that synapses onto D1+ MSNs of the direct pathway in the NAc shell express several properties distinct from those synapses onto D1- MSNs of the indirect pathway, which indicate that D1+ MSNs are intrinsically more excitable than D1- MSNs.

SYNAPTIC RESPONSES OF D1+ AND D1- MSNS TO BURSTS OF AFFERENT STIMULI

A previous study demonstrated an increase in spontaneous excitatory neurotransmitter release onto NAc MSNs following a burst of afferent stimuli designed to mimic the synaptic excitation necessary to elicit the depolarized up state characteristic of both striatal and accumbal MSNs (Lape and Dani, 2004). We next examined whether the increased basal excitability of D1+ MSNs hindered the direct pathway synapses from significantly amplifying glutamate release onto these neurons. Immediately after a 10-Hz, 15-stimuli train, D1+ MSNs did not show a significant increase in spontaneous synaptic activity (pre-train, 5.55 ± 1.21 Hz; post-train, 7.01 ± 1.05 Hz; n=6; $p>0.05$; Fig. 12a), while D1- MSNs responded with a significant increase in sEPSC frequency (pre-train, 3.49 ± 0.55 Hz; post-train, 5.12 ± 0.51 Hz; n=7; $p<0.05$; Fig. 12b). Both D1+ and D1- MSNs failed to show a significant change in sEPSC amplitude (pre-train, 14.0 ± 1.72

pA; post-train, 13.0 ± 1.36 pA and pre-train, 13.09 ± 1.31 pA; post-train, 13.86 ± 1.16 pA for D1+ and D1- MSNs respectively), which suggested that the increase in post-train synaptic activity in D1- MSNs was solely presynaptic in origin.

In addition, the 10-Hz burst of stimuli produced a facilitation of EPSCs in D1- MSNs, indicated by the second to fourth stimuli of the train evoking larger currents than the first one, while D1+ MSNs responded with a persistent depression of EPSCs throughout the stimulus train (Fig. 12c). The average normalized EPSC amplitudes of D1+ MSNs were significantly depressed (71.06 ± 2.34%; n=8; $p<0.01$) compared to the first stimulus of the train, whereas D1- MSNs showed a significant enhancement of EPSC amplitude throughout the 10-Hz train (111.51 ± 2.72%; n=6; $p<0.01$; Fig. 12c).

NMDA RECEPTOR-MEDIATED 1-HZ LTD IS PREFERENTIALLY EXPRESSED BY D1+ MSNS IN THE NAC SHELL

Previous reports, including one from our own lab, have shown that low frequency stimulation (500 pulses @ 1 Hz) paired with postsynaptic depolarization to -50 mV induces an NMDA receptor-dependent LTD of AMPA-mediated responses in the NAc (Thomas et al., 2000; Brebner et al., 2005; Jeanes et al., 2011). We utilized an identical stimulation protocol while recording from either D1+ or D1- MSNs and observed a robust LTD of synaptic responses in the D1+ MSNs (64.02 ± 5.63% of baseline; n=8; $p<0.001$ vs baseline). Neither depression nor potentiation was observed in the D1- MSNs (90.99 ± 4.23% of baseline; n=7; $p<0.01$ vs D1+ MSNs; Fig. 13a). The 1 Hz-LTD in D1+ MSNs was completely blocked in the presence of the NMDA receptor antagonist, DL-APV (100 µM), (95.35 ± 1.56% of baseline; n=6; $p<0.001$ vs D1+ control; Fig. 13b). Similarly, inclusion of ethanol (40 mM) in the bath perfusion solution entirely blocked 1

Hz-LTD in D1+ MSNs (100.29 ± 7.27% of baseline; n=7; $p<0.01$ vs D1+ control; Fig. 13c)

Activation of dopamine D1 receptors on D1+ MSNs via bath application of the selective D1-like receptor agonist, ±SKF38393 (50 μM), did not significantly alter the magnitude of LTD expression in D1+ MSNs of the NAc shell (71.86 ± 5.96% of baseline; n=5; $p<0.05$ vs baseline, $p>0.05$ vs D1+ control; Fig. 13d). However, in the presence of the selective D1-like receptor antagonist, SCH23390 (10 μM), we observed a reduced 1-Hz LTD in D1+ MSNs that was significantly different from baseline and control LTD (80.26 ± 1.71% of baseline; n=5; $p<0.001$ vs baseline, $0.05> p >0.01$ vs control; Fig. 13e).

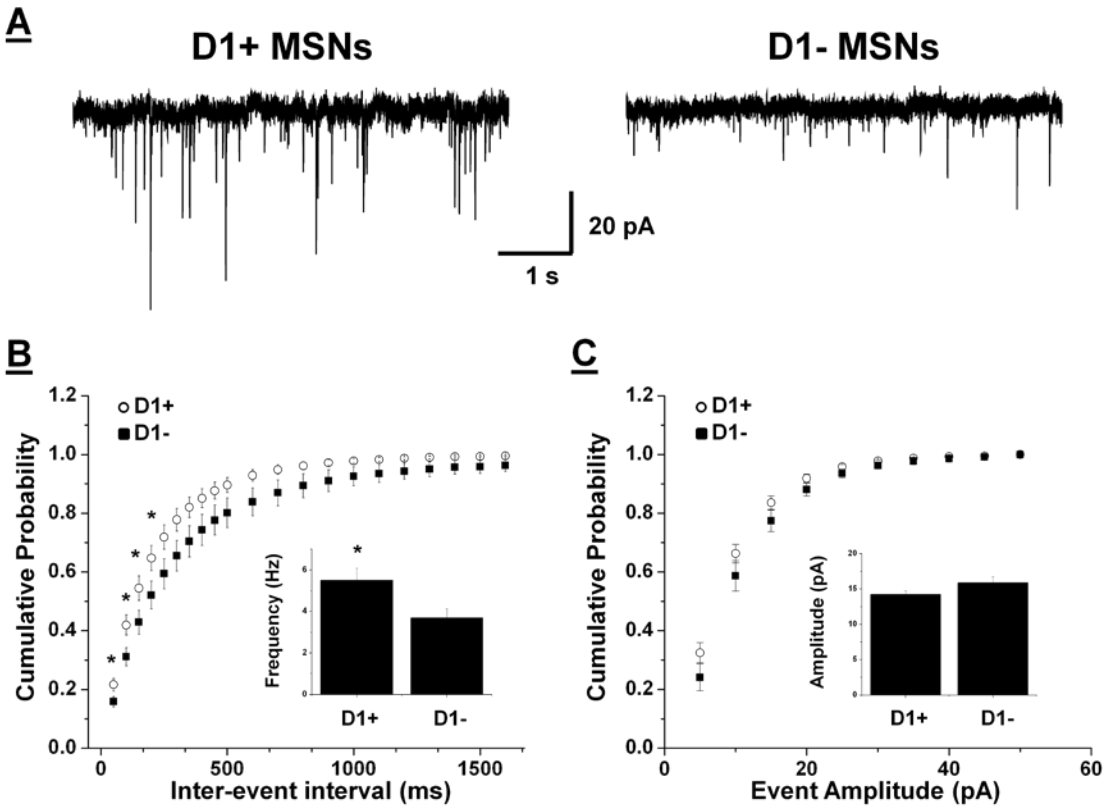
LTD EXPRESSION SWITCHES FROM D1+ TO D1- MSNS FOLLOWING IN VIVO CIE EXPOSURE AND RECOVERS OVER EXTENDED WITHDRAWAL

We previously published an observation detailing the conversion of synaptic depression to potentiation following in vivo chronic intermittent ethanol (CIE) vapor exposure and 24 hours withdrawal (Jeanes et al., 2011). We next chose to investigate whether such a synaptic potentiation would be observed following CIE and if it is preferentially expressed in either D1+ or D1- MSNs. In an air vapor control group, we observed 1 Hz-LTD in D1+ MSNs (60.82 ± 6.75% of baseline; n=5; $p<0.01$ vs baseline, $p>0.05$ vs D1+ control) but not in D1- MSNs (93.01 ± 4.57% of baseline; n=5; $p<0.01$ vs D1+ 24hr, $p>0.05$ vs D1- control; Fig. 14a) matching our findings in EtOH-naïve mice. Interestingly, we did not detect synaptic potentiation in either D1+ or D1- MSNs in slices prepared from mice 24 hours following 4 days in vivo CIE vapor exposure. Instead, we observed an occlusion of LTD in D1+ MSNs (96.31 ± 4.61% of baseline; n=7; $p<0.001$

vs Air control) and the presence of a robust 1 Hz-LTD in D1- MSNs (56.18 ± 6.97% of baseline; n=7; $p<0.01$ vs baseline, vs Air control, $p<.001$ vs D1+ 24hr; Fig. 14b).

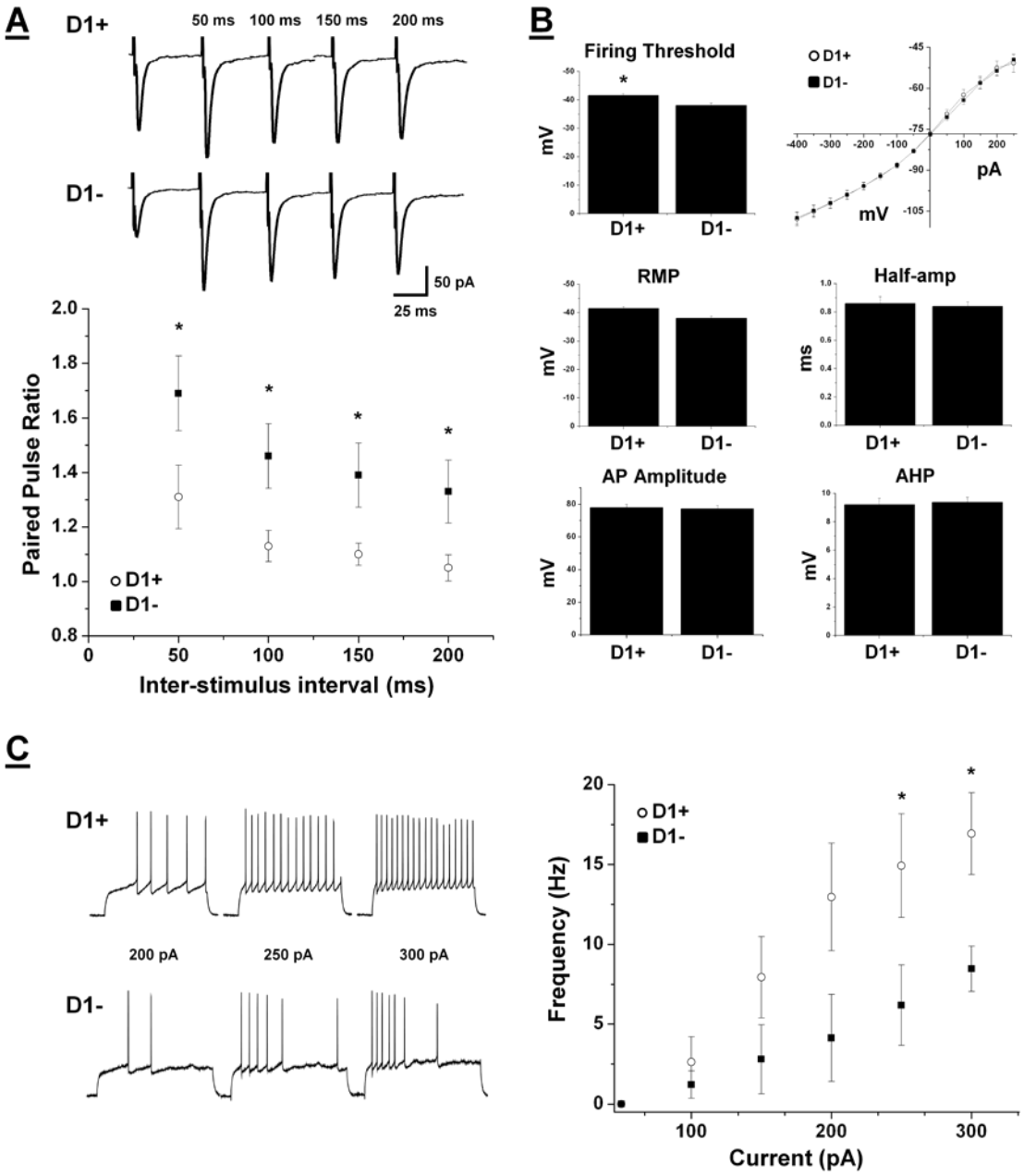
Further withdrawal (72 hours) from CIE vapor exposure resulted in modest synaptic depression for D1+ MSNs (83.95 ± 4.58% of baseline; n=5; $p<0.05$ vs baseline) that had yet to return to Air control levels, whereas 1 Hz-LTD present in D1- MSNs at 24 hours post-CIE was reduced (75.54 ± 5.16% of baseline; n=5; $p<0.01$ vs baseline; Fig. 15a). Not until one week of withdrawal did 1 Hz-LTD return close to Air control levels in D1+ MSNs (74.97 ± 5.23% of baseline; n=6; $p<0.01$ vs baseline), yet in D1- MSNs a small synaptic depression remained (80.36. ± 11.94% of baseline; n=6; Fig. 15b) that did not differ from the magnitude of LTD observed in D1+ MSNs. Given the observed emerging trend in withdrawing D1+ and D1- MSNs to return to a pre-CIE synaptic plasticity state, we chose to extend withdrawal following CIE vapor exposure even further to two weeks. Indeed, two weeks of withdrawal were necessary to reveal a robust 1 Hz-LTD in D1+ MSNs (53.32 ± 5.63% of baseline; n=5; $p<0.001$) indistinguishable from air control and ethanol-naïve groups. Likewise, we observed a transition back to control levels in D1- MSNs with a complete absence of synaptic depression (99.33 ± 14.51% of baseline; n=4, $p<0.001$ vs D1+ MSNs; Fig. 15c). A summary of the average normalized post-pairing EPSC amplitudes between minutes 40-50 in all CIE treatment groups, as well as, air control and ethanol naïve groups is shown in Figure 15d.

Figure 10. Spontaneous EPSC frequency is greater in D1+ MSNs.



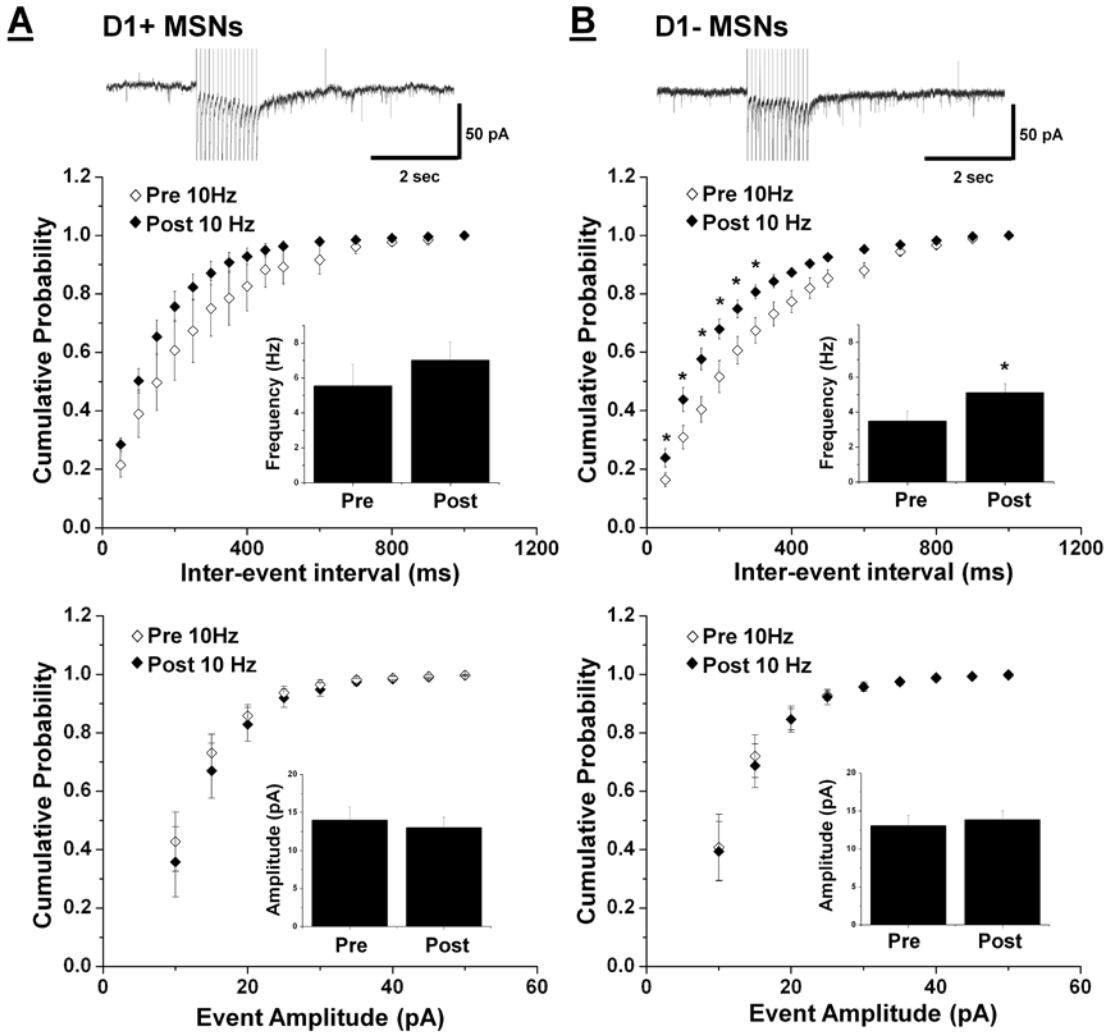
A, sample traces of sEPSCs from representative D1+ and D1- MSNs; scale bars represent 1 sec (horizontal) and 20 pA (vertical). B, cumulative probability plots of sEPSC inter-event intervals, data grouped into 50 ms bins; bar graph representing average sEPSC frequency ± SEM for D1+ (5.50 ± 0.57 Hz; n=18 neurons from 15 animals) and D1- MSNs (3.69 ± 0.45 Hz; n=16 neurons from 14 animals; *$p<0.05$ vs D1+ MSNs). C, cumulative probability plots of average sEPSC event amplitudes, data grouped into 5 pA bins, amplitudes < 5 pA were discarded; bar graph showing average sEPSC amplitude in D1+ (14.24 ± 0.58 pA) and D1- MSNs MSNs (15.87 ± 0.93 pA; $p>0.05$ vs D1+ MSNs). Recordings made in the presence of picrotoxin (50 µM).

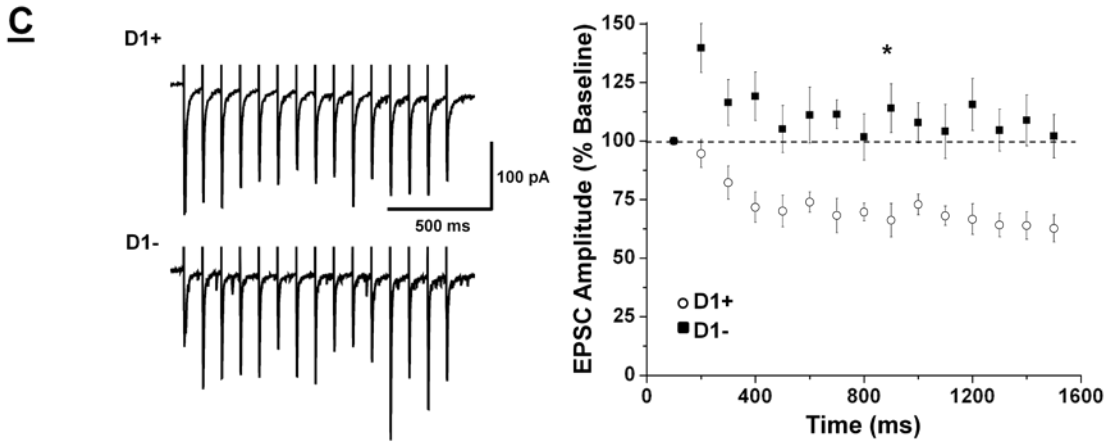
Figure 11. D1+ MSNs exhibit increased paired-pulse ratio, action potential firing, and a more hyperpolarized threshold.



A, paired pulse ratio (PPR) sample EPSC traces (inter-stimulus intervals of 50 ms, 100 ms, 150 ms, and 200 ms) from representative D1+ and D1- MSNs; scale bars represent 25 ms (horizontal) and 50 pA (vertical); graph of average PPR (determined by taking ratio of EPSC 2/EPSC 1) ± SEM for each inter-stimulus interval for D1+ (50ms, 1.31 ± 0.12; 100 ms, 1.13 ± 0.06; 150ms, 1.10 ± 0.04; 200ms, 1.05 ± 0.05; n=7 neurons from 5 animals) and D1- MSNs (50ms, 1.69 ± 0.14; 100 ms, 1.46 ± 0.12; 150ms, 1.39 ± 0.12; 200ms, 1.33 ± 0.12; n=5 neurons from 5 animals; *$p<0.05$, vs D1+ MSNs, for each inter-stimulus interval). B, bar graph representing average basal cellular parameters ± SEM, with only significant difference in action potential firing threshold determined from fixed current injections in D1+ (-41.53 ± 0.69 mV; n=12 neurons from 12 animals) and in D1- MSNs (-38.01 ± 0.85 mV; n=11 neurons from 11 animals; *$p<0.05$, vs D1+ MSNs). C, sample traces of action potential firing subsequent to fixed current injections in representative D1+ and D1- MSNs; graph representing average frequency ± SEM of action potential firing in response to depolarizing current injections (50-300 pA, 50 pA steps) in D1+ (250 pA, 14.93 ± 3.24; 300 pA, 16.93 ± 2.56 Hz; n=10 neurons from 7 animals) and D1- MSNs (250 pA, 6.19 ± 2.52 Hz; 300 pA, 8.47 ± 1.43 Hz; n=7 neurons from 5 animals; *$p<0.05$, vs D1+ MSNs). Paired-pulse recordings made in the presence of picrotoxin (50 µM), but AP firing were made in normal ACSF.

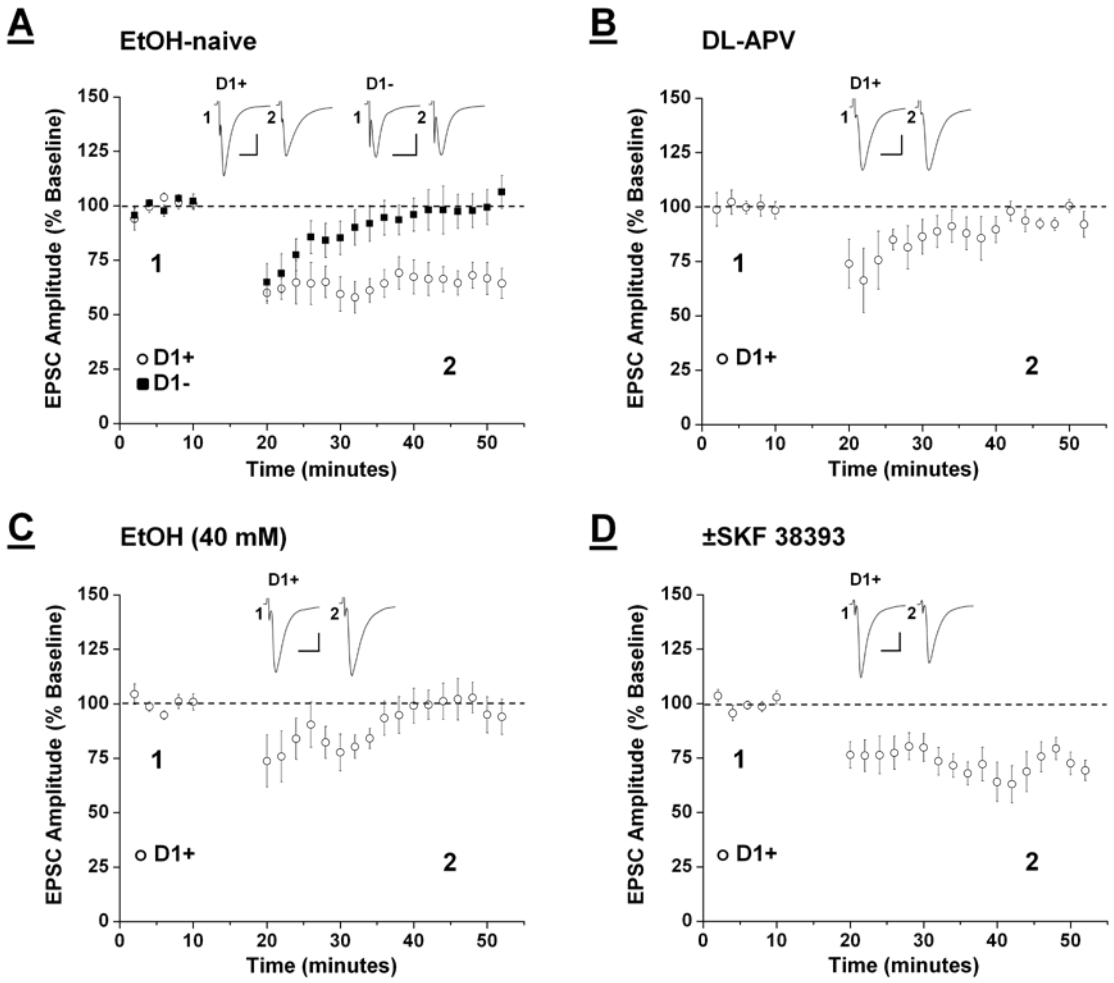
Figure 12. 10 Hz burst stimulation does not increase spontaneous or evoked excitatory synaptic input onto D1+ MSNs.

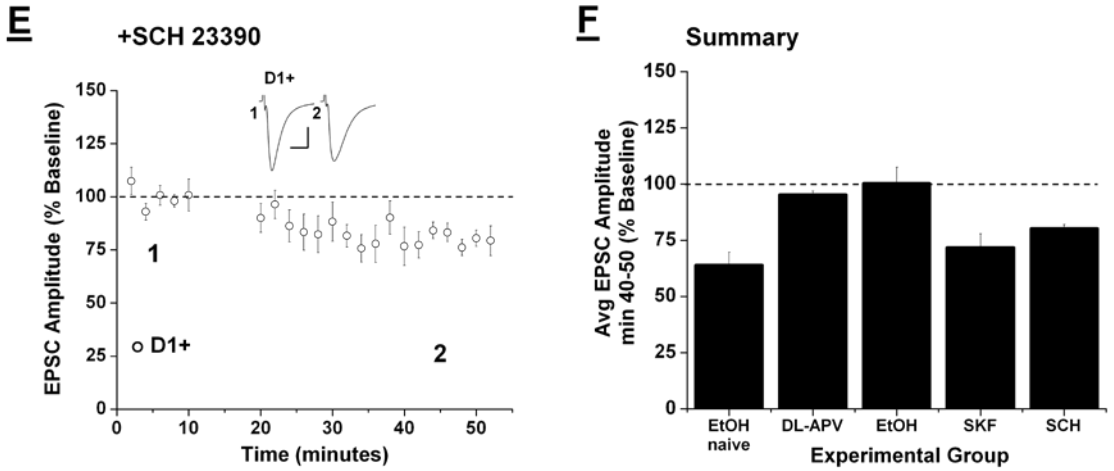




A, sample trace of sEPSCs before and after 10 Hz burst stimulation in a representative D1+ MSN; cumulative probability plots of sEPSC inter-event intervals, data grouped into 50 ms bins; bar graph representing average sEPSC frequency ± SEM (pre-train, 5.55 ± 1.21 Hz; post-train, 7.01 ± 1.05 Hz; n=6 neurons from 6 animals) cumulative probability plots of average sEPSC event amplitudes, data grouped into 5 pA bins, amplitudes < 5 pA were discarded; bar graph showing average sEPSC amplitudes (pre-train, 14.0 ± 1.72 pA; post-train, 13.0 ± 1.36 pA). B, sample trace of sEPSCs before and after 10 Hz burst stimulation in a representative D1- MSN; cumulative probability plots of sEPSC inter-event intervals, data grouped into 50 ms bins; bar graph representing average sEPSC frequency ± SEM (pre-train, 3.49 ± 0.55 Hz; post-train, 5.21 ± 0.51 Hz; n=7 neurons from 6 animals; *p<0.05, pre-train vs post-train); cumulative probability plots of average sEPSC event amplitudes, data grouped into 5 pA bins, amplitudes < 5 pA were discarded; bar graph showing average sEPSC amplitudes (pre-train, 13.09 ± 1.31 pA; post-train, 13.86 ± 1.16 pA). C, sample traces of evoked EPSCs during 10 Hz burst stimulation of representative D1+ and D1- MSNs; average normalized EPSC amplitude (% of first EPSC in 15 stimulation burst) ± SEM during the 10 Hz burst in D1+ (71.06 ± 2.34%; n=8 neurons from 6 animals; **p<0.01, vs 1st EPSC in train) and D1- MSNs (111.51 ± 2.72%; n=6 neurons from 6 animals; **p<0.01, vs 1st EPSC in train, vs corresponding D1+ EPSC).

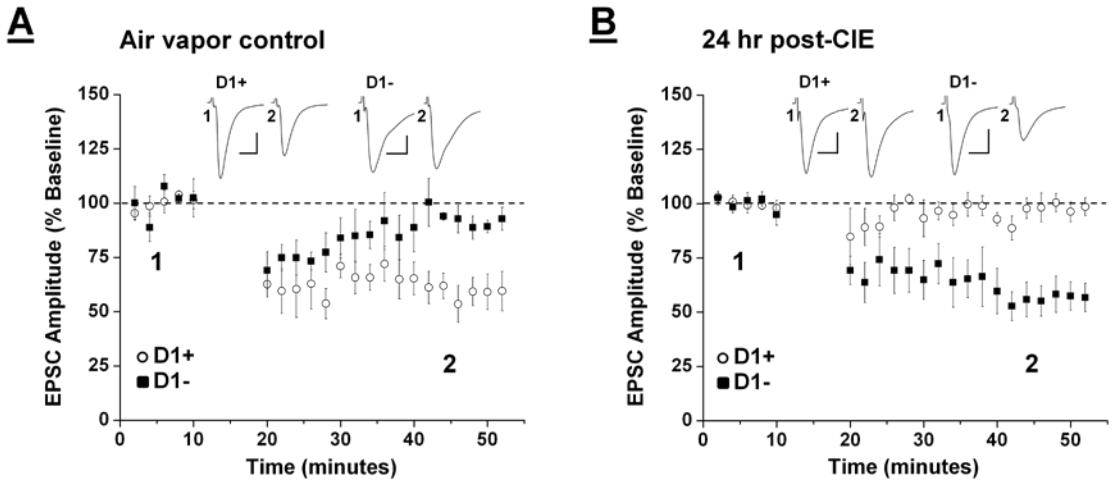
Figure 13. D1+ MSNs express NMDAR-dependent LTD in the NAc shell.





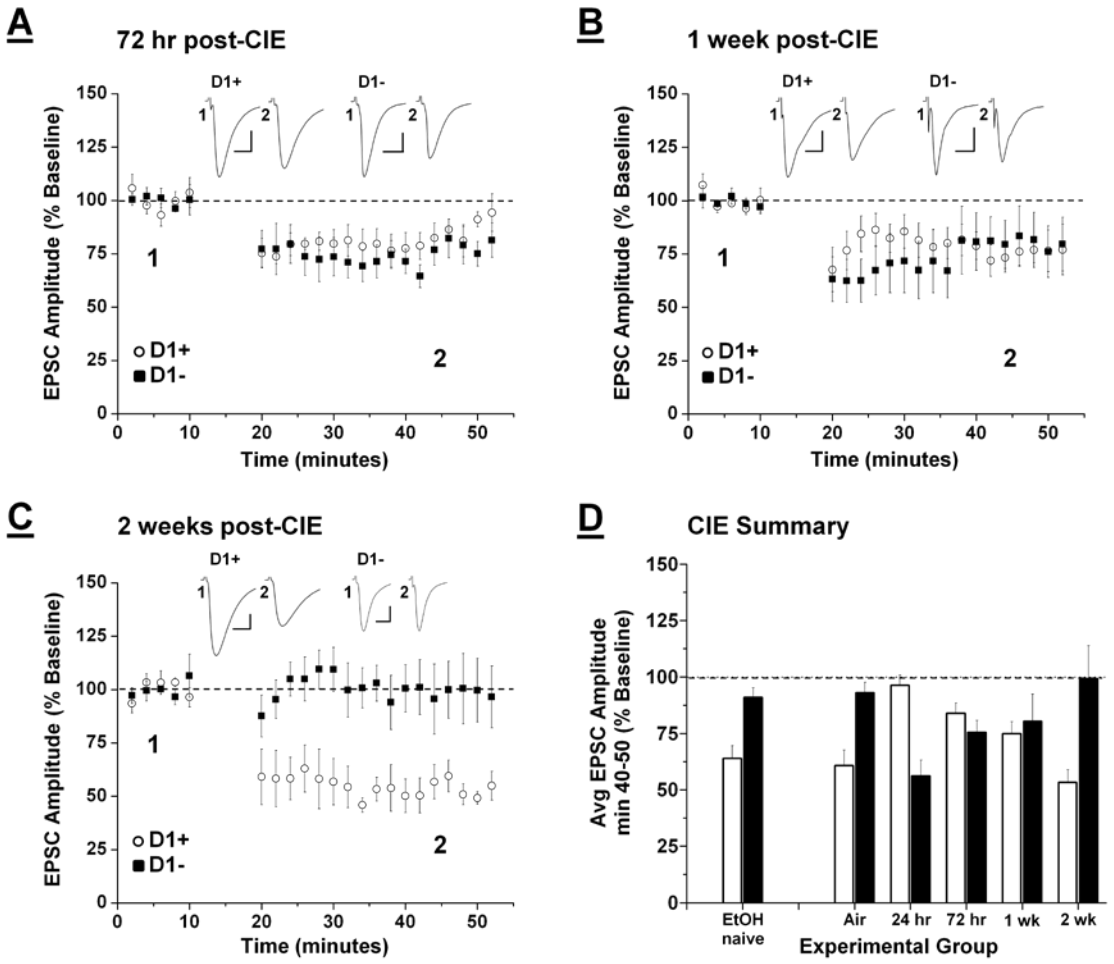
A, conditioning stimulation (500 pulses @ 1Hz with concurrent depolarization to -50 mV, denoted as "pairing") induces long-term depression of evoked AMPA receptor-mediated EPSCs in NAc shell of D1+ (64.02 ± 5.63% of baseline, n=8 neurons from 8 animals) but not D1- MSNs (90.99 ± 4.23% of baseline, n=7 neurons from 7 animals). Each data point represents an average of 12 consecutive normalized EPSC amplitudes condensed into 2 minute bins (% baseline ± SEM) from each neuron studied at that time point. B, pairing stimulation, in the presence of DL-APV (100 μM), in D1+ MSNs did not elicit LTD (95.35 ± 1.56% of baseline, n=6 neurons from 5 animals). C, pairing stimulation, in the presence of ethanol (40 mM), in D1+ MSNs did not elicit LTD (100.29 ± 7.27% of baseline, n=7 neurons from 5 animals). D, pairing stimulation, in the presence of the D1-like agonist, ±SKF38393 (50 μM), in D1+ MSNs did not affect LTD induction (71.86 ± 5.96% of baseline, n=5 neurons from 5 animals). E, pairing stimulation, in the presence of the D1-like antagonist, +SCH23390 (10 μM), in D1+ MSNs reduced but did not completely block LTD expression (80.26 ± 1.71% of baseline, n=5 neurons from 5 animals). Sample EPSC traces of averaged baseline "1" (min 0-10) and post-pairing "2" (min 40-50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 10 ms (horizontal) and 50 pA (vertical) in all traces. F, bar graph representing the percentage change ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each experimental group.

Figure 14. CIE vapor exposure switches 1 Hz-LTD expression from D1+ to D1- MSNs after 24 hr withdrawal.



A, 24 hrs following 4 consecutive days of air vapor exposure (16 hr on, 8 hr off, with pyrazole only injections) pairing stimulation induces LTD in D1+ (60.82 ± 6.75% of baseline, n=5 neurons from 5 animals, p>0.001, vs baseline) but not in D1- MSNs (93.01 ± 4.57% of baseline, n=5 neurons from 5 animals) similar to ethanol-naïve controls. B, 24 hrs following 4 consecutive days of ethanol vapor exposure (16 hr on, 8 hr off, with 1.5 g/kg 20% ethanol and pyrazole injections) pairing stimulation does not elicit LTD in D1+ (96.31 ± 4.61% of baseline, n=7 neurons from 7 animals, p>0.001 vs D1+ air vapor) but does induce LTD in D1- MSNs (56.18 ± 6.97% of baseline, n=7 neurons from 7 animals, p>0.001, vs baseline). Sample EPSC traces of averaged baseline "1" (min 0-10) and post-pairing "2" (min 40-50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 10 ms (horizontal) and 50 pA (vertical) in all traces.

Figure 15. Extended withdrawal from CIE exposure reveals a gradual return of cell type-specific synaptic plasticity in the NAc shell.



A, 72 hrs following CIE vapor exposure, pairing stimulation induces modest LTD in D1+ (83.95 ± 4.58% of baseline, n=5 neurons from 5 animals, $p<0.05$, vs baseline) and a greater depression in D1- MSNs (75.54 ± 5.16% of baseline, n=5 neurons from 5 animals). B, 1 week following CIE exposure LTD is nearly fully recovered in D1+ (74.97 ± 5.23% of baseline, n=6 neurons from 6 animals) but a modest LTD remains in D1- MSNs (80.36 ± 11.94% of baseline, n=6 neurons from 6 animals). C, 2 weeks following CIE exposure LTD in completely recovered in D1+ MSNs (53.32 ± 5.63% of baseline, n=5 neurons from 5 animals) and absence of LTD is present in D1- MSNs (107.17 ± 17.02% of baseline, n=4 neurons from 4 animals). D, bar graph representing the percentage change ± SEM for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each experimental group. Sample EPSC traces of averaged baseline "1" (min 0-10) and post-pairing "2" (min 40-50) EPSCs (60 sweeps, 10 min) of a single representative neuronal recording from each drug exposure group. Scale bars represent 10 ms (horizontal) and 50 pA (vertical) in all traces.

Discussion

The present study sought to determine whether nucleus accumbens shell D1+ and D1- medium spiny neurons differed in basal or post-CIE synaptic transmission and plasticity. This report includes the following novel observations. First, D1+ MSNs of the NAc shell are inherently more excitable than those lacking D1 receptors. Second, indirect pathway synapses onto D1- MSNs, but not direct pathway synapses onto D1+ MSNs, increase spontaneous excitatory neurotransmitter release subsequent to a 10Hz burst stimulation of synaptic afferents. Third, synaptic depression of glutamatergic excitatory transmission in the NAc shell is induced solely in D1+ but not D1- MSNs. Fourth, 24 hours following a repeated regimen of in vivo chronic intermittent ethanol vapor exposure 1 Hz-LTD can be induced solely in D1- MSNs, while LTD is completely occluded in D1+ MSNs. Complete recovery of 1 Hz-LTD expression, or lack thereof, back to controls levels in both D1+ and D1- MSNs respectively does not occur until 2 weeks of withdrawal from CIE vapor exposure. To our knowledge, this is the first demonstration of a reversal in the cell type-specificity of synaptic plasticity in the NAc shell, as well as, the gradual recovery of the pre-drug exposure plasticity state following extended withdrawal. We believe these observations highlight the adaptability of NAc MSNs to the effects of long-term ethanol exposure.

SYNAPTIC INPUTS ONTO D1+ AND D1- MEDIUM SPINY NEURONS

We found that D1+ MSNs of the NAc shell were more active than D1- MSNs; these findings directly contrast the previously described differences between these neuronal subtypes in the striatum and NAc core (Cepeda et al., 2008; Grueter et al., 2010). Our observations for D1+ MSNs were consistent across several parameters-

increased spontaneous excitatory presynaptic neurotransmitter release, lower paired-pulse ratio, more hyperpolarized AP threshold, and enhanced AP firing in response to fixed current injections. These results suggest an important functional difference between the synapses within subregions of the NAc. Given that administration of most drugs of abuse, including ethanol, lead to an increase in extracellular dopamine concentrations in the NAc (Imperato and Di Chiara, 1986; Weiss et al., 1993), cell type-specific synaptic differences between the NAc core and shell may underlie the different aspects of reward processing. In fact, increased dopamine release subsequent to burst firing of VTA neurons has been shown to occur solely in the NAc shell but not the NAc core or dorsal striatum (Zhang et al., 2009). In addition, we have shown a significant increase in spontaneous excitatory synaptic transmission in response to afferent 10 Hz burst stimulation only in D1- MSNs, which is exhibited by an increased post-burst sEPSC frequency and increased EPSC amplitude of the 15 stimulations within the burst itself. We feel these data are supported by the idea that synapses with a low initial release probability tend to show short-term facilitation, while high release probability synapses display short-term depression (Thomson, 2000). Taken together, our data suggest that D1+ and D1- MSNs in the NAc shell receive different magnitudes of excitatory afferent input that is opposite of that in the NAc core and striatum.

1 Hz-LTD in the NAc shell is specific to D1+ MSNs

From our basic electrophysiological characterization of D1+ and D1- MSNs in the NAc shell, we expected D1+ MSNs to express LTD due to the increased spontaneous activity of their afferents and propensity to exhibit short-term synaptic depression during burst stimulation. Indeed, we were able to detect 1 Hz-LTD only in the D1+ MSNs.

Interestingly, our findings that the specificity of LTD in the NAc shell is to D1+ MSNs differ from both the dorsal striatum and NAc core where LTD is specific to D2+ MSNs (Kreitzer and Malenka, 2007; Grueter et al., 2010). This discrepancy may be explained by the different induction protocols used (high frequency (100 Hz) in striatum and 10 Hz in NAc core), or the difference between using mice expressing eGFP under either the D1 or D2 receptor promoter (report in NAc core analyzed D2+ and D2- MSNs). Given that the NAc core is continuous with the dorsal striatum and core MSNs differ from the shell in their cellular morphology, neurochemistry, projection patterns, and functions (Heimer et al., 1991; Zahm and Brog, 1992; Meredith, 1999), it is not surprising that our findings regarding LTD expression differ as well. In fact, we consider this idea central to our focus on NAc shell plasticity and ethanol exposure, as the shell is more prominently associated with drug reward (Carlezon et al., 1995; Rodd-Henricks et al., 2002; Sellings and Clarke, 2003; Ikemoto, 2007). We have previously shown that afferent stimulation (1 Hz) paired with postsynaptic depolarization reliably produces LTD in the NAc shell, and that this effect was NMDAR-dependent. In the current study, we found that this LTD could only be induced in D1+ MSNs, that like our previous report was found to be NMDAR-dependent and could be occluded by a physiologically relevant concentration of ethanol (40 mM). Interestingly, although LTD is unaffected by prior D1-like receptor activation, it is significantly reduced in the presence of the D1-like receptor antagonist, suggesting that a basal dopaminergic tone facilitates the expression of 1 Hz-LTD; and that removal of this dopaminergic tone on D1 receptor-expressing MSNs results in a decreased synaptic depression subsequent to afferent stimulation. D1 receptor antagonists have been shown to reduce operant responding for ethanol (Rassnick et al., 1993; Samson et al., 1993), which could implicate the reduction and/or omission of LTD

in D1+ MSNs as a synaptic mechanism through which the NAc circuitry is altered to facilitate ethanol consumption.

CHRONIC INTERMITTENT ETHANOL EXPOSURE AND WITHDRAWAL AND 1 HZ LTD EXPRESSION

Our lab was the first to demonstrate that 1 Hz-LTD in the NAc shell is converted to synaptic potentiation in CIE vapor-exposed mice (Jeanes et al., 2011). Based on the initial electrophysiological differences observed between MSN subtypes, one goal of the current study focused on whether this transition from LTD to LTP was cell type-specific. We discovered a remarkable switch from only D1+ MSNs expressing 1 Hz-LTD under control conditions to only D1- MSNs expressing it following a 4 day CIE vapor exposure. The CIE vapor exposure model has been extensively demonstrated to stably increase voluntary ethanol consumption in mice following withdrawal and is thought to induce a dependent state (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009b). In our own hands, we observed an increase in voluntary ethanol consumption following a single 4 day CIE exposure, which is identical to the method used in the current study (Jeanes et al., 2011). However, an important distinction between the current study and those previous is the mouse strain used, as C57BL/6J mice are known to voluntarily consume ethanol while Swiss Webster (SJL) mice drink very little by comparison (Belknap et al., 1993). We believe this important phenotypic difference could explain why we observed an occlusion of LTD rather than a conversion to synaptic potentiation in NAc shell D1+ MSNs. Yet, the implications of this discrepancy are quite exciting, as perhaps those genetic determinants that confer the high drinking phenotype to C57BL/6J mice may also contribute to the extensive change in synaptic plasticity polarity observed following CIE exposure. However, in Swiss Webster mice, two recent studies

report that either intra-NAc or intraperitoneal administration of the specific dopamine D1 antagonist (SCH-23390) blocked the expression of behavioral sensitization to ethanol (Abrahao et al., 2011; Camarini et al., 2011). Although the mouse strain used in this study lacks a significant ethanol drinking phenotype, certain important ethanol-related behaviors are intact. Future studies using our current BAC transgenic mouse line backcrossed onto the C57BL/6J background will address these questions.

To our knowledge, this is the first report to demonstrate a switch in the specific dopamine receptor-expressing MSNs that exhibit LTD following CIE exposure and 24 hours withdrawal. In addition, we have shown the gradual recovery of baseline plasticity expression over several time periods until complete recovery at two weeks of withdrawal. Our data suggest that a vital period of neuroadaptation exists following ethanol exposure in which the NAc shell integrates afferent stimuli in a manner opposite to that before drug experience. In fact, multiple bouts of CIE and withdrawal are typically necessary for subsequent escalation of voluntary ethanol consumption in mice (Griffin et al., 2009a). We posit that further CIE experience would cement these synaptic alterations in the NAc shell, which could contribute to the complex neuronal modifications that underlie increased voluntary ethanol consumption following CIE. Our data support this notion, as we observed a steady increase in LTD expression in D1+ MSNs and a similarly gradual decline in LTD expression in D1- MSNs as withdrawal from CIE exposure progressed.

Drugs of abuse, mainly psychostimulants, have been proposed to elicit LTD in the NAc, which is associated with the expression of behavioral sensitization (Thomas et al., 2001; Brebner et al., 2005; Martin et al., 2006; Kourrich et al., 2007). Additionally, in response to cocaine administration, D2+ MSN-specific alterations of a different form of LTD were shown in the NAc core (Grueter et al., 2010), while D1+ and D2+ MSNs

displayed bidirectional changes in basal excitatory transmission in the NAc shell (Kim et al., 2011). Optogenetic activation of direct pathway D1+ MSNs or indirect pathway D2+ MSNs facilitates and inhibits cocaine-induced conditioned place preference, respectively (Lobo et al.). The chronic cocaine-induced inability to induce NMDAR LTD in the NAc is thought to contribute to the transition to addiction (Kasanetz et al., 2010). We believe that cell type-specific alterations in the expression of synaptic plasticity in the NAc shell are likely to represent cellular mechanisms that contribute to the development of ethanol dependence. In particular, our observation that D1+ MSN specific LTD expression gradually returns over two weeks of withdrawal suggests that permanent changes in D1+ and D2+ MSN synaptic plasticity expression may underlie the maintenance of the aberrant voluntary ethanol consumption that is a hallmark of dependence.

CHAPTER 4:

CONCLUDING REMARKS AND FUTURE STUDIES

A major challenge for researchers in the alcohol field is that decades of basic and clinical neuroscience are attempting to elucidate the biological mechanisms of a human behavior developed over several millennia. Alcohol use has been integrated into our culture to such an extent that it is the only legal addictive substance still universally consumed without significantly detrimental stigmas or guilt attached to it. It doesn't appear that the elimination of alcohol use from our society will occur in the near future, and so too will alcoholism continue to be a substantial concern. However, because alcohol use is so prevalent and addiction is so multi-faceted, investigation into the biological mechanisms underlying alcoholism is supremely intriguing. Myriad experimental strategies have been employed to explore the neurobiology of addiction. One useful strategy is determining how the transition from a drug-naïve to an addicted state occurs. The findings presented in this dissertation primarily focused on this initial period of drug exposure and the neuroadaptations that result.

Firstly, we investigated the neuroadaptive alterations in excitatory synaptic plasticity in the nucleus accumbens (NAc) shell in response to *in vivo* chronic intermittent ethanol (CIE) exposure. The results from this study indicate that CIE exposure modulates a pre-existing equilibrium between synaptic depression (LTD) and potentiation–where potentiation of synaptic transmission occurs in response to afferent stimuli that normally induce depression. The role of the NAc is well-established as an integrator of novel stimuli, and it is also involved in processing the incentive-salience of these new stimuli for the animal. These findings suggest that after exposure to ethanol this brain region exhibits drastic synaptic adaptations that cause the neurons to respond in

an opposite manner to exactly the same afferent stimuli. Such a reversal of synaptic processing may help explain the initial neural signaling alterations that prepare the animal for subsequent drug experiences. NAc neurons have now been primed (significantly altered synaptic transmission), and upon further drug exposure, they will respond differently than in the drug-naïve state. The reversal of synaptic plasticity to potentiation after 24 hours is lessened after 72 hours, but LTD remains occluded. These data suggest that following the first bout (3 days) of ethanol exposure significant synaptic changes occur, but these alterations begin to recover as withdrawal from the exposure increases. It is possible that subsequent ethanol exposures serve to cement the synaptic adaptations resulting from initial drug experience, perhaps further increasing the likelihood of synaptic potentiation. The observation that synaptic plasticity completely switches polarity following CIE exposure represents a drastic alteration in how the NAc is processing afferent input.

Since these neuronal recordings are conducted in brain slices, functional implications of these data are mostly speculative. However, these data were collected in an identical method with the only difference being CIE exposure; so, at the very least, the observed change in synaptic plasticity in the NAc represents a snapshot of the generalized synaptic adaptations occurring in this brain region as a result of ethanol exposure. These results highlight the fact that CIE exposure seriously augments information processing in the NAc, which could lead to inappropriate associations being made concerning the molecular effects of ethanol in the brain. These aberrant correlations could manifest as overemphasis on the rewarding properties of ethanol. Then, once ethanol is metabolized and excreted from the animal's blood, a significant association has been made between the physiological effect of ethanol and the environment surrounding its procurement. These associations may be encoded in NAc

neurons as changes in synaptic plasticity, and further ethanol exposure could strengthen these synaptic adaptations potentially leading to a change in the animal's behavior. The transition to addiction is primarily defined by a change in an animal's drug taking behavior, from controlled to compulsive and uncontrolled. Perhaps these changes in synaptic plasticity in the NAc are the genesis of the behavioral modifications that contribute to the addicted state.

However, in order to support this claim, future studies are necessary to correlate changes in NAc synaptic plasticity with ethanol-related behaviors. We did choose a form of ethanol exposure (CIE) that has been demonstrated by several groups, including our own data in this study, to result in increased voluntary ethanol consumption in mice. Although the CIE vapor treatment was a passive administration of ethanol, the two-bottle choice studies suggest that the mice were choosing to consume more ethanol after CIE exposure than before. Thus, if the synaptic changes we observed in slice recordings are present *in vivo*, we would then be able to draw a more direct cause/effect relationship. Yet, until these studies are conducted, therapeutic implications are tempered and must remain speculative. Nonetheless, it is tempting to hypothesize that these synaptic modifications do contribute to the increased drinking behavior, thereby opening up the possibility that if normal synaptic functioning could be recovered, either pharmacologically or physiologically, then the drinking behavior would return to pre-CIE levels.

Finally, we chose to further investigate these alterations in NAc synaptic plasticity to determine if they are expressed in a cell type-specific manner. The benefits of such an exploration are to more extensively characterize which neurons are changing in response to ethanol and how these changes develop over time. Medium spiny neurons (MSNs) within the NAc belong to two opposing pathways based on their expression of dopamine

receptors that differentially modulate an animal's behavior. The study above outlined data collected from both neuronal populations, while in this study we were able to specifically record from either MSN subtype. The first major finding of this report is that D1 dopamine receptor-expressing (D1+) MSNs of the NAc shell express LTD, while D2 dopamine receptor-expressing (D1-) MSNs do not. The primary significance of this finding is that the cell type-specific plasticity in the NAc shell is opposite from that reported in the NAc core, where D2+ MSNs solely express LTD (Grueter et al., 2010). This difference may be vital to our understanding of how the NAc processes reward-related information. It is believed that the shell and core belong to an ascending spiral of information processing that leads an animal from developing associations with certain stimuli to then initiating appropriate behaviors that will increase the likelihood that those stimuli are experienced again. Thus, it is important to know how both populations of MSNs are functioning prior to drug exposure.

Another important observation in this study demonstrates, for the first time, a complete reversal in cell type-specific synaptic plasticity following CIE exposure. Whereas, in air vapor control and ethanol-naïve animals, we observed LTD expression in D1+ MSNs but not in D1- MSNs, the opposite was true 24 hours following CIE exposure. This result is quite fascinating because it directly establishes a connection between ethanol exposure and a significant disruption in normal synaptic activity in the NAc in a cell type-specific manner. Following drug exposure, these two opposing pathways now function in an opposite manner than in the drug-naïve state. Implications for this finding are similar to those described above. The plastic changes in the neuronal signaling within the NAc could explain the aberrant associations that an animal makes during drug exposure. Given that the NAc shell is thought to be one of the first brain regions to integrate novel reward-related stimuli, these data could be a remarkable

representation of this idea on a synaptic level. As in the previous study, the only missing component to this report is to directly determine the behavioral consequences of such a change in synaptic plasticity. Perhaps these synaptic alterations are necessary for the development of abnormal learning associated with drugs of abuse. Or, these findings may simply characterize an attempt by NAc synapses to counteract ethanol-induced changes and maintain homeostasis. That is, we may have simply observed the result of this synaptic homeostatic process rather than a direct representation of how these neurons are processing afferent input. Either way, these findings demonstrate a stark difference in synaptic transmission before and after ethanol exposure in a brain region well-known to participate in reward-related informational processing.

The final significant aspect of this study concerns the gradual recovery of pre-CIE levels of cell type-specific synaptic plasticity expression after two weeks of withdrawal. I speculated after the first study that additional ethanol exposures could cement the conversion of LTD to synaptic potentiation in these neurons. While this result does not directly support that conclusion, it does provide additional support to the notion that after the first bout of CIE (4 days in this study) normal synaptic plasticity returns if ethanol exposure does not recur. Indirectly, this observation suggests that given an appropriate amount of time, the NAc will return to a normal state of synaptic functioning (at least in our measure of synaptic function–glutamatergic plasticity). To my knowledge, this is the first demonstration of a reversal in synaptic plasticity following CIE exposure that then reverts back to the drug-naïve state over time. These data appear to lend support to idea that repetitive drug experience is necessary to make the neuroadaptations that underlie aberrant drug taking behaviors permanent. In addition, these data also reinforce the notion that repetitive drug experience-induced neuroadaptations can be overcome by periods of drug abstinence. This notion is not new as it is well-known that the only true

cure for addiction is abstinence. Yet, these findings do reveal a remarkable synaptic level view of how the brain is actually changing as the animal is removed further and further from the time when the drug was on board. Similarly, it is possible that with additional bouts of CIE exposure, these mice would require longer and longer periods of withdrawal in order for the experimenter to observe a return of synaptic plasticity expression to normal levels. Future studies are needed to explore this interesting possibility.

Drug-induced changes in NAc synaptic plasticity could establish an overt alteration of the smooth information flow in the reward-related neuronal network. These same observations may be interpreted as a 'metaplastic' modification induced by those drugs, rather than a loss of function. The concept of metaplasticity comprises all those biochemical changes occurring at the synaptic level, which, although not expressed in terms of changes in synaptic strength, are capable of affecting the susceptibility of the same synapses to LTP or LTD, in response to subsequent stimuli (Abraham and Bear, 1996). Perhaps aberrant reward-related learning is encoded through synaptic metaplasticity in the NAc, which would shape the potential of the same synapses to change their efficacy. The capability of expressing opposing forms of synaptic plasticity, or to resist any change in EPSC amplitude, in response to a similar pattern of afferent stimulation, may be, by itself, a property of these synapses. Therefore, LTP may be induced instead of LTD, or , alternatively, either one of the two forms of plasticity is prevented, when a particular normal or neuropathological state occurs. An occlusion of LTD following CIE exposure could then be interpreted as a resistance to change of synaptic function or as a scenario where LTD was already saturated from CIE exposure resulting in no further depression. As in the second study, the appearance of LTD could represent an increase in activity of glutamatergic synapses on D1- MSNs following CIE exposure, so that now synaptic depression is possible. It is not entirely clear which

interpretation best fits these findings. Separate neuronal subsets may be regulated by persistent modifications in the amplitude of synaptic signals (LTP or LTD) through specific patterns of afferent activity, in such a way that, in an anatomically uniform area like the NAc, a local balance of excitation and inhibition may be achieved within distinct neuronal micro domains. Following drug exposure, this balance appears to be shifted between D1+ and D1- MSNs, so that perhaps the output of the NAc shell has changed sufficiently so as to cause dysfunction in downstream target brain regions.

An important question that remains concerns future usefulness of the findings outlined in this dissertation to the scientific community. As discussed, a major hindrance of these studies are that they have not be directly correlated with ethanol-related behaviors, but rather conducted using a ethanol exposure model known to increase voluntary ethanol consumption in mice. Speculation as to whether reversing or blocking the changes in synaptic plasticity could counteract a change in drinking behavior will remain as such until sufficient experimental data is collected. Yet, one possible utility of these data is that a change in NAc synaptic plasticity may be an engram of alcohol dependence. Perhaps the detection of synaptic potentiation in response to LTD-inducing stimuli could accurately diagnose whether that animal was ethanol dependent. Again, future studies are needed to explore this concept. In particular, NAc synaptic plasticity must be tested in mice that underwent CIE exposure and exhibited an increase in voluntary ethanol consumption. Then, if potentiation rather than depression is consistently observed in animals with that phenotype, we would at least have a post-hoc method of confirming the phenotype on a synaptic level. Moving up the evolutionary ladder, it is even more tempting to speculate that these plastic changes are present in the human alcoholic brain. If so, figuring out a way to detect such synaptic changes non-invasively could provide a novel diagnostic procedure for alcohol dependence. One

method of potential use is transcranial magnetic stimulation, which has been used to treat depression in patients resistant to other treatments. If there was a way to establish a reliable readout of synaptic functioning (or neural ensemble activity) then it could be used to diagnose the presence of altered synaptic activity which may indicate the patient is alcohol dependent. Better diagnostic techniques are crucial for substance dependence, in part, because it is extremely difficult for patients to receive insurance-covered healthcare for treatment. If we were to possess a more accurate measure of drug dependence that does not rely on complicated psychosocial criteria then perhaps it would be easier for patients to get treatment covered by insurance, and thus probably make it more likely they would seek treatment.

One final issue to discuss concerns the best avenues through which to pursue more effective treatments for alcoholism. Unfortunately, none of the medications currently used to treat alcoholism are effective in all or even most patients. The challenge, then, in deciding what pharmacological treatment options to investigate for research purposes is whether or not relapse prevention or abstinence recovery should be the only primary outcome. The only cure for addiction is abstinence, this much is clear. Yet, for other drugs of abuse, it is quite obvious that they cannot be used in a socially acceptable manner leaving abstinence as the endgame. In the back of an alcoholic's mind, I believe he/she is troubled by the fact that so much of our population can use alcohol without becoming addicted. Thus, I believe the most effective treatment option to reach the largest number of patients with alcohol use disorders will focus on harm reduction as the main goal. I don't anticipate the development of a pharmacotherapy that will convince alcoholics that they no longer need to drink when that realization does not already exist within them. Drugs that reduce cravings or blunt the withdrawal-negative-affect stage of alcoholism will certainly help an alcoholic resist relapse, but the

psychological component to addiction will always need to be overcome in order to achieve permanent remission. The decision to seek help is central to the success of any psychosocial intervention. In addition, the installation of hope, empathy, and therapeutic alliance gleaned from these types of interventions, such as twelve-step programs, can greatly enhance the efficacy of abstinence as the treatment goal, where abstinence rates of 67-75% 6 months after treatment and 60-68% 12 months after treatment have been found (Ouimette et al., 1999). In basic science, however, the need to ask questions and pursue the answers experimentally has and will continue to drive innovations in treatment; but, it is necessary to focus these efforts towards a common treatment outcome if viable options are to be developed for a large number of alcoholic patients.

One effective strategy is to determine how the transition to drug dependence occurs. Presumably, the process of excessive, uncontrolled drug use is gradual and not immediate upon first drug experience, although many addicts can remember the first time they tried their drug of choice. As researchers, it is possible to use animal models to observe the neuroadaptations that occur through the transition to addiction. The power of animal research is that we control when we want to take a snapshot of the brain at a particular point of the animal's addiction process. Thus, over time, we should be able to understand a great deal about how the brain changes, and more importantly, which changes are necessary to the expression of the abnormal behaviors central to addiction. The bulk of the work outlined here is directed toward this strategy. Once the specific changes necessary for the transition to uncontrolled drug use are elucidated, perhaps scientists will then be able to target treatments to those specific neuroadaptations. If successful, such treatments would be more applicable to a larger number of patients who are not yet severely affected with the chronic, relapsing nature of addiction. Thus, in patients who seek treatment and desire to remain abstinent anti-craving

pharmacotherapies will be beneficial. If possible, other patients not yet willing to pursue abstinence would have another avenue of treatment aimed at reversing the neuroadaptations within their brains that led to the development of their uncontrolled drug taking behaviors. Only time will tell whether such an outcome is even possible, but with advances in deep brain stimulation and transcranial magnetic stimulation perhaps the actual rewiring of aberrant synaptic activity is achievable. In fact, a recent study just demonstrated that one week following cocaine exposure, *in vivo* optogenetic depotentiation of potentiated synapses in the NAc could abolish cocaine-induced locomotor sensitization (Pascoli et al., 2012). This report is a valuable proof-of-principle that reversing drug-induced changes in NAc synaptic plasticity can prevent the augmented behavioral responses to subsequent drug exposures.

Research into the mechanisms underlying the development of alcohol dependence has significantly advanced our knowledge of the biological substrates of the disease. Tremendous breakthroughs in elucidating the basic neurobiology of addiction and in developing and validating behavioral and pharmacological treatment of alcoholism have been achieved over the past half century. However, as of today, no widely effective pharmacological treatment exists for alcohol dependent patients. One major hindrance that basic science and clinical researchers must overcome is determining which direction research efforts should be focused on a disease state that possesses numerous contributing factors from the molecular effects of ethanol itself to the psychosocial environment of the addict. In short, the answer to this problem is that all aspects of the disease must be investigated simply because it is such an immensely complicated disorder. The findings presented in this dissertation address only a small component of the overall disease, yet we believe they may represent a significant advancement toward our understanding of the biological underpinnings of alcohol dependence.

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