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ETHANOL EXPERIENCE ELICITS CIRCUIT SPECIFIC ADAPTATIONS OF VENTRAL HIPPOCAMPAL-ACCUMBENS GLUTAMATERGIC SIGNALING

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by

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Dedication

To Margaret, the light of my life, without her steadfast support and love this work may never have been completed. To Ruby, an amazing friend and faithful companion; you will be missed.

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The University of Texas at Austin, 2017

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The purpose of this study was to determine the effects of ethanol exposure and consumption on the expression of plasticity of D1 dopamine receptor expressing (D1R) medium spiny neurons (MSNs) in the nucleus accumbens (NAc) shell receiving glutamatergic input solely from the ventral hippocampus (vHipp). Drd1a-tdTomato mice on a C57BL/6J background were injected bilaterally in the vHipp with a viral vector in order to express channelrhodopsin (ChR2) in vHipp terminals that synapse onto shell D1R-MSNs. Blue LED light stimulation was used to selectively depolarize ChR2 expressing vHipp terminals in the NAc shell, resulting in light-evoked EPSCs originating from vHipp. In voltage clamp experiments, we found that an induction protocol pairing 1 Hz blue light stimulation with postsynaptic membrane depolarization produced LTD in the vHipp to NAc circuit that is NMDA receptor dependent. In this study we found acute application of ethanol in vitro uniquely alters plasticity in the vHipp to NAc circuit. Low to moderate intoxicating concentration of ethanol blocked light evoked LTD expression which is in contrast to previous observations. Ethanol consumption in rodents impaired vHipp-Shell plasticity as well as resulted in altered glutamatergic signaling, and the insertion of Ca2+ permeable AMPA receptors (CPARs) D1R-MSNs post synaptic

membranes. These findings suggest that the vHipp to NAc circuit is highly sensitive to ethanol treatment and may constitute a critical neuroadaptation that leads to the expression of ethanol dependence.

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CHAPTER 1:

BACKGROUND

ALCOHOL

The alcohols are a class of organic hydrocarbon compounds which contain a hydroxyl moiety (e.g. functional group). Ethanol, or ethyl alcohol, is frequently referred to by the lay public as “alcohol” and consists solely of two-carbon alcohol. It is a highly volatile, flammable, colorless liquid with a slight characteristic odor and is the sole alcohol fermented and distilled for alcoholic beverage consumption by humans. Ethanol is a psychoactive drug which humans have produced and consumed since the Neolithic Era over 7000 years ago (Chrzan, 2013). Alcohol consumption plays an extraordinarily important behavioral, pharmacological, economic and societal role and constitutes one of one of the most widely used and abused psychoactive agents by humankind.

ALCOHOL DEPENDENCE

Drug dependence is a chronically relapsing disorder that is characterized by three main domains (1) compulsion to seek out and take a drug, (2) loss of control in limiting intake of that drug, and (3) the emergence of a negative emotional state (e.g., dysphoria or anxiety) typical of a motivational withdrawal syndrome when drug access is prevented (Koob & Volkow, 2010). Loss of control of drug use is a hallmark of drug dependence and is clinically separate from limited, occasional use, of an abusable drug (Koob & Le Moal, 1997). It is important to note that excessive drug use (i.e. alcohol abuse) does not always lead to alcohol dependence. Alcohol abuse and dependence exist on a continuum

whereby excessive alcohol intake in alcohol abusing individuals can lead to alcohol dependence in those individuals. Alcohol 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 & Agatsuma, 2005) and (2) the abuse component, whereby excessive alcohol consumption leads to dysregulation or alteration of the function of brain signaling systems that manifest themselves into the drug dependent behavioral state.

According to the Center for Disease Control (CDC, 2012) more than 50% of Americans over the age of 18 consume alcohol regularly and approximately 1/3 of 18-24 year olds meet the criteria for an alcohol use disorder (AUD) (National Institute on Alcohol Abuse and Alcoholism [NIAAA], 2005). As of 2012 around 17 million adults ages 18 and older had an AUD and an estimated 855,000 adolescents could be diagnosed with an AUD (NIAAA). According to the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-V) a person can be diagnosed with an AUD if they exhibit behavior across a number of loci. These loci include: 1) prolonged or excessive alcohol use, 2) persistent desire or unsuccessful efforts to control use, 3) excessive time allocated to use and seeking of alcohol, 4) craving or a strong desire to use, 5) neglecting responsibilities in order to use, 6) continued use despite negative consequences, 7) giving up normal activities in order to use, 8) use during physically hazardous conditions, 9) use despite physical or psychological issues caused or exacerbated by use, 10) formation of tolerance to the effects of alcohol, and 11) expression of withdrawal symptoms. Anyone meeting any two of these 11 criteria during the same 12 month period can receive a diagnosis of an AUD. The severity of an AUD can be classified as mild (2-3 symptoms), moderate (4-5), or severe (6 or more) depending on the number of criteria met.

SOCIETAL IMPACT

Excessive alcohol consumption is associated with a number of serious health related problems that include but are not limited to: liver cirrhosis, forms of cancer, unintentional injuries, violence, and fetal alcohol spectrum disorder. According to the CDC (2004) excessive alcohol consumption is responsible for on average 79,000 deaths, and 2.3 million years of potential life lost each year in the US. Deaths from alcohol use include over 19,000 from alcoholic liver disease and over 30,000 alcohol-induced deaths, excluding accidents and homicides that occur per year (CDC, 2004). All told alcohol related deaths are the third leading cause of preventable death in the United States (Mokdad et al., 2004). In addition to the lives lost due to excessive alcohol consumption there is a high economic burden produced by excessive use. Some of these costs are related to increased healthcare costs, increased crime and criminal justice system costs, and lost worker productivity via missed work or diminished work output. The economic cost of excessive drinking has been conservatively estimated to be over $220 billion dollars per year, on a per capita basis costing approximately $746 per person (Bouchery et al., 2011). These costs have significantly increased compared to prior estimates of $148 billion and $184.6 billion from 1992 and 1998 (Harwood et al., 1999) suggesting that costs are increasing year to year. Given the high societal cost of AUD in the United States, our government should devote more resources and funding towards both treating individuals who suffer from an AUD and working to prevent excessive alcohol intake.

CURRENT TREATMENT APPROACHES

As stated above approximately 17 million adults in the United States have been diagnosed with an AUD. Unfortunately only a paltry 20% of AUD sufferers seek treatment for their alcohol abuse or dependence (Cohen et al., 2007; Dawson et al., 2005;

Huebner & Kantor, 2011). A significant impediment to treatment of alcohol dependent individuals is that complete termination of alcohol use, or going "cold turkey", can produce seizures in these dependent individuals. Ethanol is a potent modulator of inhibitory GABA signaling within the brain (Kumar et al., 2009). Repeated excessive ethanol use results in a downregulation of inhibitory signaling. During withdrawal from ethanol there is an upregulation of excitatory signaling coupled with a dearth of inhibitory signaling which results in a signaling imbalance that predisposes an ethanol dependent individual to seizures. Benzodiazepines, allosteric modulators of $GABA_A$ receptors, are the primary treatment given to dependent individuals to prevent withdrawal induced seizures. While these pharmacological agents are effective at preventing withdrawal seizures they are not effective at maintaining abstinence from alcohol and their use is contraindicated with alcohol consumption as they have a potentially synergistic effect on inhibitory signaling. This synergistic effect can lead to enhanced effects of alcohol, in particular inhibition of the respiratory system leading to death.

There have been several pharmacotherapies that have at least some efficacy in promoting abstinence from ethanol consumption in humans. Currently there are three FDA approved pharmacotherapies to treat AUD patients. These therapies fall into two main types of treatments: aversive medications, a drug in this category is meant to deter a patient from drinking, and anti-craving drugs, which are meant to reduce a patients desire to drink.

Disulfiram (Antabuse) is an alcohol dehydrogenase inhibitor and is the primary aversive medication used in treatment. When disulfiram is onboard subsequent consumption of alcohol will result in a build-up of acetaldehyde causing a negative aversive reaction in the user. Unfortunately the effectiveness of this treatment is limited

to compliant patients (Chick et al., 1992) as many patients will stop taking disulfiram due to its negative effects.

One of the two main anti-craving drugs is acamprosate (Campral) which is believed to be a partial agonist for the N-methyl-D-aspartate (NMDA) receptor; however the exact mechanism of therapeutic action is in debate. Acamprosate has been approved for use in Europe since 1989 and was approved by the FDA in 2004. There is some evidence that Acamprosate treatment has some ability to aid the maintenance of abstinence although findings have been mixed (Bouza et al., 2004; Mann et al., 2004). The other main anti-craving agent is naltrexone which is a mu opioid antagonist that was FDA approved in 1994. Naltrexone is believed to suppress the rewarding properties of alcohol via its effect on the opioid receptor. naltrexone treatment has been shown to reduce the number of drinks consumed as well as heavy drinking days (Anton et al., 2006; O'Malley et al., 1992; Pettinati et al., 2006). This suggests that naltrexone may be a useful adjunct in a harm reduction strategy employed for mild AUD sufferers or individuals that abuse ethanol. However, evidence suggests that Naltrexone is less effective for the maintenance of alcohol abstinence (Assanangkornchai & Srisurapanont, 2007).

Another therapeutic approach has been to use a combination of these approved drugs; for example the combination of acamprosate and naltrexone may be a more effective treatment then either alone (Anton et al., 2006; Kiefer et al., 2003). Acamprosate and disulfiram is another combination therapy that appears to be more effective than using a single drug (Besson et al., 1998). While all three of the FDA approved drugs listed here have shown some efficacy in a subpopulation of patients that suffer from an AUD, these drugs leave much to be desired in the ability to prevent

relapse and maintain abstinence. New pharmacological targets are needed in order to find effective pharmacotherapies that assist AUD patients in preventing relapse.

MODELS OF ALCOHOL DEPENDENCE

Human Alcohol Use Phenotypes

Alcoholism is a neuroadaptive disorder; repeated series of exposure and withdrawal from ethanol produce neural adaptations that lead to a persistent negative affective state as well as an alteration in the function of key motivation systems within the brain. These adaptations underlie excessive alcohol drinking and relapse following periods of abstinence. In the United States the most common drinking pattern seen in individuals who abuse ethanol is binge drinking. According to the NIAAA (2004) binge drinking is the consumption of 5 or more drinks for men and 4 or more drinks for women within a 2 hour period. This pattern of consumption readily produces blood ethanol concentrations (BECs) greater than 0.08% or 80 mg/dL which is considered the legal limit for intoxication within the United States. Excessive alcohol use is a hallmark exhibited in human AUD and any animal model employed should exhibit an excessive ethanol intake phenotype.

In humans, acute withdrawal from alcohol use produces a generalized nervous system hyperexcitability. This hyperactivity within the brain can lead to tremors, seizures, and autonomic nervous system hyperactivity. Following withdrawal, individuals can undergo periods of abstinence where they may display anxiety, low mood, and disturbed sleep. In protracted abstinence minor or normally insignificant challenges can produce negative affect and craving which can lead to relapse (Sinha & Li, 2007).

Laboratory Animal phenotypes

The critical phenotypes that animal models of AUD should exhibit are excessive voluntary intake of ethanol to a level that produces intoxication and relapse, or behaviors that mimic relapse following periods of withdrawal or abstinence. In the past a major impediment to producing valid animal models of AUD is that many rodents will not voluntarily consume sufficient quantities of ethanol to generate the blood ethanol concentrations necessary to produce dependence. However, in the past few decades there have been a number of approaches to generate animals that model excessive alcohol intake. Some examples of animal models that display these phenotypes include ethanol exposure procedures designed to increase self-administration of ethanol and selective breeding for ethanol intake,

In animal and clinical studies the amount and duration of alcohol exposure play a critical role in the development of dependence and the expression of withdrawal (Finn & Crabbe, 1997). One of the major behavioral approaches used to increase volitional intake is through limited access paradigms. These paradigms involve animals being given repeated access to ethanol for a limited amount of time determined by the experimenter. There is evidence that a greater severity of withdrawal symptoms is consistently observed when chronic ethanol exposure is delivered in an intermittent rather than continuous fashion (Becker, 1998). These restricted access paradigms typically occur during some portion of the animals dark cycle as rodents are nocturnal and consumption behaviors are greatest during their dark cycle (Rhodes et al., 2005). Drinking in the dark (DID) is a limited access model of binge drinking. Animals are given access to a 20% ethanol solution for a 2 hour period that starts 3 hours into their dark cycle. Ethanol consumption is measured for 3 consecutive days and on the 4^{th} day animals have their access increased

to a 4 hour period. BECs are measured immediately after the 4 hour access on the final day. Using this model animals that consume ethanol will achieve BECs greater than 100mg/dL and exhibit behavioral evidence of intoxication (Thiele & Navarro, 2014).

Another approach to generating laboratory animals that consume high quantities of ethanol is via selective breeding. Selective breeding for a high drinking phenotype has been used to successfully produce lines of mice that display some aspects of alcohol dependence such as excessive ethanol consumption or high BECs. The C57BL/6J mouse strain in particular has been well established to voluntarily consume high doses of ethanol, whereas other strains such as the DBA/2J strain will avoid alcohol (Rosenwasser & Fixaris, 2013; Shelton & Grant, 2002; Yoneyama et al., 2008). Another line of selectively bred mice that exhibit high BECs are the high drinking in the dark (HDID) mice. Mice that exhibited high BECs following the DID procedure were selectively bred together. Subsequent generations of these mice consume excessive quantities of ethanol in the DID paradigm and have high BECs following limited accesses to ethanol (Barkley-Levenson & Crabbe, 2014), these mice may be useful for genetic analysis of the binge drinking phenotype.

While the drinking models discussed thus far are capable of analyzing a dependent individual's progress to sustained high levels of ethanol consumption, they have not been designed to determine how the subjects become dependent. One approach to inducing dependence in laboratory animals is through the use of ethanol vapor inhalation, termed chronic intermittent ethanol (CIE) vapor exposure. CIE involves an intermittent pattern of four 16 hour bouts of ethanol vapor exposure interspersed by 8 hour withdrawal periods. This approach has been shown to reliably model the escalation in ethanol intake that occurs in humans who suffer from AUD and is considered a model

for the formation of alcohol dependence in laboratory animals. (Becker & Lopez, 2004; Griffin et al., 2009; Griffin Iii et al., 2009). Several studies in mice have demonstrated increased alcohol responding or drinking in dependent compared with non-dependent animals (Chu et al., 2007; Dhaher et al., 2008; O'Dell et al., 2004; Rimondini et al., 2002; Roberts et al., 2000; Sommer et al., 2008).

The enhanced ethanol consumption during withdrawal in dependent animals is capable of producing BECs and brain ethanol levels that are directly comparable to levels that are seen following forced chronic procedures (Griffin Iii et al., 2009; Roberts et al., 2000). Similar to what has been observed in human AUD, animals with prior ethanol dependence exhibit exaggerated sensitivity to the effect of ethanol associated cues as well as stressors that enhance alcohol-seeking behavior (Xiu Liu & Weiss, 2003; Sommer et al., 2008). This exaggerated sensitivity is persistent and has been observed up to several months after the last ethanol exposure (Becker & Lopez, 2004; Sommer et al., 2008; Valdez et al., 2002). These findings support the idea that during periods of abstinence, there is an enhancement in the biological substrates that underlie relapse which in turn perpetuates the addicted state.

MESOCORTICOLIMBIC SYSTEM AND REWARD

Dopamine

Dopamine is a neurotransmitter released from neurons in the midbrain region called the ventral tegmental area (VTA). The VTA has extensive projections to regions known to be involved in reward processing and guiding goal-directed behaviors (Grace et al., 2007; Ikemoto, 2007; Wise, 2004). There are 5 dopamine receptor subtypes; the signaling of each is mediated by G-protein coupled receptors (GPCRs). The dopamine

receptor subtypes are grouped into 2 main classes based on the G-protein that the receptor is coupled with (Neve et al., 2004). The Dopamine D1-like family, which includes the D1 and D5 receptors, is coupled to G_s and G_{olf} proteins. Activation of these receptors leads to the activation of adenylyl cyclase (AC) and subsequent increases in protein kinase A (PKA) activity, increases in in PKA activity are generally excitatory. The other family of dopamine receptors is the D2-like family, which includes the D2, D3, and D4 receptors. These receptors are coupled to $G_{i/o}$ proteins. Activation of D2-like receptors leads to inhibition of AC and subsequent decreased PKA activity which is generally inhibitory. D2 receptors G-protein activation can also lead to the activation of G protein coupled inwardly rectifying potassium (GIRK) channels. Activation of GIRK associated D2 receptors leads to the opening of the GIRK channel resulting in potassium influx into a cell thereby hyperpolarizing the cell. One of the main targets of VTA dopamine projections is the nucleus accumbens (NAc). It has been shown that activation of D2 receptors potently inhibits neurons within the NAc (O'Donnell & Grace, 1996). It has also been shown that D1 receptor stimulation potentiates glutamatergic, the main excitatory neurotransmitter, drive onto NAc neurons thereby increasing activation of these neurons (C. Cepeda et al., 1998). In addition, locally applied antagonist for the dopamine receptors indicate that D2 antagonism increases NAc neuron firing whereas D1 antagonism decreases cell excitability in the NAc (West & Grace, 2002).

Nucleus Accumbens

The NAc is part of the ventral striatal complex and is the site where cortical and alocortical information is integrated with motivational signals derived from limbic regions. The NAc's output is responsible for regulating appropriate goal-directed behaviors (Groenewegen et al., 1999; Mogenson et al., 1980; Nicola et al., 2000; Wise,

2004). The NAc receives excitatory glutamatergic afferents primarily from the hippocampus, basolateral amygdala (BLA), cerebral cortex, and thalamus. The primary projection from the NAc is to the ventral pallidum. The NAc can be divided into two main sub sections, the core and the shell (Humphries & Prescott, 2010b).

The core is the central region surrounding the anterior commissure (AC) and is located beneath and is morphologically similar to the dorsal striatum (Zahm & Brog, 1992). The projections from the core are primarily to the dorsolateral portion of the ventral pallidum, the entopeduncular nucleus and to the substantia nigra. The core is innervated by the dorsal portions of the prelimbic cortex and the dorsal agranular insular areas (Brog et al., 1993). The core also receives projections from the anterior portion of the BLA (Groenewegen et al., 1999) and the dorsal subiculum (Brog et al., 1993). The shell of the NAc occupies the most ventral and medial portions of the region (Zahm & Brog, 1992).

The shell projects to VTA dopamine cells that in turn project to the core. The shell is innervated by the ventral portion of the prelimbic, infralimbic, medial orbital, and agranular insular corticies (Berendse et al., 1992; Brog et al., 1993). The shell also receives input from the posterior portion of the BLA (Wright et al., 1996) as well as a preferential projection from the ventral subiculum of the hippocampus (Brog et al., 1993; Groenewegen et al., 1987). There is 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 circuitry (i.e. moving from NAc shell, to core, and finally to the dorsal striatum) (Haber et al., 2000; Susan R. Sesack & Grace, 2010).

There are four main cell types expressed in the NAc: 1) giant aspiny cholinergic neurons, 2) medium aspiny GABAergic neurons which are fast spiking neurons

colocalized with parvalbumin, 3) low threshold spiking neurons that express nitric oxide, somatostatin, and neuropeptide Y (NPY), and 4) Medium Spiny Neurons (MSNs). MSNs are GABAergic and are the primary neurons expressed in the shell and core, composing roughly 90-95% of the cells in these regions (Meredith, 1999). Projecting axons, from MSNs, have local collaterals that contact dendrites of neighboring MSNs and are capable of lateral inhibition (Wilson & Groves, 1980). A critical characteristic of MSNs is their bistable membrane potential which includes both an "upstate" at about -50 mV and a "downstate" at about -80 mV (O'Donnell & Grace, 1995). Inputs from the hippocampus are a critical driver for depolarization of the membrane of the cell towards the -50 mV "upstate". This change in potential is crucial for the cell to fire. Population activity in the ventral hippocampus (vHipp) is correlated with the upstate in NAc neurons (Goto & O'Donnell, 2001b). In the depolarized upstate, MSNs display a greater excitability. Excitatory transmission from other inputs such as the prefrontal cortex (PFC) and the amygdala are capable of initiating action potentials (APs) during the upstate generated by vHipp activity (O'Donnell et al., 1999). It has been postulated that ensembles of NAc neurons simultaneously go into the depolarized upstate whenever a situation arises that demands the attention of the animal, this ensemble activation being the direct result of activation of attention related dopamine pathways (Goto & O'Donnell, 2001a).

There are two distinct sub families of MSNs within the NAc depending on dopamine receptor expression. These sub families include the D1 dopamine receptor expressing (D1R-MSNs) family and the D2 dopamine receptor expressing (D2R-MSNs) family. The D1R-MSNs are co-localized with the signaling peptides dynorphin and substance P, whereas D2R-MSNs are co-localized with enkephalin (X. Y. Lu et al., 1998; Zhou et al., 2003). D1R-MSNs and D2R-MSNs of the NAc have a similar projection

pattern to the direct and indirect pathways of the dorsal striatum (Humphries & Prescott, 2010a; Susan R. Sesack & Grace, 2010; Smith et al., 2013). In this pattern D1R-MSNs project to the substantia nigra pars reticulata and the VTA from the core and shell respectively, as well as to the ventral pallidum. Accumbens D2R-MSNs from the core and shell project primarily to the ventral pallidum. However, there have been recent findings that suggest that the classical direct and indirect pathway observed in the dorsal striatum is not exactly replicated in the NAc. This architecture does not apply to mouse NAc projection to the ventral pallidum (Kupchik et al., 2015).

DRUG REWARD

Most drugs of abuse, including ethanol, activate the mesocorticolimbic dopamine system and lead to an increase in extracellular dopamine in the NAc (Doyon et al., 2003; Imperato & Di Chiara, 1986; Weiss et al., 1993). Dopamine signals released by a given stimulus are thought to exert their influence on responses to subsequent stimuli that follow the initial triggering one. All drugs of abuse elicit, to some extent, an incentive arousal state as a result of their ability to increase extracellular dopamine in the NAc shell (Di Chiara et al., 2004). Dopamine released from the ventral tegmental area (VTA) has been described in a number of ways over the years. For instance it is believed that mesolimbic dopamine has a role as a motivational learning signal (Spanagel & Weiss, 1999); that dopamine is a signal of pathological associative learning in addiction (Di Chiara, 2002); is a neural substrate of incentive salience (Berridge & Robinson, 2003); or is a signal that informs about the predictability of reward related to cues associated with drug availability (Fiorillo et al., 2003).

The mesocorticolimbic system is arranged in such a way that cortical neurons are likely promoters of goal-directed behaviors. In this general scheme, information from the vHipp provides spatial, contextual information, and emotional memories, the PFC provides

executive control (including the ability of task switching and response inhibition), and the BLA communicates information regarding conditioned associations and emotional valance (Ambroggi et al., 2008; Gruber et al., 2009; Ito et al., 2008; Kalivas et al., 2005; M. E. Wolf, 2002). Therefore, cortical activation of the NAc direct circuits leads to disinhibition of appropriate action plans that facilitate reward acquisition whereas, cortical activation of indirect circuits is likely to inhibit motor plans that are maladaptive for obtaining reward (Mink, 1996; Redgrave et al., 1999, 2011).

The shell subregion of the NAc is critically involved in various aspects of drug reward (Ikemoto, 2007; Rodd-Henricks et al., 2002; Sellings & Clarke, 2003). The shell of the NAc can be considered the entryway for input into the striatum. There is evidence that the transition to an addicted state follows neural adaptations that occur first in the shell, then the NAc core, and finally in the dorsal striatum. The striatum of non-human primates is organized into a series of parallel circuits that are linked in an ascending spiral to the dorsal striatum (Haber et al., 2000). The architecture of these circuits is such that shell neurons project to core neurons which in turn project to dorsal striatum neurons; adaptations of these circuits produced by drugs of abuse are likely responsible for the transition from goal-directed to habitual behaviors during the development of drug dependence. Therefore, improving our understanding of the synaptic adaptations that underlie these neural circuit modifications could greatly improve our understanding of how these complex habitual behaviors are formed and expressed.

SYNAPTIC PLASTICITY AND DRUG DEPENDENCE

Synaptic plasticity of neural circuits allows for the reorganization of synapses in response to an ever changing environment. The two best characterized forms of synaptic plasticity are long-term potentiation (LTP) or long-term depression (LTD). Both are

mechanisms for modulating synaptic strength in an experience-dependent manner. LTP involves a long lasting increase in synaptic strength. LTP was first described in the hippocampus where high frequency stimulation of the performant pathway inputs to the dentate gyrus resulted in a long-lasting enhancement of excitatory synaptic transmission (Bliss & Lomo, 1973). LTD is a long-lasting decrease in synaptic strength and the first reliable means of inducing this form of plasticity were described in 1992 involving depression of CA1 hippocampal cells following low frequency stimulation of the Schaffer collateral projection to CA1 (Dudek & Bear, 1992). Both of these processes are believed to be involved in learning and memory. The long lasting adaptations that are responsible for LTP can reinforce some inputs whereas the adaptations that underlie LTD can reduce inputs. Some of the major forms of synaptic plasticity, including their induction mechanisms, which have a known relationship to drug exposure, will be discussed below. These forms of plasticity have been observed in various brain regions of mouse, rats, and non-human primates.

Synaptic Plasticity Mechanisms

Excitatory Signaling

The majority of synapses that undergo LTP and LTD communicate using the excitatory amino acid, L-glutamate, as their neurotransmitter. Glutamate acts on a number of different receptors including: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs), N-methyl-D-aspartate (NDMA) receptors (NMDARs), kainate receptors, and metabotropic glutamate receptors (mGluRs) (Collingridge et al., 2009). The majority of fast excitatory synaptic transmission is mediated by AMPARs. AMPARs are

ionotropic receptors composed of tetramers of the GluA1-4 subunits. Most AMPARs contain the GluA2 subunit which undergoes RNA editing that produces a glutamine (Q) to arginine (R) switch at the Q/R site of the pore-lining region. This RNA editing at the Q/R site is critical in the electrical properties of these receptors. GluA2-containing AMPARS have the positive arginine in the pore-line region that prevents calcium (Ca^{2+}) influx as well as intracellular polyamines from blocking the pore at positive potentials which results in a linear (ohmic) current voltage relationship. In contrast, AMPARs that lack GluA2 subunits are Ca^{2+} permeable, have a higher conductance, and are blocked by endogenous intracellular polyamines in at high voltage potentials resulting in an inward rectifying current voltage relationship.

NMDARs are voltage activated cation channels which have slower kinetic properties compared to AMPARs. NMDARs are heterotetramers composed of two obligatory GluN1 subunits and two GluN2 subunits, of which there are 4 subtypes (GluN2A-D). NMDARs require binding of both glutamate and the co-agonist glycine in order for the channel to open. At resting membrane potentials, extracellular magnesium (Mg^{2+}) blocks the receptor pore. Depolarization of the cell removes the Mg^{2+} block allowing cations including Ca^{2+} to enter the cell. In this way NMDARs act as coincident detectors; two inputs are required for NMDAR activation, sufficient postsynaptic depolarization and synaptic release of glutamate. Activation of NMDARs and the elevation of intracellular Ca^{2+} are critical for the induction of many forms of plasticity. NMDARs are triggers for synaptic plasticity; however, they do not determine the direction of change in synaptic efficacy. Activation of NMDARs can lead to LTP or LTD (C. Lüscher & Frerking, 2001).

Long-term Potentiation

NMDAR-dependent LTP is induced in experimental conditions via high frequency stimulation (HFS; 100 Hz), traditionally via afferent electrical tetanus stimulation. This stimulation pattern is believed to drive sufficient glutamate release to result in AMPAR mediated depolarization of the post synaptic neuron sufficient to remove the Mg^{2+} blockade and allow NMDARs to be activation. Activation of NMDARs during HFS allows Ca^{2+} to enter the postsynaptic cell. Elevation in postsynaptic Ca^{2+} leads to an intracellular signaling cascade that includes the activation of Ca^{2+}-calmodulin kinase type II (CAMKII). Activation of CAMKII by Ca^{2+} ultimately results in the insertion of postsynaptic AMPRs into the membrane resulting heightened excitability and potentiation of the synapse (C. Lüscher & Frerking, 2001; Nicoll & Malenka, 1999) (Illustration 1).

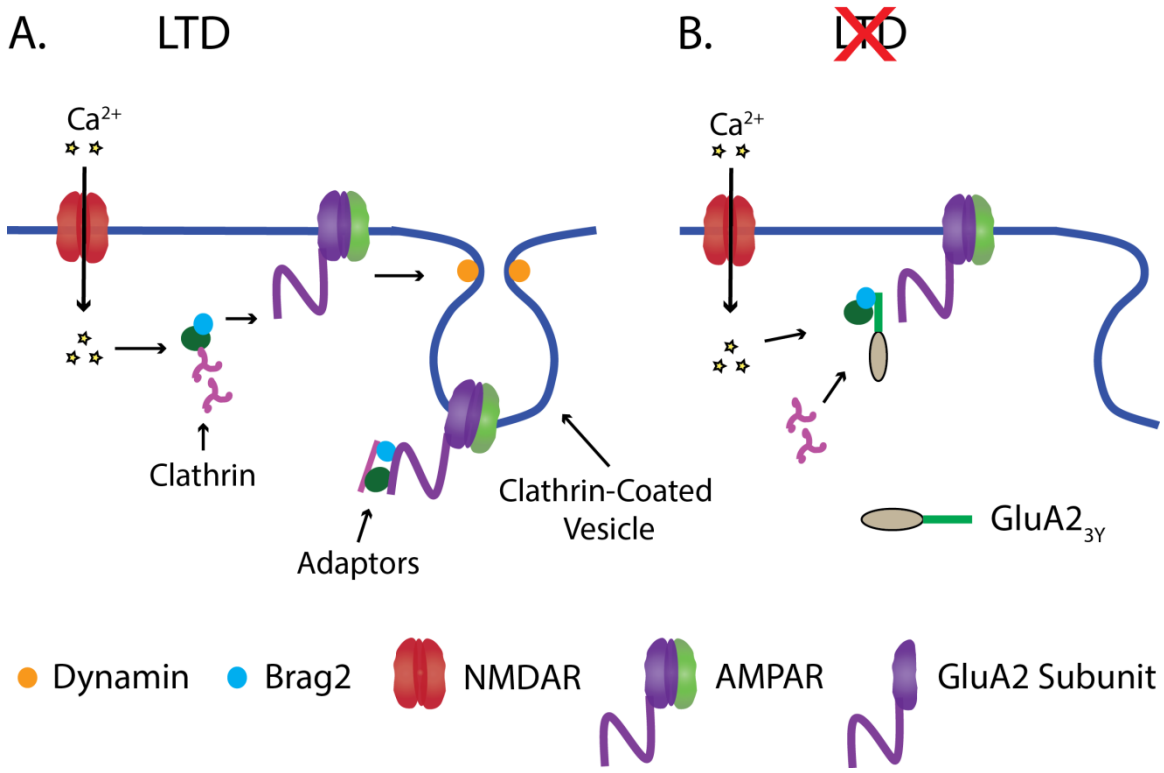
Long-term Depression

NMDAR-dependent LTD can be experimentally induced using a weak activation of NMDARs, via low frequency stimulation (LFS, 1-3 Hz), traditionally using afferent electrical stimulation. LFS results in a small to moderate rise in intracellular Ca^{2+} levels (lower levels then HFS) which leads to a distinct intracellular signaling cascade than that of LTP. Ca^{2+} that enters the postsynaptic terminal via NMDARs, binds to calmodulin and leads to the activation of protein phosphatase 2B (PP2B, aka calcineurin). Calcineurin in turn dephosphorylates inhibitor-1, leading to the activation of protein phosphatase 1 (PP1) (Mulkey et al., 1993). Active PP1 is then capable of dephosphorylating serine845 on AMPAR subunit GluA1, which leads to the subsequent removal of these AMPRs from the synapse via a clathrin- and dynamin-dependent process (C. Lüscher et al., 1999; Selig et al., 1995).

Active PP1 is also capable of targeting GluA2 subunit containing AMPARs for removal (Stockwell et al., 2016). The decrease in synaptic strength is due to the removal of postsynaptic AMPARs (Illustration #1). In the NAc the most well described form of NMDAR-LTD involves the removal of GluA2 subunit containing AMPARs. Following activation of PP1 Clathrin adaptor proteins, such as Brag2, bind to the C-terminus of the GluA2 subunit. The GluA2 expressing AMPAR is then internalized via a clathrin-coated vesicle with the assistance of dynamin proteins (Beattie et al., 2000; Malenka, 2003). This interaction of the adaptor proteins with the intracellular C-terminus of GluA2 subunits is critical for the expression of NMDAR-dependent LTD in the NAc. Disruption of this interaction is capable of preventing the expression of LTD (Brebner et al., 2005b; Jeanes et al., 2014a; Scholz et al., 2010).

A second major form of postsynaptic LTD is mGluR-dependent LTD. The canonical signaling pathway for this form of plasticity is via activation of group I mGluRs (G_q protein signaling) which leads to the hydrolysis of phosphatidylinositol to generate inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 and DAG can trigger a number of intracellular cascades including activation of protein kinase C (PKC) and release of Ca^{2+} from intracellular stores. This can lead to a decrease in synaptic strength through a variety of distinct mechanism including the removal of AMPARs form the postsynaptic membrane (Kauer & Malenka, 2007).

Illustration 1. Induction and expression of NMDAR-dependent synaptic depression.



A) Low-frequency conditioning stimulation paired with postsynaptic depolarization produces LTD of AMPAR-mediated EPSCs. This form of LTD relies on activation of NMDARs and influx of Ca2+. Ca2+ influx recruits clathrin and adaptor proteins to bind to the intracellular tail of the GluA2 subunit C-terminal, resulting in internalization of GluA2-containing AMPARs. (B) The artificial peptide GluA23Y mimics this tail of the GluA2 subunit, saturates adaptor proteins, prevents AMPAR internalization, and therefore occludes formation of LTD. Adapted with permission from *Renteria et al. Int. Rev. Neurobiology 126 (2016) 441-465.*

Presynaptic LTD

Presynaptic plasticity involves a long term decrease in the probability of neurotransmitter release. One of these forms of presynaptic LTD that is commonly observed in the NAc involves the activation of endocannabinoid (eCB) CB1 receptors (CB1Rs). CB1Rs are $G_{i/o}$ linked GPCRs and are the most abundant GPCR expressed in the brain. A rise in postsynaptic Ca^{2+} via activation of postsynaptic voltage gated Ca^{2+} Channels (VGCCs) or release from intracellular stores by activation of postsynaptic mGluRs leads to the production of eCB (Kauer & Malenka, 2007). These eCBs produced in the postsynaptic neuron are released and act as a retrograde signal that activates presynaptic CB1Rs. Activation of presynaptic CB1Rs results in a reduction in adenylyl cyclase and PKA activity that decreases vesicular release of neurotransmitter. In the NAc a prolonged moderate efferent electrical stimulation (13 Hz) induces eCB-LTD (Hoffman et al., 2003; Robbe, Kopf, et al., 2002).

Plasticity in the Nucleus Accumbens

NMDAR-dependent LTD is the most frequently reported form of synaptic plasticity observed in the NAc. Different patterns of LFS of synapses on NAc MSNs are capable of producing at least three forms of LTD in the NAc; induced by the different mechanism discussed above. The first form is the most commonly seen NMDAR-LTD (aka 1 Hz-LTD), requires activation of NMDARs, is induced via LFS (500 stimuli at 1 Hz) paired with postsynaptic depolarization to -50 mV. The reduction in synaptic strength is expressed via a reduction in postsynaptic AMPAR expression, in particular AMPARs expressing GluA2 subunits (Brebner et al., 2010; Jeanes et al., 2011a; Thomas & Morrisett, 2000). Two forms of presynaptic LTD, independent of NMDAR activation, have been observed in the NAc: 1) requires eCB signaling generated by postsynaptic

group I mGluR activation (Robbe, Kopf, et al., 2002); 2) is mediated by presynaptic mGluR2/3 activation that leads to the inhibition of Ca2+ channels via cAMP/PKA pathway and subsequent reduction in transmitter release (Robbe, Alonso, et al., 2002). The presynaptic forms of LTD are expressed as a decrease in glutamate release and are induced by higher frequency of LFS (10-13 Hz) than that used in 1Hz NMDAR-LTD.

These various forms of LTD and LTP have been reported at glutamatergic NAc MSN synapses (Cooper et al., 2017; Hikida et al., 2016; Christian Lüscher & Malenka, 2011a; Malenka, 2003; Morisot & Ron, 2017; Scofield et al., 2016; van Huijstee & Mansvelder, 2015; Marina E. Wolf, 2010), and it is believed that these long-term adaptations in efficacy of glutamatergic signals to the NAc contribute to optimization and shaping of its functions. According to the Artola-Brocher-Singer rule, induction of LTP or LTD depends on the ability of the postsynaptic membrane to reach different sequential thresholds that lead to the induction of either LTP or LTD (Artola & Singer, 1993). In the case of NMDAR-mediated increases in postsynaptic Ca2+ levels at least three potential concentrations can be reached: high Ca2+ (leading to LTP induction mechanisms), intermediate (no plasticity induction, or maintenance of current synaptic strength), or low Ca2+ (leading to LTD induction) (Cho et al., 2001). In this configuration glutamatergic input to the NAc can lead to various states of plasticity that may control the final output of signals from the NAc that function to gate salient information and ignore unwanted impulses through the activation or depression of target MSNs (Berretta et al., 2008). Abnormal behavior may result from a shift in the overall synaptic plasticity equilibrium present in the NAc produced by repeated exposure to drugs of abuse.

Nucleus Accumbens Plasticity and Drug Dependence

Synaptic plasticity in the NAc appears to be critical for encoding behavioral responses produced by repetitive drug exposure. As stated previously NAc MSNs are normally quiescent neurons whose activity depends predominantly on excitatory inputs from cortical and limbic regions. Given the accumbens role as the entry point for information into the striatum, alterations in glutamatergic synaptic plasticity onto MSNs in the NAc can have pronounced impact on the output of striatal circuits.

Psychostimulants

Some of the earliest work examining drug induced alterations in the primary observed form of synaptic plasticity within the NAc, NMDAR-LTD, was first described by Thomas et al. in 2001 who discovered occlusion of NMDA-LTD in mice sensitized to the locomotor response to psychostimulants following repeated daily IP injections of cocaine. Furthermore, a variety of glutamatergic synaptic changes were observed including a decrease in the AMPA/NMDA ratio, the ratio AMPAR-mediated to NMDAR-mediated currents, and a decrease in AMPAR mediated miniature excitatory post synaptic current (EPSC) amplitude, together suggesting a decrease in the number of postsynaptic AMPARs present at the synapse. NMDAR-LTD was likely occluded due to those AMPARs that would have been internalized by the LFS having already been internalized during the repeated cocaine exposure. In abstinent (21 days) mice that had previously self-administered cocaine via operant condition also exhibit a long lasting disruption in the expression of NMDAR-LTD in the NAc core (Martin et al., 2006a). Repeated exposure to psychostimulants can produce an increased density of dendritic spines in NAc MSNs (Martin et al., 2006a) and this increase in spine density may potentially be more robust in D1 receptor expression MSNs following cocaine exposure

(Dobi et al., 2011; Kim et al., 2011; Xiuwu Zhang et al., 2007). In addition to the alteration in synaptic spines, cocaine-induced increases in miniature EPSC frequency, an indirect measure in postsynaptic AMPAR density, have been observed in D1-MSNs of the NAc shell (Kim et al., 2011) but not in the core (Dobi et al., 2011).

Shortly following repeated cocaine exposure an increase in the number of "silent" synapses, those lacking detectable AMPARs but containing NMDARs, has been observed in the NAc shell (Huang et al., 2009). These "silent" synapses are likely synaptic spines that have undergone AMPAR removal leaving only the NMDARs present at the membrane. The increase in "silent" synapses may play a role in the decreased AMPA/NMDA ratio observed at a similar time point of exposure (Kourrich et al., 2007a) and appears to peak towards the end of exposure and disappears following withdrawal from drug exposure (Huang et al., 2009). These silent synapses are likely not lost during withdrawal but are instead "unsilenced" by the reinsertion of AMPARs onto the membrane (Conrad et al., 2008a; Dobi et al., 2011). Furthermore, during this unsilencing process, a specific subtype of AMPAR is being selectively inserted into the membrane of synapses during protracted withdrawal. Specifically calcium permeable AMPARs lacking GluA2 subunits (CPARs) are being inserted onto the postsynaptic membrane following withdrawal from cocaine (Conrad et al., 2008a). These GluA2 lacking CPARs are identified by their unique current voltage relationship at depolarized potentials using a rectification index (ratio of AMPA mediated current at negative membrane potential to the current at a positive membrane potential). The insertion of these CPARs likely increases synaptic drive onto NAc MSNs during abstinence as evidence suggest that selectively blocking CPARs with an intra-NAc infusion of the specific antagonist NAPSM attenuates drug seeking in response to cocaine-associated cues (Conrad et al.,

2008a). Finally, cue induced craving for drug intensifies during withdrawal and that this incubation of craving involves the accumulation of CPARs s (Loweth et al., 2014).

Disrupting the internalization of AMPARs from synapses (thereby preventing LTD expression) prevents the expression of behavioral sensitization to amphetamine (K. Brebner et al., 2005b) and selective activation of D1-MSNs in the NAc appears to enhance cocaine sensitization and conditioned place preference (CPP) for a cocaine paired environment. In contrast selective activation of D2-MSNs reduced behavioral sensitization and CPP (Conrad et al., 2008a). The importance of the alteration in NAc LTD produced by psychostimulants is further supported by a study of self-administration of cocaine in rats (Kasanetz et al., 2010a). Rats that self-administered cocaine were divided into two groups, those that would reliably self-administer (addicts) and those that would not (non-addicts) decided by their score on three addiction-like behaviors. LTD in the NAc was impaired but gradually recovered in non-addict animals but was persistently impaired in addicted animals. Selective in vivo depotentiation of cocaine-induced potentiation of excitatory transmission in D1-MSNs abolished cocaine-induced locomotor sensitization (Vincent Pascoli, Turiault, Lüscher, et al., 2012).

In sum, within the psychostimulant literature there appears to be a modest reduction in spine density during exposure to psychostimulants mediated by a reduction in AMPAR expression, the formation of “silent” synapses, and the occlusion of LTD expression at synapses on NAc MSNs. Following withdrawal and abstinence from drug exposure there is an enlargement of spines which is associated with the insertion off CPARs, and increased excitatory tone onto NAc MSNs; which may contribute to escalations of drug seeking observed during periods of protracted withdrawal (Kasanetz et al., 2010a). A challenge does of psychostimulants results in the reemergence of silent

synapses and the loss of LTD expression at NAc MSN synapses (Scott J. Russo et al., 2010).

Ethanol

While there is a large body of evidence for the neuroadaptive responses produce by psychostimulants, it is critical to note that neural adaptations produced by chronic ethanol exposure are likely due to both shared and distinct mechanisms. Indeed ethanol is a potent modulator of plasticity and both *in vitro* and *in vivo* exposure to ethanol can disrupt the expression of plasticity (McCool, 2011). Ethanol has a unique inhibitory action at NMDARs (Lovinger et al., 1989, 1990). Prior work from our lab indicates that ethanol is capable of inhibiting NMDARs in the NAc (Maldve et al., 2002; Tao A. Zhang et al., 2005); this effect on NMDARs likely influences the neuroadaptive changes in NAc glutamatergic function produced by ethanol exposure which are distinct from the psychostimulants previously discussed. Our lab was the first to report a disruption in NAc shell NMDAR-LTD following *in vivo* ethanol exposure. Chronic intermittent ethanol (CIE) vapor exposure reverses the polarity of synaptic plasticity in the NAc shell; a LFS induction protocol that normally produces NMDAR-LTD instead produced synaptic potentiation 24 hours following CIE exposure that persist for up to 72 hours into withdrawal from vapor (Jeanes et al., 2011b).

NMDAR-LTD is also attenuated in mice that exhibit behavioral sensitization to the locomotor effects of ethanol (Karina Possa Abrahao et al., 2013), a subset of Swiss Webster mice showed locomotor sensitization to daily IP ethanol injections and an attenuation of NMDAR-LTD was observed in the NAc only in ethanol sensitized mice but was not observed in non-sensitized mice. This effect was believed to be due to a

dampening of NMDAR function as measured by a decrease in the expression of NMDAR NR1 subunits in the sensitized animals.

Additionally the NMDAR-LTD has been observed to be disrupted in the NAc of ethanol dependent rats (Spiga et al., 2014). Rats were given an ethanol enriched diet for 20 days and following withdrawal from this ethanol exposure a disruption in NAc MSN plasticity was observed in conjunction with a loss of long thin dendritic spines and a decrease in NMDAR function. In sum, ethanol is a potent modulator of synaptic activity within the NAc and this effect is likely due to ethanol's ability to inhibit NMDARs.

Differential role of MSN subtypes in plasticity and addiction

With the development of bacterial artificial chromosome (BAC) transgenic mouse lines that express a fluorophore linked to either the D1 or D2 dopamine receptor promotor (Matamales et al., 2009; Valjent et al., 2009) researchers may now begin to tease apart the role that each subpopulation plays within the NAc. In the dorsal striatum the electrophysiological properties of D1 MSNs differ from that of D2 MSNs (Carlos Cepeda et al., 2007; Day et al., 2008; Valjent et al., 2009). Similarly in the NAc core, which is believed to be morphologically similar to the dorsal stratum, the D1- and D2-MSNs have different electrophysiological and synaptic properties (Grueter et al., 2010b) including excitability, the basal frequency of miniature EPSCs, and the NMDA/AMPA current ratio was higher in D2-MSNs than in D1-MSNs

With a difference in the basal electrophysiological properties observed between D1- and D2-MSNs it would be no surprise that differences in synaptic plasticity would be observed between these two sub families. In the NAc core, a LFS protocol produced LTD, dependent on the activation of CB1Rs and the vanilloid transient receptor potential channels TRPV1, only in D2-MSNs and not in D1-MSNs (Grueter et al., 2010a). This

TRPV1-dependent LTD in D2-MSNs was insensitive to occlusion by cocaine treatment (unlike NMDAR-LTD).

A single dose of cocaine also differentially modulates NMDAR-LTP in D1-and D-2 MSNs of the NAc shell (Vincent Pascoli, Turiault, & Lüscher, 2012). In mice that are cocaine naïve, a HFS protocol can produce NMDAR-LTP in both D1-and D2-MSNs. After a single cocaine IP injection LTP is disrupted only in D1-MSNs. Using an optogenetic approach, with blue light activation of channelrhodopsin (ChR2) to selectively stimulate particular cells of interest, LTD induced through LFS of excitatory efferents to the NAc and reverse the cocaine induced LTP. Disrupting this synaptic adaptation produced by cocaine prevented the expression of cocaine induced behavioral sensitization.

Our lab previously reported that CIE vapor exposure disrupts NMDAR-LTD in the NAc shell MSNs of C57Bl/6J mice. Twenty four hours after a single four day bout of vapor exposure (16 hours on, 8 hours off) a LFS induction protocol that normally induces NMDAR-LTD, instead produced LTP (Jeanes et al., 2011b). To determine if this effect by ethanol is differentially expressed in MSN subtypes DRD1a-eGFP transgenic mice on a Swiss Webster background were obtained (Jeanes et al., 2014) thereby allowing the selective recording from D1 receptor expressing MSNs or D1 receptor lacking MSNs believed to be D2-MSNs. NMDAR-LTD was expressed only in D1-MSNs in the NAc shell and not in the D1 lacking MSNs. Twenty four hours after ethanol vapor exposure there was a reversal in the expression of plasticity for both cell types. Specifically, the LFS pairing protocol had no effect in vapor exposed D1-MSNs but did elicit LTD in vapor exposed D2-MSNs that did not exhibit LTD in the naïve state. Over a two week period, the expression of plasticity was gradually restored to levels observed in naïve

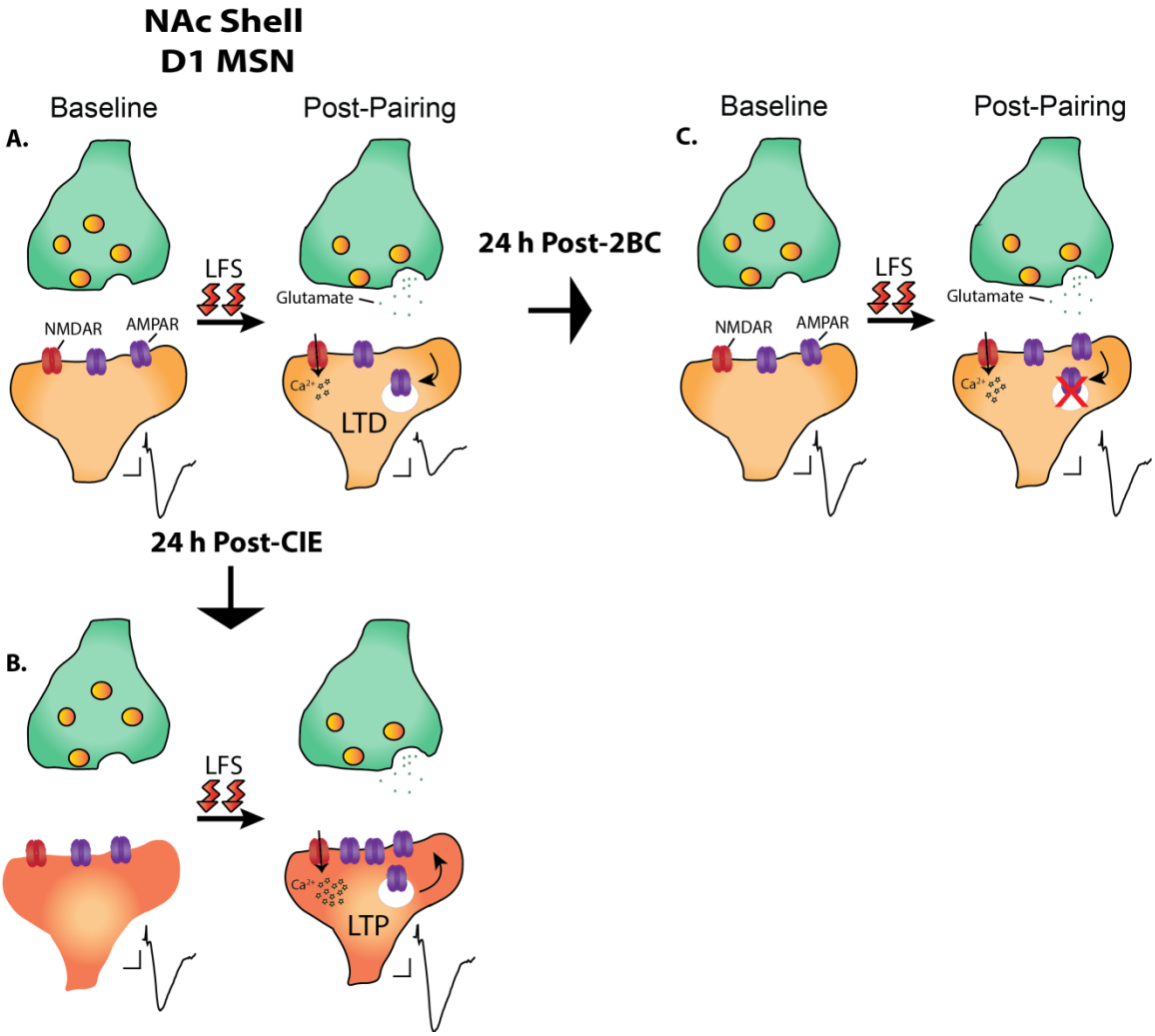
animals; a gradual return of LTD following LFS in D1-MSNs and a gradual loss of LTD expression following LFS in D2-MSNs. Given the difference in expression of plasticity observed between the C57Bl/6J and DRD1a-eGFP mice on a Swiss Webster background (flip in expression of LTP instead of LTD following CIE in C57Bl/6J mice and merely the loss of LTD in Swiss Webster mice), coupled with the difference in drinking observed between these strains; we have postulated that according to the Artola-Brocher-Singer rule the sequential thresholds needed to reach plasticity differ between these mouse strains. We also suspect that the difference in neural adaptations produced by ethanol underlie the differences observed in drinking between these two mouse lines.

Selective manipulation of D1- and D2-MSNs has been observed to differently modulate drug related behaviors. D1-and D2-MSNs in the NAc have distinct roles in reward and aversive behaviors (Hikida et al., 2010, 2013; Lobo et al., 2010). CPP for cocaine or food reward was significantly reduced by selective blockade of D1-MSNs, which suggest a crucial role of these neurons for reward learning. Selective blockade of D2-MSNs prevents the expression of aversive behaviors. Activity of these subtypes of neurons have also been observed to have opposing roles in drug induced locomotor sensitization, where inhibition of D1-MSNs or and activation of D2-MSNs prevents the expression of sensitization to the locomotor effect of cocaine (Chandra et al., 2013).

More recently, work from our lab indicates that a single 4 day bout of CIE vapor (16 hours on, 8 hours off) produces differential alterations in MSNs from td-Tomato mice expressing a fluorophore under the D1 receptor promotor on a C57Bl/6J background (Renteria et al., 2017). Following 24 hour withdrawal from a single bout of vapor the two neuronal subtypes exhibited a reversal in the expression of plasticity; the induction protocol that produced LTD in D1-MSNs of naïve mice instead produced LTP following

vapor withdrawal. In D2-MSNs of naïve mice our pairing protocol produced no change in current amplitude and instead produced LTD following withdrawal from vapor. In addition to withdrawal induced alterations in synaptic plasticity a general increase in excitability as well as NMDAR function was observed in D1-MSNs following vapor exposure. Together, these findings and information discussed above suggest that ethanol treatment initially suppresses NMDAR function followed by a rebound enhancement in NMDAR function during withdrawal which is likely in part responsible for the change in plasticity and excitability of D1-MSNs observed during withdrawal.

Illustration 2. Alterations in accumbal plasticity following different ethanol experiences.



CIE-induced metaplasticity of glutamatergic transmission differs between NAc shell D1 MSNs following exposure to CIE Vapor (left panel) or CIE Vapor and 2BC consumption (right panel). (A) In slices from ethanol–naïve mice, NMDAR-dependent LTD is observed in D1 MSNs following low-frequency stimulation (LFS) paired with postsynaptic depolarization to -50 mV. LTD is expressed via removal of AMPARs from the postsynaptic membrane. (B) Following in vivo CIE exposure the same LTD induction protocol that was applied to ethanol–naïve slices induces LTP of glutamatergic transmission onto D1 MSNs, presumably via insertion of AMPARs into the postsynaptic membrane. (C) Following CIE Vapor exposure that produces ethanol dependence (escalation in volitional intake) the ability to elicit LTD is lost. Adapted with permission from *Renteria et al. Int. Rev. Neurobiology 126 (2016) 441-465.*

THE VENTRAL HIPPOCAMPUS

The hippocampus likely does not act as a unitary structure (Moser & Moser, 1998). Instead it appears that the dorsal and ventral portions of the hippocampus take on different roles. Anatomical studies reveal that the input and output connections of the dorsal hippocampus (dHipp) and the ventral hippocampus (vHipp) are distinct (Swanson & Cowan, 1977). In addition, spatial memory appears to depend on dHipp and not vHipp (Moser et al., 1995); whereas, vHipp and not dHipp lesions alter stress responses and emotional behavior (Henke, 1990). Manipulations of vHipp tend to decrease fear and anxiety (Kirsten G. Kjelstrup et al., 2002; Maren & Holt, 2004), and increase motivation for food (Ferbinteanu & McDonald, 2001).

For a comprehensive review characterizing the differences between the vHipp and the dHipp see Fanselow and colleagues (2010). Briefly, the ventral CA1 and ventral subiculum (vSub) share massive bi-directional connectivity with nuclei of the amygdala. Both send direct projections to the central nucleus of the amygdala which may have the potential to mediate the vHipp contribution to fear learning (Maren & Holt, 2004) as well as the expression of anxiety (Herman et al., 2005; McEwen et al., 1997; Rodrigues et al., 2009). The large reciprocal connection with the amygdala structures implicates the vHipp in emotional responses and memory related to emotion. The ventral CA1 as well as the vSub and the medial band of the lateral and medial entorhinal cortical areas, gives rise to projections to the shell of the NAc (Groenewegen et al., 1996) which plays an important role in reward processing (Wassum et al., 2009b) and motivation of feeding behavior (Kelley, Baldo, & Pratt, 2005; Kelley, Baldo, Pratt, et al., 2005).

The use of modern techniques to selectively target these subregions indicates a role for the vHipp in contextual retrieval of memory in reward related contexts. The

consolidation of contextual memory was only impaired when inactivating the vHipp indicating that vHipp principle neurons are a key driver for synaptic consolidation of memory (Zhu et al., 2014). Robust deficits have also been observed in the retrieval and expression of contextual biconditional discrimination memory following selective and transient post-acquisition inactivation of the vHipp but not the dHipp. The loss of retrieval during inactivation of the vHipp was accompanied by a decrease in anxiety relevant behaviors and indicates that the vHipp is necessary for the contextual retrieval and expression of cue-reward memories (Riaz et al., 2017b).

Information from the hippocampus is involved in mediating drug-seeking behaviors (Fuchs et al., 2005; Riaz et al., 2017b). Lesions of the vHipp decrease sign-tracking behavior and increase goal-tracking behavior during the acquisition of Pavlovian conditioned approach training (Fitzpatrick et al., 2016). The loss of glutamatergic input from the vHipp to MSNs in the NAc may be responsible for this decrease in sign-tracking behavior by disrupting gating and outflow of NAc signals (French & Totterdell, 2002) suggesting that enhanced glutamatergic signaling from the vHipp could promote sign-tracking like behavior. Striking similarities exist between behaviors produced by Pavlovian sign-tracking procedures and key symptoms of drug abuse (T. W. Robbins & Everitt, 2002; Tomie et al., 2008; Tomie & Sharma, 2013). In addition to the change in Pavlovian conditioning, inactivation of the ventral CA1 selectively inhibits cocaine seeking without affecting food-induced reinstatement or locomotor behavior (Lasseter et al., 2010; Rogers & See, 2007). The ventral CA1 region is a likely site at which the persisting ‘memories that sustain addiction’ may be encoded (Nestler, 2001). Indeed, persistent changes in synaptic transmission and LTP occur in the CA1 region of the vHipp following extended access to cocaine self-administration in rats (Keralapurath et

al., 2017). These results are consistent with the conclusion that withdrawal from extended access to cocaine self-administration results in a chronically potentiated state within the ventral hippocampus that is mediated by an increase in AMPAR at Schaffer collateral synapses onto CA1 region of the vHipp. This increased activity at the CA1 likely has important functional implicates for the activity of vHipp projections to other brain regions following repeated drug exposure.

Ventral hippocampal input to the nucleus accumbens and adaptations to drug exposure

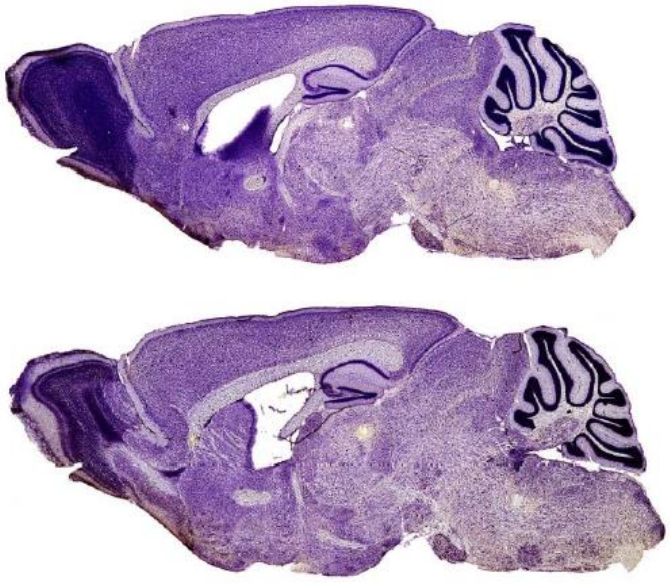
The main glutamatergic input to the nucleus accumbens originates from the vHipp (Britt et al., 2012). This projection is potentiated following extended withdrawal from non-contingent (Britt et al., 2012) or contingent (Pascoli et al., 2012) cocaine exposure. The adaptations in glutamatergic signaling from the vHipp to the shell of the accumbens following cocaine self-administration was specific to D1 expressing MSNs and was not apparent in D2 expressing MSNs. When assessed following a short (3 day) withdrawal from repeated non-contingent cocaine exposure a depression of vHipp input onto D1R-MSNs was observed (MacAskill et al., 2014). Regardless of the input most studies show that exposure to cocaine potentiates excitatory input onto D1R-MSNs but not D2R-MSNs (Bertran-Gonzalez et al., 2008; Bock et al., 2013; MacAskill et al., 2014; Pascoli et al., 2012). Overexpression of ΔFosB increases behavioral responses to cocaine but decreases excitatory input onto D1R-MSNs (Grueter et al., 2013). This decrease in excitatory drive has been hypothesized to be due to an increase in silent synapses, with potentiation occurring after the silent synapses mature (Lee & Dong, 2011). Together this suggests that that there is depotentiation of glutamatergic input from the vHipp onto shell D1R-MSNs that occurs briefly after the initiation of withdrawal from cocaine and is followed

by a potentiation in glutamate following extended withdrawal that is driven by the maturation of silent synapse. To date no research has been conducted to examine the impact of ethanol exposure on vHipp input to the shell of the NAc.

REPRESENTATIVE BRAIN SLICES FOR ELECTROPHYSIOLOGICAL RECORDINGS

From each animal typically two sagittal brain slices that contained the NAc shell were prepared from each hemisphere. Care was taken to ensure that similar thickness (230-250µm) and orientation was maintained for the slices on each experimental day. Recordings were conducted in the shell of the NAc in slices that did not contain dorsal striatal tissue and in the most rostral and ventral areas away from the anterior commissure. We believe that the recordings represent MSNs only from the NAc shell subregion and in particular the more lateral regions of the shell.

Illustration 3. Representative sagittal brain slices containing the nucleus accumbens



Hypothesis and Aims

Overall Project Rationale

Glutamatergic function within the brain's reward system is altered by exposure to drugs of abuse. Drug craving persists for long after cessation of drug taking and can even be enhanced during periods of prolonged abstinence (Grimm et al., 2001; Lin Lu et al., 2004). This increased craving for the drug can lead to relapse, which is a hallmark of addiction. Craving and the subsequent risk of relapse are likely encoded by drug induced alterations of synaptic function that occur within the brain's reward system following chronic exposure. The NAc, and in particular the shell subregion, is a critical constituent of the reward circuit. The NAc has an important role as an integrator of cortical and subcortical information whose output influences behavior. Neuroadaptations produced within the NAc contribute to the formation of drug dependence (Christian Lüscher & Malenka, 2011b) and drug induced adaptations of this brain region can subsequently influence reward seeking behavior as well as the motivational aspects of reward. The most commonly observed form of glutamatergic synaptic plasticity observed in the NAc is NMDAR-LTD. Several reports have shown that *in vitro* and *in vivo* drug or alcohol exposure alters the expression of this form of LTD; indicating that this form of plasticity is highly sensitive to drug and ethanol exposure and that synaptic neuroadaptations in the NAc produced by ethanol and other drugs of abuse likely underlies the formation of dependence (Abrahao et al., 2013; Brebner et al., 2005; Jeanes et al., 2014; Jeanes et al., 2011b; Kasanetz et al., 2010a; Mao et al., 2009; Martin et al., 2006a; Renteria et al., 2017; Shen & Kalivas, 2012; Spiga et al., 2014; Thomas et al., 2001).

Expression of NMDAR-LTD is disrupted following *in vivo* ethanol exposure in a cell subtype specific manner (see above). This initial characterization was completed

using DRD1a-eGFP transgenic mice on a Swiss Webster background that do not voluntarily consume ethanol (Jeanes et al., 2014). Therefore, work in our lab switched to using a reporter mouse line on a C57Bl/6J background, as there is an abundance of literature supporting the voluntary consumption of ethanol in these mice. DRD1a-tdTomato mice on a C57Bl/6J background were chosen to allow us to better understand how alterations in NMDAR-LTD contribute to an escalation in volitional ethanol intake.

The chronic intermittent ethanol (CIE) exposure paradigm has been well-established to induce ethanol dependence and increase volitional ethanol intake (Becker & Lopez, 2004; Griffin et al., 2009; Griffin Iii et al., 2009). CIE was used to induce an escalation in ethanol volitional intake in tdTomato mice and alterations in NMDAR-LTD were examined in the NAc following an increase in ethanol drinking in a cell specific manner. Animals underwent two bottle choice (2BC; 2 hour access to 15% EtOH or H_2O) drinking interspersed with three bouts (4 days, 16 hours on, 8 hours off) of CIE ethanol vapor or air (control) exposure. Animals exposed to ethanol vapor significantly escalated their 2BC ethanol intake. NMDAR-LTD was assessed at 24 hours, 1-2 weeks, and 3 weeks after the last 2BC drinking session. It is critical to note that prior work examined alterations in NMDAR-LTD 24 hours after CIE vapor exposure (Jeanes et al., 2014; Renteria et al., 2017). Twenty four hours after the last drinking session a LFS pairing protocol failed to induce NMDAR-LTD expression in NAc shell D1-MSNs. This loss in LTD expression persisted for up to 2 weeks and was recovered after 3 weeks which correlates to the duration of increased volitional ethanol intake following CIE vapor exposure (Renteria et al., 2017). There was no change in NAc core D1-MSN plasticity during any of these time periods following last ethanol consumption. A significant increase in sEPSC frequency and decrease in the paired pulse ratio was also observed in

D1-MSNs of the shell, indicating an increase in presynaptic release of glutamate at those synapses. A significant decrease in the rectification index was observed in D1-MSNs of the NAc shell was observed at 24 hours after last ethanol intake that persisted for up to two weeks and returned to air control levels after three weeks. This rectification of the current voltage (IV) relationship of AMPAR mediated EPSCs suggests the insertion of CPARs into D1-MSN shell synapses following withdrawal in mice that escalated their volitional intake. Adaptions produced by CIE-induced enhancement in ethanol intake were not observed in NAc core D1-MSNs suggesting that the shell and not the core is critical for these initial adaptions associated with drinking escalation.

The NAc shell D1-MSNs receive dopamine input from the VTA which encodes salience of environmental stimuli (Humphries & Prescott, 2010a) as well as excitatory glutamatergic inputs from the vHipp, mPFC, and BLA. These glutamatergic inputs provide specific and distinct information in the context of reward-related situations. Specifically the vHipp signals contextual relevance, the BLA provides emotional valence, and the mPFC conveys action outcome information (Berridge & Kringelbach, 2013; Robbins & Everitt, 1996). Alterations in NAc LTD produced by repeated drug exposure, as discussed above, are critical for drug related behaviors. Manipulations of drug induced plasticity in accumbens MSNs can prevent the expression of drug related behavior. Blocking drug induced alterations of NMDAR-LTD in MSNs prevents the expression of behavioral sensitization to the motor effects of amphetamine (Brebner et al., 2005; Choi et al., 2014) and disrupts escalation in volitional ethanol intake following CIE (see chapter 3). In addition, optogenetic manipulation of plasticity in D1R-MSNs in the NAc has been shown to disrupt behavioral sensitization to cocaine (Pascoli et al. 2012). These

findings further corroborate the critical importance of drug induced alterations of NMDR–LTD in NAc MSNs to the formation of drug related behavior and dependence.

The findings discussed above were the result of experiments that utilized electrophysiological techniques involving electrical stimulation of glutamate release from all available inputs to the NAc. However, drug induced adaptations in glutamatergic signaling of a single glutamatergic input may be masked during these experiments. The wide adoption of optogenetic tools allows for isolation of specific neural circuits and will allow us to examine plasticity in an input specific manner. Currently there is still a dearth in research examining input specific adaptations of glutamatergic projection to the NAc following ethanol exposure. Recently an elegant study conducted by Pascoli and colleges (2014) used optogenetic tools to isolate the vHipp and mPFC glutamatergic inputs to the accumbens and examined synaptic alterations following cocaine self-administration and withdrawal. Extended withdrawal from cocaine produce input specific alterations in NAc MSNs including an increase in the AMPA/NMDA ratio in MSNs receiving isolated vHipp input, indicating an increase in the AMPAR component that was not observed in isolated mPFC inputs. A 1 Hz stimulation protocol (NMDAR-LTD) in slice normalized (abolished) cocaine-evoked increases in the AMPA/NMDA ratio at this input. In addition, *in vivo* optogenetic LTD induction protocols were conducted prior to cue induced reinstatement procedures. One Hz stimulation of the vHipp-D1R-MSNs produced a reversal of cocaine withdrawal-evoked potentiation of glutamatergic signaling and resulted in a significant reduction in cue-associated lever pressing. These findings indicate the likelihood that repeated drug exposure and withdrawal produces input specific synaptic adaptations and reversal of these adaptations may alter drug associated behaviors.

It is currently unknown if ethanol exposure produces distinct input specific synaptic adaptations of glutamatergic transmission in the accumbens. Previous work in the cocaine literature indicates that exposure and withdrawal produces input specific alterations in glutamatergic transmission in the shell of the accumbens. However, the mechanism of action of cocaine and alcohol is different and it is highly likely that input specific neuroadaptations produced by these two drugs will differ. The NAc shell receives the densest glutamatergic input from the vHipp and this input produces larger EPSCs at MSNs than input from either the mPFC or BLA (Britt et al., 2012; O'Donnell & Grace, 1995). In addition photostimulation of this pathway is capable of reinforcing instrumental behavior (Britt et al., 2012). Also as discussed above the vHipp plays a role in reward processing and is critical for the expression of reward context associations. For this reason we propose selectively targeting the vHipp input to NAc shell by using optogenetic tools to determine if binge-like ethanol exposure is capable of producing synaptic alterations in this isolated circuit. If ethanol exposure produces synaptic alterations in this isolated circuit it would be initial evidence to support our hypothesis that glutamatergic input from the vHipp plays an important role in the development of ethanol dependence and the expression of ethanol dependent behaviors. To our knowledge using optogenetic methods to isolate input specific neuroadaptations produced by chronic ethanol exposure has not been attempted.

SPECIFIC AIMS

Specific aim 1: Establish whether vHipp to NAc shell synapses show properties similar to those previously documented via standard local shNAc electrical stimulation *in vitro*.

Findings discussed above indicate that synaptic alterations in the NAc are critical for drug related behaviors. The use of optogenetic tools will allow us to dissect the neural

adaptations that occur in an isolated glutamatergic input to the NAc shell circuit in order to examine how this circuit is altered by alcohol exposure. Exposure and withdrawal to other drugs of abuse produces input specific synaptic alterations of the NAc and *in vivo* manipulations of synaptic plasticity in these isolated inputs prevents the expression of drug related behavior (Hearing et al., 2016a; Joffe & Grueter, 2016; Pascoli et al., 2014). However, it is unknown if alcohol selectively alters excitatory transmission or plasticity to the NAc shell in an input specific manner.

Aim 1.1: Verify that activation of selective glutamate release from terminals from the vHipp is capable of eliciting plasticity in NAc shell D1-MSN synapses.

Aim 1.2: Characterization of synaptic plasticity in the isolated vHipp to NAc shell D1-MSN synapses following acute *in vitro* ethanol exposure.

Specific Aim 2: Determine the neural adaptations in isolated vHipp to NAc shell D1R-MSN synapses elicited by chronic ethanol experience and withdrawal**.**

Research conducted in our lab indicates that withdrawal from two bottle choice (2BC) ethanol exposure interspersed with CIE exposure increases the rectification index (insertion of GluA2-lacking AMPARs) and results in a loss of LTD expression for up to two weeks after last exposure (Renteria et al., 2017). However, this work examined global changes in excitatory inputs to D1R-MSNs following CIE exposure. We are interested in the expression of ethanol induced adaptations in isolated glutamatergic inputs. Using the viral insertion of channelrhodopsin into vHipp excitatory output neurons with terminals present in the NAc shell we will be able to selectively stimulate

glutamate release from these terminals locally and determine whether specific neural adaptations are produced in this circuit following *in vivo* ethanol exposure and withdrawal.

Aim 2.1: Determine alterations in NMDAR-LTD in the vHipp to NAc shell D1-MSNs circuit after an escalation in ethanol drinking.

Aim 2.2: Determine changes in glutamatergic signaling in vHipp to NAc shell D1-MSNs circuit after an escalation in ethanol drinking.

Chapter 2:

Ventral Hippocampal-Nucleus Accumbens Shell Glutamatergic Signaling Is Altered By In *Vitro* and *In Vivo* Ethanol Exposure

This chapter written in the style for submission to Addiction Biology.

Authors: Daniel Kircher, Heather Aziz, Richard Morrisett. Daniel Kircher created the study design and conducted all experiments. Heather Aziz conducted breading of animals and assisted with animal behavior. Richard Morrisett contributed to the study design.

ABSTRACT

The purpose of this study was to determine the effects of ethanol exposure and consumption on the expression of plasticity of D1 dopamine receptor expressing (D1R) medium spiny neurons (MSNs) in the nucleus accumbens (NAc) shell receiving glutamatergic input solely from the ventral hippocampus (vHipp). Drd1a-tdTomato mice on a C57BL/6J background were injected bilaterally in the vHipp with a viral vector in order to express channelrhodopsin (ChR2) in vHipp terminals that synapse onto shell D1R-MSNs. Blue LED light stimulation was used to selectively depolarize ChR2 expressing vHipp terminals in the NAc shell, resulting in light-evoked EPSCs originating from vHipp. In voltage clamp experiments, we found that an induction protocol pairing 1 Hz blue light stimulation with postsynaptic membrane depolarization produced LTD in the vHipp to NAc circuit that is NMDA receptor dependent. In this study we found acute application of ethanol in vitro uniquely alters plasticity in the vHipp to NAc circuit. Low to moderate intoxicating concentration of ethanol blocked light evoked LTD expression which is in contrast to previous observations. Ethanol consumption in rodents impaired vHipp-Shell plasticity as well as resulted in altered glutamatergic signaling, and the insertion of Ca2+ permeable AMPA receptors (CPARs) D1R-MSNs post synaptic

membranes. These findings suggest that the vHipp to NAc circuit is highly sensitive to ethanol treatment and may constitute a critical neuroadaptation that leads to the expression of ethanol dependence.

INTRODUCTION

Chronic drug and alcohol exposure produces alterations in glutamatergic signaling and plasticity within the nucleus accumbens (Christian Lüscher & Malenka, 2011b; McCool, 2011). One of the most critical forms of plasticity that has been observed to be altered by drug exposure within the NAc is NMDAR-dependent long-term depression (LTD). A growing body of literature suggests that chronic exposure to psychostimulants, heroin, and alcohol can result in altered or impaired expression of NMDAR-dependent LTD in the NAc (Abrahao et al., 2013; Jeanes et al., 2014b; Jeanes et al., 2011b; Kasanetz et al., 2010a; Mao et al., 2009; Martin et al., 2006b; Renteria et al., 2017; Renteria et al., 2017; Shen & Kalivas, 2012; Spiga et al., 2014; Thomas et al., 2001). The mechanisms by which drugs of abuse produce neuroadaptations of glutamatergic signaling likely overlap with the mechanisms necessary for the expression of NMDAR-LTD.

The alcohol dependence model currently employed by our lab is the chronic intermittent ethanol (CIE) vapor exposure model. This model reliably induces alcohol dependence, as indicated by an increase in voluntary ethanol consumption (Becker & Lopez, 2004; Jeanes et al., 2011a; Renteria et al., 2017). Ethanol vapor exposure alone is capable of altering the excitability of D1-MSNs as well as increasing NMDAR activity 24 hours after last vapor exposure (Renteria et al., 2017). CIE vapor exposure also disrupts plasticity in a cell subtype and strain dependent manner. Twenty four hours after

vapor exposure NMDAR-LTD is occluded in D1-MSNs from mice on a Swiss Webster background; whereas an identical pairing protocol produces LTP in D1-MSNs from mice on a C57BL/6J background. This difference in LTD expression is likely related to the fact that one group of animals readily consumes ethanol (C57BL/6J) while the other does not (Swiss Webster). Interestingly, in both strains of animals D2-MSNs that show no LTD in the naïve state instead expressed LTD 24 hours after last vapor exposure Jeanes et al., 2014; Renteria et al., 2017).

The CIE vapor model readily increases volitional ethanol consumption, associated with a loss in LTD expression in D1-MSNs of the NAc shell, but not core. Disruptions in plasticity persisted for up to two weeks and returned to air control levels three weeks after the last ethanol drinking session (Renteria et al., 2017). This alteration in LTD expression mirrors the time course of escalated drinking observed following CIE exposure (Becker & Lopez, 2004; Griffin et al., 2009). The loss of LTD was also accompanied by a change in the rectification index of AMPAR mediated currents in D1-MSNs of the shell. 24 hours after the last drinking session D1-MSNs from ethanol vapor exposed animals exhibited rectified AMPAR currents at positive holding potentials (Renteria et al., 2017). The presence of rectification is a possible indication that GluA2 subunit lacking, also referred to as Ca2+ permeable, AMPARs (CPARs) have been inserted into the synapse. This changed in rectification was observed for up to two weeks after the last drinking session and returned to control levels after 3 weeks. Changes in rectification as well as insertion of CPARs into the synapse is also produced by withdrawal from cocaine administration (Conrad et al., 2008b; Loweth et al., 2014; Marina E. Wolf & Tseng, 2012).

The above studies were conducted using electrical stimulation of all available glutamatergic inputs to the NAc MSNs. Recent work in the psychostimulant field indicates that selective adaptations are produced by drug exposure depending on which glutamatergic input to the NAc is assessed (Joffe & Grueter, 2016; Pascoli et al., 2014; Pascoli, et al., 2012; Stuber et al., 2012). Our primary goal was to characterize neuroadaptations in an isolated glutamatergic input from a single brain region projecting to the shell that may contribute to excessive ethanol consumption. We chose to initially asses glutamatergic input from the ventral hippocampus (vHipp) due to that regions strong anatomical and functional relationship with the NAc. The NAc receives major inputs from the hippocampal ventral subiculum (Groenewegen et al., 1987) as well as the ventral CA1 (Britt et al., 2012) to the shell of the accumbens. The accumbens requires hippocampal stimulation in order for its neurons to depolarize and assume an activated state (O'Donnell & Grace, 1995). Neurotransmission in accumbens-hippocampal circuitry has been implicated in triggering relapse in addiction (Kalivas et al., 2005).

In this study, we used Drd1-tdTomato mice on a C57BL/6J background injected with a viral vector to express the light activated cation channelrhodopsin (ChR2) in the primary output (glutamatergic) neurons of the ventral hippocampus in order to selectively stimulated glutamate release from a single input onto D1-MSNs in the shNAc. In the present study we aimed to determine how alterations in AMPAR mediated signaling and the disruption of NMDAR-LTD in an isolated glutamatergic projection to the shell contributes to excessive alcohol consumption.

METHODS

Animals

Mice were heterozygous BAC transgenic mice in which the tomato fluorophore expression was driven by dopamine D1 receptor (Drd1a-tdTomato from Jackson Laboratories) gene regulatory elements. All animals used were male. Transgenic mice were back crossed in the C57BL/6J line. Mice were single housed after surgery. All animals were kept in a temperature and humidity controlled environment with a 12 h light/ 12 h dark cycle (lights on at 0700 h). Animals were given a minimum of 3 days of recovery from surgery prior to light reversal (lights on at 2100 h). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Stereotaxic Injections

AAV2-CaMKIIa-hChR2 (H134R)-eYFP or AAV2-CaMKIIa-eYFP (for control mice) produced at the University of North Carolina (Vector Core Facility) was injected into the vHipp of 5- to 6-week-old mice. Anesthesia was induced at 3% and maintained at a range of 1.5-2% isoflurane (w/v) (Animal Health International) as determined by depth of animal breathing. The animal was placed in a stereotaxic frame (Kopf) and bilateral craniotomies were performed using stereotaxic coordinates adapted from a mouse brain atlas and then refined by sham injections (for vHipp: anterior-posterior = -2.8; medial-lateral = ± 2.8; dorsal-ventral (from the surface of the brain) = -4.2). Injections of virus (0.55 µl per injection site) were made using graduated pipettes (Drummond Scientific Company), broken back to a tip diameter of 10-15 µm, at an infusion rate of ≈0.05 µl per min. A subset of animals was given two weeks to recover prior to two bottle choice drinking. Another subset of animals was ethanol naïve and remained in their home cage

until electrophysiological recordings. Naïve animals were individually housed at the same time course as the drinking animals and recordings were conducted at the same age as drinking animals. Optogenetic manipulations typically occurred no sooner than 10 weeks after the injection.

Two Bottle Choice Drinking

Two bottle choice (2BC) drinking is a limited access (2 hrs.) paradigm used to measure changes in volitional consumption (Griffin, 2014). Thirty minutes prior to the beginning of the dark cycle (light out 0930 hours) mice are given access to two bottles, one containing a 15% ethanol and tap water solution and the other containing tap water alone. Animals and bottles were weighed prior to 2BC and bottles were weighed after two hour access. Bottle were compared to give the amount consumed (g alcohol per kg animal body weight/2hrs). Two weeks after virus injection a subset of animals underwent 21 days of consecutive 2BC dinking. The last 5 days of this drinking was measured as baseline ethanol consumption. Following this 21 days of drinking animals underwent chronic intermittent ethanol (CIE) vapor exposure (EtOH vapor or air control). Seventy two hours after each bout of vapor exposure 2BC drinking was conducted for 5 days to measure CIE induced changes in ethanol consumption. Drinking data was analyzed using a repeated measures four-way ANOVA with EtOH vapor and Air groups as well as ChR2 and eYFP virus injections as the between subject variables and the drinking session and drinking day as the repeated measures. Further analysis using Bonferroni post hoc tests or one way ANOVAs was used to determine differences in drinking between groups at each drinking session and changes in drinking session vs baseline drinking within each group.

Chronic Intermittent Ethanol

Mice were exposed to CIE vapor or air (control) using a previously established model that has been shown to lead to increases in volitional ethanol consumption in mice (Becker & Lopez, 2004). Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 3.5 liter/min. Ethanol vapor was then combined with a separate air steam to give a total flow rate of approximately 4 liters/min. Ethanol enriched air (or air alone for the control group) was delivered to mice in special mouse chamber units with airtight tops (Allentown Inc., Allentown NJ). Each bout of vapor consisted of 16 hours of ethanol vapor exposure followed by 8 hours of withdrawal, repeated for 4 consecutive days. Mice were injected (i.p.) with a loading dose of ethanol (20% v/v, 1.5 g/kg) and pyrazole (68.1 mg/kg) suspended in 0.1M PBS prior to vapor exposure. Air control mice were handled the same as ethanol exposed mice but received an injection of only pyrazole suspended in PBS.

Blood Ethanol Concentrations

Following the completion of each 16 hour vapor exposure tail blood samples of approximately 5 µl were collected upon removal from the vapor prior to withdrawal. BECs were measured using gas chromatography with a Bruker 430-GC (Bruker Corporation, Fremont CA) equipped with a flame ionization detector and CombiPAL autosampler. For each mouse two samples of tail blood were taken and each was added to a 10 ml vial containing 45 µl supersaturated sodium chloride solution. Samples were warmed to 65°C and the solid-phase micro extraction fiber (SPME; 75 µm CAR/PDMS, fused silica; Supelco, Bellefonte, PA) was used to absorb ethanol vapor from each sample. Ethanol peaks determined by GC were analyzed using CompassCDS Workstation software (Bruker Corporation, Fremont CA). BECs of tail blood were

calibrated against ethanol peaks from prepared ethanol standards (100-400 mM). Ethanol vapor flow rates and ethanol and loading injections were adjusted to maintain mice within a BEC range of 150-200 mg/dl (37-47 mM) following each 16 hour chambering period.

Brain Slice Preparation

Mice were at least 13 weeks of age at the time of slice preparation. Parasagittal slices (230-240 μm thick) containing the NAc were prepared using a Leica vibrating microtome. Mice were anesthetized by inhalation of isoflurane and the brains were rapidly removed and placed in 4°C oxygenated artificial cerebrospinal fluid (ACSF) containing the following in (mM): 210 sucrose, 26.2 $NaHCO_3$, 1NaH_2PO_4, 2.5 KCl, 11 dextrose, bubbled with 95% O_2/ 5% CO_2. Slices were transferred to a non-sucrose based ACSF solution for incubation containing the following (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 2.4 $MgCl_2$, 1.8 $CaCl_2$, 10 dextrose, were continuously bubbled with 95% O_2/ 5% CO_2; pH 7.4, 32°C, and were maintained in this solution for at least 60 minutes prior to recording.

Patch Clamp Electrophysiology

Whole cell voltage clamp recordings were conducted in the nucleus accumbens shell. Cells were identified using a BX50 microscope (Olympus) mounted on a vibration isolation table. Recordings were made in ACSF containing (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 0.9 $MgCl_2$, 2.0 $CaCl_2$, and 10 dextrose, bubbled with 95% O_2/ 5% CO_2. ACSF was continuously perfused at a rate of 2.0 mL/min and maintained at a temperature of ≈ 32°C. Picrotoxin (50μM) was included in the recording ACSF to block $GABA_A$ receptor-mediated synaptic currents. The NMDAR antagonist D-

APV (50 µM) was added to the recording solution for Rectification Index recordings in order to isolate AMPAR mediated currents. Strontium chloride ($SrCl_2$, 6 mM) was substituted for $CaCl_2$ in the recording solution for asynchronous EPSC (asEPSC) recordings. 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 3-6 MΩ. For Plasticity, NMDA/AMPA ratio, Rectification Index ratio sEPSC, and asEPSC recordings, electrodes were filled with (in mM): 120 CsMeSO4, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4 Mg-ATP, 0.3 Na-GTP, 0.1 spermine, and 5 QX-314-Cl. For all recordings, input and access resistances were monitored throughout the experiments. Cells in which input and access resistance varied more than 20% were not included in analysis.

Data Acquisition and Analysis

Whole-cell voltage-clamp recordings were conducted on NAc shell D1-MSNs surrounded by ChR2 expressing terminals. D1-MSNs were identified by the presence of td-Tomato and ChR2 expressing terminals were identified by the presence of eYFP using fluorescent light. Excitatory postsynaptic currents (EPSCs) were acquired using an amplifier (Dagan) filtered at 1 kHz, and digitized at 10-20 kHz with a Digidata 1440B interface board using pClamp 10.6 (Axon Instruments). Light-EPSCs were evoked with local 4 ms blue light pulses from an optic fiber directed at the NAc shell through the microscope objective. A blue light LED (Plexon) was coupled to the objective via a fiber coupler (IS-OGP/OLY, Siskiyou Corporation). Light intensity was adjusted for each recording to elicit EPSCs in a range from 150-250 pA. In experiments for long term plasticity, light-EPSCs were evoked by 4 ms blue light stimulation for at least 10 minutes every 15 seconds, to ensure a stable baseline. To induce LTD, a conditioning stimulus of

500 light pulses (4 ms) at 1 Hz was paired with continuous postsynaptic depolarization to -50 mV. Light-EPSCs were then monitored for 30 minutes after pairing. The magnitude of LTD was calculated by averaging normalized (to baseline) EPSC values from 20-30 minutes post pairing and comparing that to the average normalized EPSCs during the 10 minute baseline. Our criterion for plasticity required that the difference between averaged EPSCs from the baseline and post pairing be greater than 2 standard deviations. Data from each neuron within a treatment group was combined and represented as percent baseline values. Summary data are presented as mean ± SEM. Data was analyzed using a one-way ANOVA and Bonferroni post hoc test or Student's t test in which statistical significance from baseline for within each group was defined as $p < 0.05$.

RESULTS

Optogenetic isolation of the ventral hippocampal to NAc shell input is capable of expressing LTD and this opto-LTD is NMDA receptor dependent.

Injection of AAV2-CaMKIIa-hChR2(H134R)-eYFP or AAV2-CaMKIIa-eYFP into the vHipp of male Drd1-tdTomato mice (PD35-PD42) resulted in selective expression of ChR2-eYFP in glutamatergic vHipp output neurons and their terminals present in the NAc shell (Fig. 1 A). The use of the CaMKIIa promotor on the viral vector for ChR2 ensures that the expression of ChR2 is limited to the principle glutamatergic projection neurons from the vHipp. Activation of ChR2 present in glutamate terminals from a single input to the NAc is capable of eliciting EPSCs in MSNs (Britt et al., 2012; Pascoli et al., 2014) and plasticity has been observed at these isolated synapses. In the present experiment we conducted whole cell patch clamp recordings on D1R-MSNs identified via tdTomato expression. Blue light stimulation of ChR2 expressed in vHipp

terminals in the NAc shell resulted in light evoked EPSCs. A summary diagram of the electrophysiological experiments can be seen in Figure 1 B.

We have previously seen that NMDAR-dependent LTD is expressed only in D1R-MSNs (Jeanes et al., 2014b; Renteria et al., 2017). A pairing protocol that combined light stimulation (4ms pulses at 1 Hz) with depolarization to -50 mV produced an optogenetic LTD (Figure 1 C-D, 51.14 ± 0.728 percent of baseline, n = 7, five animals). a single input onto D1R-MSNs is capable of exhibiting plasticity following an induction protocol similar to the electrical stimulation induction protocol that activates all available inputs used in our prior work. We also observed that the perfusion of the NMDA receptor antagonist D-APV onto the slice prior to and during our optical pairing protocol prevented the expression of LTD (Figure 1 C-D, 99.48 ± 1.629 percent of baseline, n = 5, five animals) confirming that this form of plasticity is dependent on the activation of NMDA receptors.

Acute *in vitro* ethanol exposure inhibits ventral hippocampal-accumbal glutamatergic plasticity

Ethanol is a well-known inhibitor of NMDA receptors and prior work from our lab indicates that it is capable of differentially inhibiting the expression of LTD in the NAc when stimulating glutamate release from all available inputs using electrical stimulation (Jeanes et al., 2014; Jeanes et al., 2011; Renteria et al., 2017; Renteria, Maier et al., 2017). Our aim was to verify that pharmacologically relevant concentrations of ethanol are capable of disrupting LTD of the vHipp to shell D1R-MSN circuit.

Bath application of a moderately to strongly intoxicating concentration of ethanol (40 mM), equivalent to ≈ 0.2 blood alcohol concentration (BAC) in a person and within the range of our target BECs during *in vivo* CIE vapor exposure (described below),

completely inhibited LTD expression (Figure 2, 101.9 ± 2.998 percent of baseline, n = 8, seven animals). Bath application of a low to moderately intoxicating concentration of ethanol (20 mM), equivalent to ≈ 0.1 BAC, also resulted in a loss of LTD expression. A slight decrease in EPSC amplitude was observed but was well below our threshold for plasticity expression (Figure 2, 92.5 ± 1.776, n = 8, six animals). A one way ANOVA revealed a significant difference in EPSC amplitude as a percent of baseline between both ethanol treatment groups and naïve animals as well as a significant difference in EPSC amplitudes as a percent baseline between 20 mM and 40 mM EtOH treated groups (ANOVA: $F_{(2, 15)} = 172.7$, $P < 0.0001$, Bonferroni *post hoc* test $P < 0.0001$, 20 mM EtOH vs naïve and 40 mM EtOH vs naïve, $P < 0.05$ 20 mM EtOH vs 40 mM EtOH0). These findings are in contrast to our previous report using electrical stimulation that did not observe the loss of LTD expression at the low to moderate concentration of ethanol (Jeanes et al., 2011).

CIE vapor exposure induced escalation of volitional ethanol intake in mice

Our initial *in vitro* findings suggest that the vHipp-Shell circuit may be sensitive to ethanol exposure. In order to examine the neural adaptations produced by *in vivo* ethanol exposure within this circuit, animals were allowed to freely consume ethanol and were then subjected to either ethanol vapor or air exposure. An outline of the *in vivo* behavioral experiments is shown in Figure 3a. Two weeks after stereotaxic injection of the viral vector for ChR2-eYFP or control-eYFP mice received daily limited access two bottle choice (2BC) drinking for 21 days prior to the first vapor (or air control) exposure. The last 5 days of drinking were measured as the baseline of consumption and was stable for both air and ethanol vapor exposed groups. CIE vapor exposure produced an escalation in ethanol consumption (g/kg) in the vapor treated animals following each bout

of chambering as compared to baseline intake. No escalation in ethanol consumption was observed in the air exposed mice. There was also no change in intake between animals injected with channelrhodopsin or eYFP (control) virus (Figure 3b). This is supported by repeated measures four-way ANOVA with corrections of sphericity using Greenhouse-Geisser that indicated a significant main effect of vapor exposure ($F_{(2.389, 78.841)} = 5.293$ P < 0.001), a significant main effect of drinking session ($F_{(2.389, 78.841)} = 10.439$ P <0.0001), a significant treatment group by drinking session interaction ($F_{(2.389, 78.841)} = 4.371$, P = 0.011), and no main effect of virus injected ($F_{(2.389, 78.841)} = .310$, P = 0.772). Animals injected with a virus that expressed eYFP but not ChR2 were used as a behavioral control and also underwent the same drinking and vapor or air exposure protocol. Those animals that underwent ethanol vapor exposure exhibited an increase in drinking after each vapor session that was not observed in air exposed animals (Figure 3b). Post hoc analysis revealed that ethanol vapor-exposed mice significantly increased volitional ethanol intake versus baseline across all drinking sessions (*P < 0.05 versus baseline, ****P < 0.0001 versus baseline). In addition ethanol vapor exposed mice significantly increased their intake in the 2nd and 3rd drinking session as compared to air-exposed mice (^P < 0.05 versus air-exposed, ^^P < 0.001 versus air-exposed). CIE vapor induced increase in volitional ethanol consumption mirror those previously reported in our lab (Renteria et al., 2017).

Ethanol consumption and CIE induced escalation of intake disrupts ventral hippocampal-accumbal glutamatergic plasticity

Several reports have observed a disruption in NMDAR-dependent plasticity after *in vivo* drug or ethanol exposure that is related to drug induced behaviors (Abrahao et al., 2013; Brebner et al., 2005a; Kasanetz et al., 2010b; Pascoli et al., 2011). Prior work from

our lab indicates that persistent long term disruptions in NMDAR-LTD are produced in shell D1R-MSNs following CIE induced escalation of ethanol intake. These findings were collected following electrical stimulation of glutamate release from all available inputs to the shell of the NAc (Renteria et al., 2017). Here, we conducted plasticity recordings 24 hours following the last 2BC drinking session in Drd1-tdTomato animals that had been subjected to EtOH vapor or air exposure.

Ethanol vapor treated animals exhibited a loss of expression of NMDAR-LTD in the vHipp-shell circuit (100.3 ± 1.676 percent baseline, n = 9, seven mice, Figure 3c-d). NMDAR-LTD was expressed in air treated control mice 24 hours after last drinking session (67.64 ± 0.828 percent of baseline, n = 8, six animals, Figure 3c-d). However, we observed a reduction in the magnitude of LTD as compared to naïve control animals. A one-way ANOVA revealed a significant difference in EPSC amplitude at the 40-50 min time period between air-exposed, vapor-exposed, and naïve animals ($F_{(2, 15)} = 466$, $P < 0.0001$). Post hoc analysis revealed a significant difference between naïve and air-exposure (****$P < 0.0001$, versus naïve), naïve and vapor-exposure (****$P < 0.0001$, versus naïve), and air-exposure and vapor-exposure (^^^^$P < 0.0001$, versus air-exposure). We also observed that the amount of ethanol consumed during the last 2 hour drinking session was correlated with the magnitude of LTD. Recordings from vapor exposed animals showed no correlation ($P = 0.611$), however recordings from air exposed mice showed a significant inverse relationship between ethanol consumption and the magnitude of LTD (Spearman's $R = -0.9818$, $P < 0.001$). These findings suggest that the amount of ethanol consumed, in the absence of vapor exposure, has a negative impact on the expression of vHipp-shell NMDAR-LTD. As previously reported CIE induced

escalation in ethanol consumption results in the loss of NMDAR-LTD expression within the shell.

Ethanol consumption does not alter ventral hippocampal-accumbal NMDA receptor activity

Previous work in the lab indicates that NMDA receptor activity is enhanced twenty four hours after CIE vapor exposure as measured by NMDA/AMPA ratio recordings (Renteria et al., 2017). We examined if NMDA receptor activity was enhanced in the vHipp-shell circuit following the escalation of drinking produced by CIE vapor exposure or following 2BC consumption. We observed no change in the NMDA/AMPA ratio in D1R-MSNs from either the vapor or air treated animals ($F_{(2, 18)}$ = 0.1479, P = 0.1479, Fig 5 c-d). The NMDA/AMPA ratios for both groups were consistent with those previously shown in animals that did not undergo 2 bottle choice drinking and received air (control, naïve) exposure in a single 4 day bout of CIE treatment. This finding suggests that the enhancement in NMDA receptor activity observed twenty four hours after CIE vapor exposure is no longer expressed 8 days after the last vapor exposure in the current study (2 withdrawal days and 5 drinking days).

Ethanol consumption alters Ventral hippocampal-Accumbal glutamatergic signaling

Prior work indicates that ethanol consumption and CIE vapor exposure enhances spontaneous EPSC (sEPSC) frequency 24 hours after the last drinking session (Renteria et al., 2017). In this current cohort of animals, a one-way ANOVA ($F_{(2, 14)}$ = 8.336, P = 0.0041, Fig 4c-d) revealed a significant increase in sEPSC frequency (**P < 0.01, versus naïve; ^P < 0.05, versus air) in the vapor exposed group. Another one-way ANOAVA ($F_{(2, 13)}$ = 14.02, P = 0.0006; ***P < 0.0001 versus naïve, Fig4c-d) revealed a significant

increase in sEPSC amplitude in the vapor treated group. These vapor induced alterations are present 24 hours after the last drinking session in vapor exposed animals.

In order to examine circuit specific adaptations in glutamatergic signaling, we recorded asynchronous EPSCs (asEPSC) by using blue light stimulation to evoke quantal like events from vHipp terminals in the presence of strontium. The replacement of calcium with strontium in the recording solution acts to prolong release events and results in quantal like release events occurring for a short duration following stimulation (analysis began 30ms after stimulus offset up to 400ms). Circuit specific analysis of glutamatergic signaling revealed a significant increase in asEPSC frequency in the vapor treated group compared to naïve animals ($F_{(2, 12)} = 4.853$, *P = 0.0286; *P < 0.05 versus naïve, Fig 4a-b) suggesting that vapor exposure and an escalation in ethanol consumption leads to a presynaptic alteration in glutamatergic signaling. A one way ANOVA ($F_{(2, 12)} = 28.85$, ****P < 0.0001) revealed a significant effect of ethanol consumption on asEPSC amplitude. Post hoc analysis indicates a significant increase in asEPSC amplitude in the air treated group as compared to naïve (**P < 0.01 versus naïve) as well as significant increase in asEPSC amplitude in the vapor exposed group as compared to naïve and air exposed animals (****P < 0.0001vs naïve, ^P < 0.05 vs air, Fig 4a-b). These findings suggest that ethanol consumption is enhancing postsynaptic glutamatergic function at D1R-MSNs synapses with the vHipp, with an escalation in consumption exhibiting the largest enhancement.

In light of the observed alterations in asEPSC amplitude and frequency we attempted to recapitulate the presynaptic effect by conducting paired- pulse ratio (PPR) recordings (2 pulses, 50ms interstimulus interval) which are inversely correlated with neurotransmitter release. We observed that ethanol consumption produces a decrease in

the PPR (EPSC 2 / EPSC 1) ($F_{(2, 24)} = 13.47$, ***P = 0.001, Fig 5a-b) post hoc analysis indicates a significant reduction in PPR in both Air (**P <0.01 vs naïve) and Vapor (***P < 0.001 vs naïve) treated groups, suggesting that ethanol consumption leads to an increase in the probability of glutamate release form vHipp terminals onto D1R-MSNs of the shell. These findings together indicate that ethanol consumption is enhancing glutamatergic signaling in the vHipp-Shell circuit.

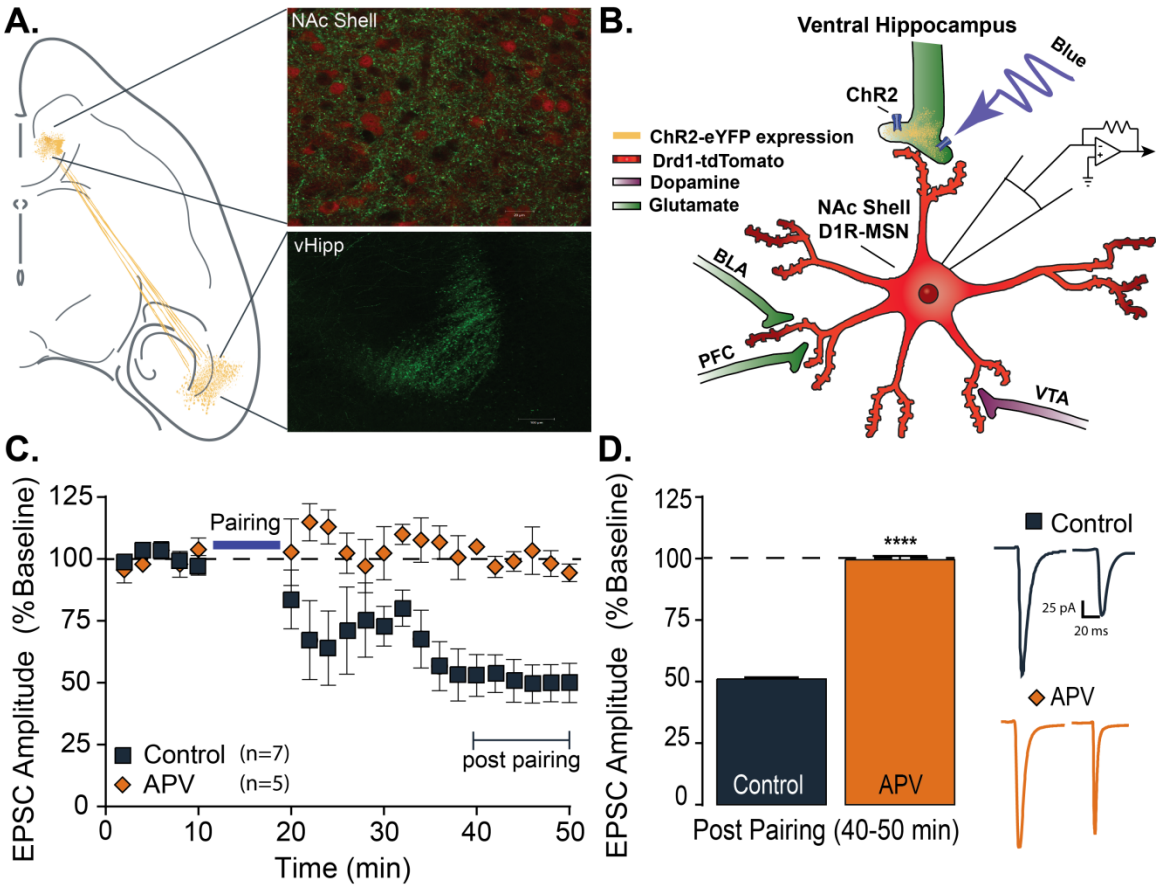
Ethanol consumption leads to the insertion of calcium permeable AMPARs into ventral hippocampus-accumbal D1R-MSN synapses

The insertion of GluA2 subunit lacking AMPA receptors which are permeable to calcium (CPARs) has been observed following exposure to and withdrawal from psychostimulants (Conrad et al., 2008b; Cull-Candy et al., 2006), opiates (Hearing et al., 2016b), and ethanol (Beckley et al., 2016; Renteria et al., 2017) The presence of these CPARs in MSNs can be observed in electrophysiological recordings by a rectification of AMPAR EPSCs generated when holding the MSN at positive holding potentials. At positive holding potentials these CPAR channels are blocked by intracellular polyamines resulting in a reduction in outward current. Prior work in our lab indicates that CIE induced escalation in ethanol consumption results in an increase in rectification of AMPAR EPSCs (Renteria et al., 2017). Electrical stimulation of all available glutamatergic inputs to D1R-MSNs exhibited rectification of AMPAR EPSCs 24 hours after the last drinking session in ethanol vapor treated animals and not in air treated animals. This change in rectification persisted for up to two weeks after the last drinking period and returned to baseline levels after three weeks.

Here we investigated whether a similar neuroadaptation occurs within the vHipp-Shell circuit following ethanol consumption. D1R-MSNs prepared from mice 24 hours

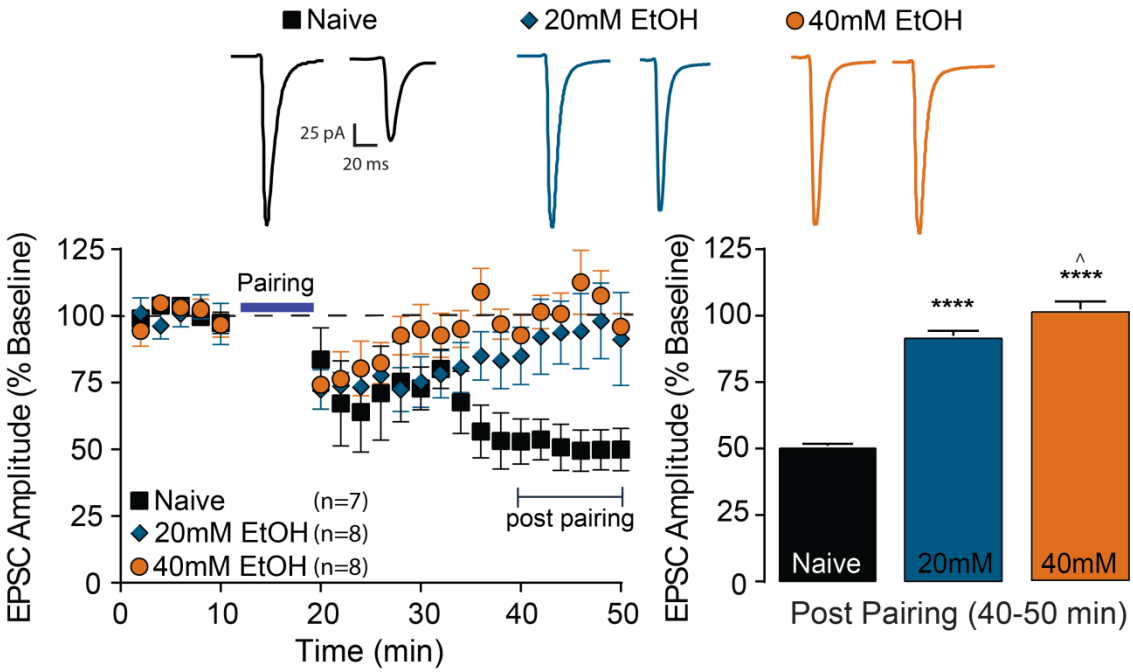
after two bottle choice drinking revealed a rectification of AMPAR EPSCs at positive holding potentials and an overall decrease in the rectification index (EPSC amplitude at +40 mV/ EPSC amplitude at -80 mV) animals ($F_{(2, 25)}$= 14.51, ****P < 0.0001, Fig 6a-b). Post hoc analysis indicates a significant reduction in the rectification index for both vapor (***P < 0.001 vs naïve) and air (***P < 0.001 vs naïve) exposed animals, suggesting that ethanol consumption results in the insertion of CPARs onto D1R-MSNs that synapse with vHipp terminals. In order to confirm that the alteration in rectification index produced by ethanol consumption is due to the insertion of CPARs, we conducted light evoked current recordings and bath applied the CPAR (GluA2 subunit lacking) selective antagonist NASPM. We observed a significant reduction in EPSC amplitudes in the presence of NASPM ($F_{(2, 72)}$ = 13.14, ****P < 0.0001, Fig 6c-d) in both air (***P < 0.001 vs naïve) and vapor (****P < 0.0001 vs naïve) exposed groups. Suggesting that the reduction in the rectification index observed after ethanol consumption is due to the insertion of CPARs.

Figure 1. A diagram of the experimental design and optical low frequency stimulation paired with membrane depolarization of D1R-MSNs induces NMDAR-LTD in vHipp-Shell circuit.



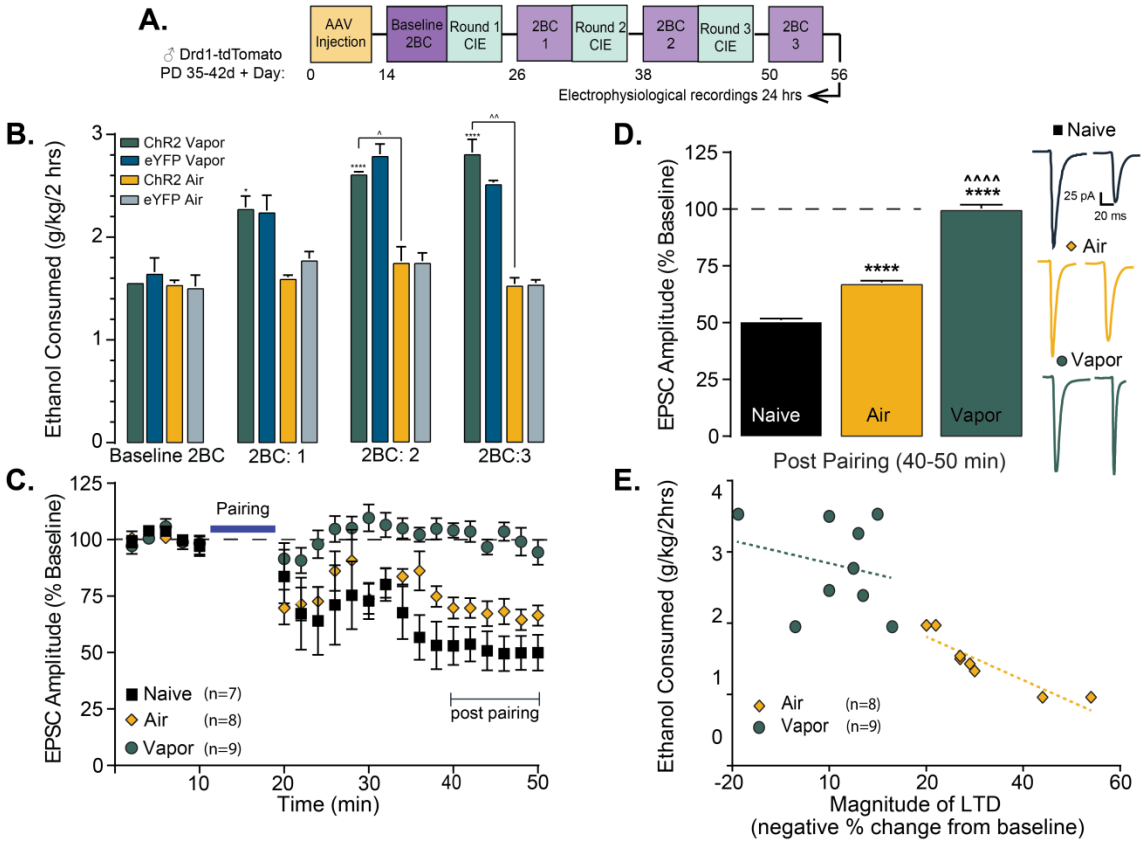
(A) Injection of AAV2-CaMKII-ChR2-eYFP or AAV2-CaMKII-eYFP into vHipp resulted in ChR2 expression in vHipp glutamatergic neurons and in the terminals within the NAc shell. D1R-MSNs were visualized by tdTomato expression. (B) Whole cell patch clamp recordings were conducted in D1R-MSNs and EPSCs were elicited by blue light stimulation of ChR2 present only in vHipp terminals within the NAc shell. (C) A pairing protocol consisting of 1 Hz light stimulation paired with depolarization to -50 mV results in LTD in D1R-MSNs receiving EPSCs only from vHipp terminals. The inclusion of the NMDAR antagonist D-APV in the bath prevents LTD expression. (D). Bar graphs representing the percentage change ± S.E.M. for average EPSC amplitudes between the baseline (min 0-10) and post-pairing (min 40-50) time point. ****P < 0.0001 (Control n= 7 cells from 5 mice; APV n=5 cells from 5 mice)

Figure 2. Acute *in vitro* ethanol exposure abolishes the expression of NMDAR-LTD in glutamatergic plasticity of vHipp-Shell D1R-MSNs.



Escalating concentrations of ethanol applied *in vitro* in the recording bath disrupt the expression of NMDAR-LTD. Maximum inhibition of LTD expression was observed following exposure to a moderate to highly intoxicating concentration of ethanol (40 mM). In the presence of a low to moderately intoxicating concentration of ethanol (20 mM) a less then maximal inhibition of LTD expression was observed. Sample traces of averaged baseline and post-pairing EPSCs (60 seeps, 10 min) of a single representative recording from each ethanol group (Naïve n= 7 cells from 5 mice; 20mM n= 8 cells from 6 mice; 40mM n=8 cells from 7 mice). Bar graph represents percentage change of baseline ± S.E.M. for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each ethanol exposure group. ****P < 0.0001, ^P < 0.05.

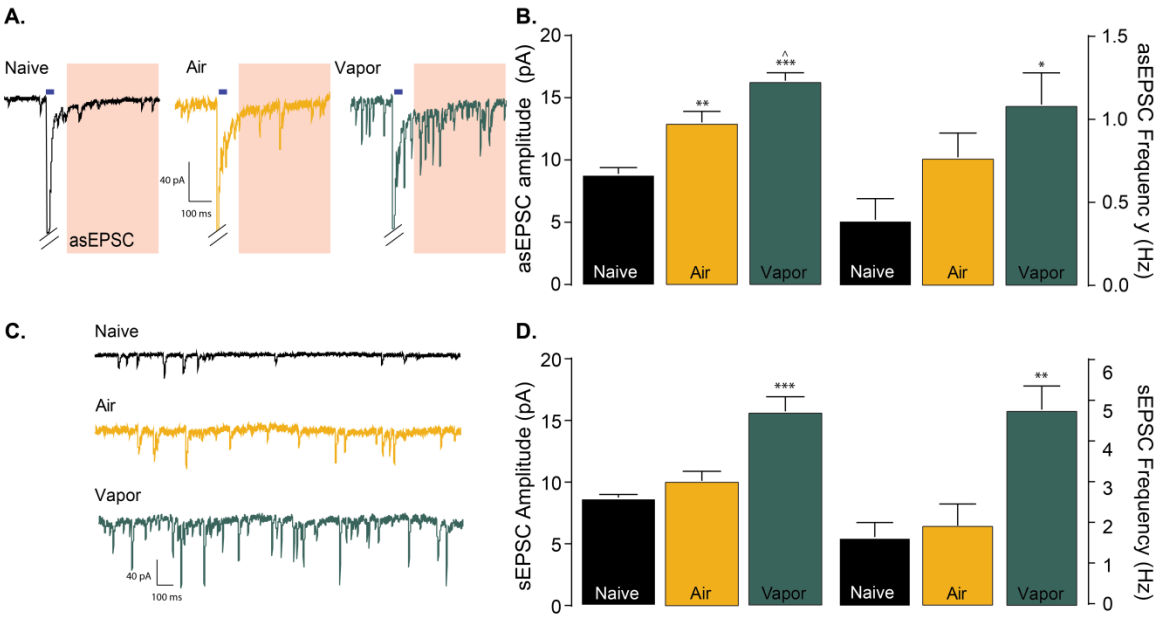
Figure 3. CIE vapor exposure induced escalation of ethanol intake. *In vivo* ethanol consumption reduced the magnitude of glutamatergic plasticity (Air) and CIE-enhanced intake abolished glutamatergic plasticity of vHipp-Shell D1R-MSNs.



(A) the CIE exposure consisted of 3 bouts of ethanol vapor (or air control) exposure separated by 3 day withdrawal and 5 day 2BC drinking (15% ethanol vs water) periods. All electrophysiological recordings were conducted 24 hours after the last ethanol drinking period. (B) Average consumption of ethanol (g/kg/2hrs) ± S.E.M. for each 2BC drinking period. Animals exposed to ethanol vapor exhibited an increase in 2BC drinking after each bout of vapor exposure regardless of virus injection. *P < 0.05 vs baseline, ****P < 0.0001 vs baseline, ^P < 0.05 vs air, ^^P < 0.01 vs air. (C) The pairing protocol resulted in LTD in naïve animals but 24 hours after the last drinking session failed to elicit LTD in the vapor exposed animals. Air exposed animals exhibit a reduction in LTD magnitude 24 hours after the last drinking period. (D) Bar graph represents percentage change of baseline ± S.E.M. for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each ethanol exposure group. ****P < 0.0001 vs naive, ^^^^P < 0.0001 vs air. (E) Positive correlation between amount ethanol consumed during

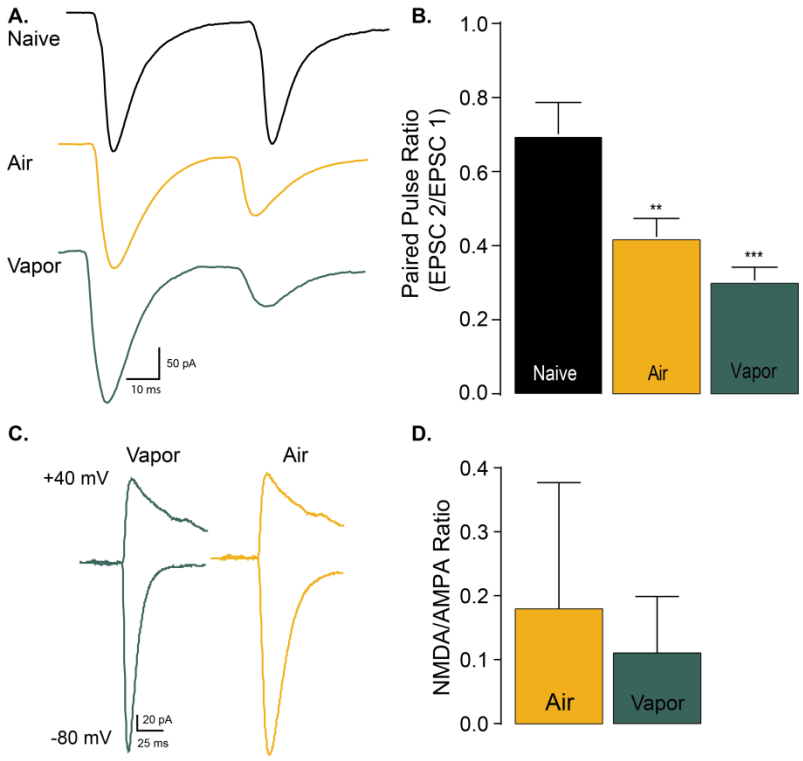
the last session with the reduction in the magnitude of LTD observed in the air exposed animals. (Air n=8 cells from 6 mice; Vapor n=9 cells from 7 mice)

Figure 4. Ethanol experience enhances glutamatergic signaling in vHipp-Shell circuit.



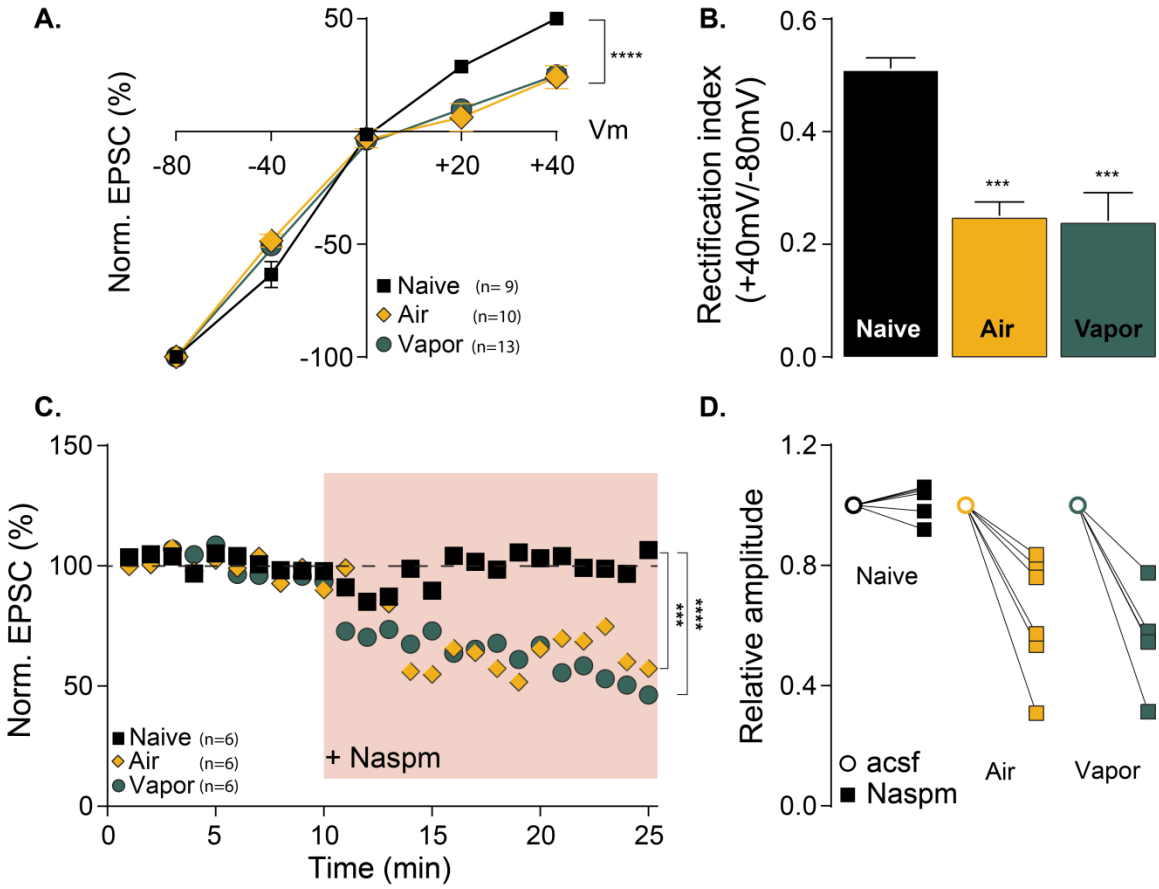
(A) Representative traces of asEPSCs; light stimulation in the presence of strontium elicit quantal like asynchronous events for a brief duration only from glutamatergic input from the vHipp. Red regions represent the time window where asEPSCs are observed (30-400 ms after light pulse). (B) Ethanol experience increased asEPSC amplitude with the largest increase observed in vapor exposed animals **P < 0.01 vs naïve, ***P <0.001 vs naïve, ^P < 0.05 vs air. An increase in asEPSC amplitude was only observed in vapor exposed animals *P < 0.05 vs naïve. (C) Representative traces of spontaneous EPSCs. (D) Ethanol vapor exposure enhanced sEPSC amplitude and frequency only in vapor exposed animals **P < 0.01 vs naïve, ***P < 0.001 vs naïve. (Naïve n=10 cells from 8 mice; Air n=12 cells from 9 mice; Vapor n=13 cells from 10 mice)

Figure 5. Ethanol experience enhances presynaptic glutamatergic signaling but not NMDAR function.



(A) representative traces of paired pule (50 ms ISI) recordings. (B) Bar graphs representing paired pulse ratio (second EPSC amplitude / first EPSC amplitude). Ethanol experience reduced the paired pulse ratio with the maximum reduction in PPR observed in the vapor exposed group **P < 0.01 vs naïve, ***P < 0.001 vs naïve. (C) Representative traces of NMDAR/AMAPR ratio recordings. AMPAR component elicited at -80 mV holding potential. NMDAR component elicited at +40 holding potential and 50 ms after stimulus offset to allow for decay of any AMPAR mediated current. (D) Bar graphs representing the NMDAR/AMPAR ratio (NMDAR mediated EPSC amplitude / AMPAR mediated EPSC amplitude). No difference was observed in this ratio between the two groups. (Naïve n=10 cells from 6 mice; Air n=12 cells from 8 mice; Vapor n=14 cells from 9 mice)

Figure 6. Ethanol experience results in the insertion of CPARs into vHipp-Shell synapses



(A) Current voltage (IV) relationship of AMPAR mediated EPSCs (in the presence of D-APV) in vHipp-Shell D1R-MSNs. Twenty four hours after drinking a significant inward rectification is observed in ethanol experienced animals but not naïve animals. (B) Rectification index (EPSC amplitude at +40 mV / EPSC amplitude at -80 mV) is decreased in both vapor and air exposed groups 24 hours after drinking ***P < 0.001 vs Naïve. (C) Bath application of the CPAR selective antagonist NASPM produced a reduction in EPSC amplitudes in both air and vapor exposed animals ***P < 0.001 vs naïve, ****P < 0.0001 vs naïve. (Naïve n=9 cells from 5 mice; Air n=10 cells from 6 mice; Vapor n=13 cells from 7 animals) (D) Averaged relative amplitudes of EPSCs before and after washing on NASPM. (n=6 cells from 6 animals for all groups)

DISCUSSION

The major finding in the present study is that by assessing the individual ventral hippocampal-accumbal input we are capable of unmasking ethanol induced adaptations that are distinct from those observed when assessing “monosynaptic” adaptations. Following acute *in vivo* exposure to ethanol the vHipp-shell circuit also appears to have enhanced sensitivity of NMDAR-LTD to ethanol exposure. Free consumption of ethanol and binge-like ethanol exposure results in alterations in vHipp-Shell plasticity and glutamatergic neurotransmission. These changes are likely associated with the formation of ethanol dependence. A particularly interesting observation was the decrease in the magnitude of synaptic depression in D1R-MSNs receiving input solely from vHipp that was negatively correlated with the volume of ethanol consumed during the last drinking session. A similar correlation between ethanol consumption and NMDAR-LTD magnitude was observed in animals that consumed higher volumes of ethanol in a 2BC drinking paradigm very similar to the one we employed (Mangieri et al., 2017). A correlation between the volume of ethanol consumed and the magnitude of LTD was not observed in the vapor treated (binge-like ethanol) group, this finding is most likely due to a floor effect. CIE-induced enhancement in ethanol consumption abolishes the ability to elicit LTD in D1R-MSNs perhaps due to alteration in the MSN shifting the threshold for the expression of plasticity.

Furthermore, this change in vHipp-Shell plasticity following ethanol consumption was associated with alterations in presynaptic glutamate signaling from the vHipp as indicated by changes in asEPSC frequency and the PPR, suggesting enhancements in release from the vHipp following drinking and in particular binge-like (vapor) exposure. Another interesting aspect of altered glutamatergic neurotransmission in this circuit

following consumption was adaptations in postsynaptic signaling as indicated by changes in asEPSC amplitude as well alterations in AMPAR subunit expression. Ethanol consumption enhanced vHipp-Shell amplitude with a more robust enhancement following binge-like exposure. We also observed the insertion of GluA2 lacking CPARs into vHipp-Shell synapses following ethanol consumption as indicated by a decrease in RI and reduction in EPSC amplitude following application of the selective antagonist NASPM.

Accumbal Plasticity as an Engram of Dependence

Alterations in accumbal plasticity are a critical neuroadaptation observed following exposure to many different drugs of abuse; and likely are critically important neuroadaptive processes underlying drug-induced behavior. These adaptations are likely critical during the early development of drug consumption and seeking. Indeed this form of plasticity appears to be altered in animals that exhibit drug induced behaviors such as sensitization to the motor enhancing effects of psychostimulants (Alcantara et al., 2011; Mao et al., 2009; Pascoli et al., 2011) or alcohol (Abrahao et al., 2014; Abrahao et al., 2013) One of the most highly reproduced forms of plasticity in the accumbens is NMDAR-LTD, which is subregion and neuronal subunit specific and is disrupted by ethanol (Jeanes et al., 2014; Renteria et al., 2017).

In the present study, we focused on recording from D1-MSNs in the shell of the NAc. Our aim was to determine input specific neural adaptations of shell D1-MSNs produced by *in vitro* and *in vivo* ethanol exposure. A recent report indicates that the vHipp input to the shell of the NAc is the most robust glutamatergic input (J. P. Britt et al., 2012). We chose to examine this dense input from the vHipp to shell to first verify that we were capable of producing NMDAR-dependent LTD of optically evoked EPSCs

in our hands, and to determine if plasticity of this isolated input onto D1-MSNs of the shell is altered by ethanol exposure.

Here we replicated our prior findings that binge-like ethanol exposure model prevents the expression of NMDAR-LTD in the shell D1R-MSNs. Our novel findings indicate that the input from the ventral hippocampus undergoes adaptations of this critical form of plasticity at much lower concentrations than observed when assessing plasticity at neurons receiving input from all available glutamatergic projections. Of particular interest is the finding that the amount of ethanol consumed correlates with the magnitude of LTD observed. This finding suggests that as animals increase their consumption there is a corresponding adaptation in plasticity within D1R-MSNs of the shell that shift these neurons away from the ability to express synaptic depression and towards a more excitable state. Prior findings indicate that a metaplastic shift occurs in D1R-MSNs 24 hours after being exposed to high concentrations of ethanol using the CIE Vapor model, a shift from synaptic depression to synaptic potentiation (Renteria et al., 2017). This same ethanol exposure that enhances the excitability and potentiation of accumbal synapses also results in a robust increase in the volitional consumption of ethanol. This is further evidence that alterations of accumbal plasticity leads towards heightened drug consumption and are likely critical for the formation of drug dependence.

Increases in ventral hippocampal-Accumbal Glutamatergic Signaling and alterations in AMPAR expression

Ethanol consumption and binge-like exposure produces enhancements in glutamatergic signaling within the vHipp-Shell circuit. We observed increases in both postsynaptic and presynaptic electrophysiological measurements of AMPAR signaling. We also observed the presence of CPARs in the synapse follow ethanol consumption. In

many brain regions AMPAR activity is largely or solely mediated through GluA2 subunit containing AMPARs; with some cells having basal CPAR activity (Conrad et al., 2008b; McGee et al., 2015; Reimers et al., 2011). CPARs are commonly enriched in GluA1 subunits and lack GluA2 subunits. This subunit composition of CPARs confers faster onset and offset kinetics as well as substantially increasing the single-channel conductance. The Calcium-permeability of these channels also likely has critical functional consequences on the synapse following their expression (Henley & Wilkinson, 2013; Liu & Cull-Candy, 2000; Wu et al., 2017). A number of types of drug exposure has been found to increase CPARs within the NAc, and predominantly within D1R-MSNs. CPAR insertion in D1R-MSNs has been reported following exposure to cocaine (Jean Terrier et al., 2016), opiates (Hearing et al., 2016b; Russell et al., 2016), and following alcohol drinking (Beckley et al., 2016) or vapor exposure (Renteria et al., 2017). Enhancements in CPAR with the NAc shell following alcohol (Beckley et al., 2016) and cocaine (James et al., 2014) exposure appears to be dependent on mTORC1 signaling. Disrupting mTORC1 signaling in the NAc suppresses ethanol intake (Neasta et al., 2010) as well as cocaine seeking (James et al., 2014). These findings suggest that insertion of CPARs in D1R-MSNs of the shell is critical for drug related behaviors. The drug-induced expression of CPARs may represent a common mechanism by which repeated drug exposure and withdrawal can regulate excitability and subsequent behavior.

Our findings would suggest that by isolating ventral hippocampal glutamatergic synapses onto NAc shell D1R-MSNs we can unmask adaptations in glutamatergic signaling that are not observed when all potential glutamatergic inputs are present during analysis. It is possible that vHipp-shell circuit has enhanced “sensitivity” to ethanol and is adapting at an earlier stage in the formation of ethanol dependence than previously

observed in non-input specific recordings. This early adaptation of CPAR expression following ethanol consumption may serve to heighten excitability of D1R-MSNs and likely is influencing the change in the expression of plasticity within this circuit. By changing the subunit composition of AMPARs in the D1R-MSN it's possible that the general conductance of these synapses is heightened and subsequent input will produce a more robust effect, driving the neuron away from being able to exhibit long last synaptic depression (LTD) and towards a more potentiated state (loss of LTD < LTP). Our finding that asEPSC amplitude is enhanced in vHipp-Shell by alcohol consumption lends credence to this hypothesis. The possible change in conductance of AMPARs to alter the excitability of D1R-MSNs may also be influenced by an increase in presynaptic glutamatergic release from the vHipp as indicated by the observed increase in asEPSC frequency and paired pulse inhibition both of which suggest an increase in presynaptic release probability. It is also possible that the expression of CPARs could lead to enhanced Ca2+ flow into D1R-MSNs during "up states" which could serve to shift the threshold for NMDAR-dependent plasticity away from LTD towards LTP, with a loss of LTD expression being an intermediate stage between depression and potentiation.

Chapter 3:

Other Works in the Lab: Accumbal Plasticity as an Engram of Alcohol Use Probability

ABSTRACT

The mesocorticolimbic system is commonly referred to as the reward system of the brain and is critical for processing reward, reinforcement, motivation and goal directed behaviors. The system is composed of the nucleus accumbens (NAc) structures the shell and core which receive dopaminergic input from neurons of the ventral tegmental area (VTA); as well as glutamatergic input from the prefrontal cortex (PFC), hippocampus (particularly the ventral regions, vHipp) and the amygdala (predominantly basolateral, BLA). A growing body of evidence suggests that various types of drugs of abuse alter glutamatergic synaptic plasticity within the NAc and in particular the Shell subregion. Alterations in plasticity within dopamine D1 receptor expressing medium spiny neurons (D1R-MSNs) produced by various drugs of abuse suggests that there is significant predictive validity in using accumbal plasticity to screen genetic models, differing animal lines, or medications in order to better understand and treat alcohol abuse and dependence. In the present studies we examined viral mediated expression of a peptide that blocks expression of NMDA receptor dependent long term depression (NMDAR-LTD) and its effect on ethanol consumption to determine if preventing accumbal plasticity prevents the formation of ethanol dependence.

We also studied high drinking in the dark (HDID) mice that were selectively bred for high blood ethanol concentrations (BECs) following drinking in the dark procedures.

INTRODUCTION

The mesocorticolimbic system is commonly referred to as the reward system of the brain and is critical for processing reward, reinforcement, motivation and goal directed behaviors (Wise, 2004). The system is composed of the nucleus accumbens (NAc) structures the shell and core which receive dopaminergic input from neurons of the ventral tegmental area (VTA); as well as glutamatergic input from the prefrontal cortex (PFC), hippocampus (particularly the ventral regions, vHipp) and the amygdala (predominantly basolateral, BLA) (Sesack & Grace, 2010). In this configuration, dopamine in the NAc acts to alert an animal to the significance of certain stimuli, so that appropriate association can be made between its surroundings and its behavior.

All drugs of abuse elicit, to differing extents, an increase in extracellular dopamine within the shell of the NAc, leading to an incentive arousal state that facilitates instrumental drug relevant behaviors (Doyon et al., 2003; Imperato & Di Chiara, 1986; Weiss et al., 1993). Repeated non-contingent exposure to drugs of abuse is believed to result in maladaptive recruitment of dopamine transmission from the VTA. Increased dopamine could produce aberrant motivational behaviors typical of adduction such as compulsive responding for drugs and perseverance on drug-related stimuli at the expense of nondrug rewards. Recent hypotheses propose that mesolimbic dopamine release acts as a motivational learning signal (Spanagel & Weiss, 1999); a signal of pathological associative learning (Di Chiara, 2002); a signal that works to inform the predictability of reward-related cues previously associated with drug availability (Fiorillo et al., 2003); or a neural substrate of incentive salience (Berridge & Robinson, 2003).

Medium spiny neurons (MSNs) of the NAc function as integrators that combine the dopamine saliency signal in the context of cognitive, sensory and emotional states

conveyed by the glutamatergic projections. Input from the prefrontal cortex supplies executive control; the amygdala conveys conditioned associations as well as emotional valence and affective drive; and the ventral regions of the hippocampus imparting spatial and contextual information (Ambroggi et al., 2008; Gruber et al., 2009; Ito et al., 2008; Kalivas et al., 2005; Wolf, 2002). The shell subregion of the NAc is particularly important for aspects of drug reward. With the transition to an addicted state following a ventral to dorsal loop with adaptations first occurring in the shell, then core of the NAc, and finally transitioning to the dorsal striatum (Ikemoto, 2007; Rodd-Henricks et al., 2002; Sellings & Clarke, 2003). Neuroadaptations within the accumbens are believed to be critical for the development and expression of addiction to various reinforcers including ethanol (Christian Lüscher & Malenka, 2011b). By gaining a better understanding of the synaptic adaptations that occur within this region following drug exposure we can better investigate and target novel therapeutic targets for intervention.

The most well characterized form of postsynaptic plasticity within the NAc is NMDAR-LTD (Thomas et al., 2001). This form of plasticity is induced by prolonged low-frequency stimulation (LFS, 1-3 Hz) of presynaptic terminals paired with postsynaptic membrane depolarization. This pairing protocol results in weak activation of postsynaptic NMDARs, leading to a moderate influx of calcium through the NMDARs, and a subsequent activation of calcineurin and protein phosphatase 1 (Mulkey et al., 1993). Activation of these adaptor proteins leads to the clathrin mediated internalization of GluA2 subunit containing AMPARs from the postsynaptic membrane which causes the long lasting decrease in synaptic strength (Beattie et al., 2000). The interaction of the adaptor proteins with the C-terminus of the GluA2 subunit is critical for the expression of NMDAR-LTD (Brebner et al., 2005a; Jeanes et al., 2014; Scholz et al., 2010).

Drug-induced disruptions of NMDAR-LTD in the NAc has been observed following exposure to various drugs of abuse and routs of administration including the psychostimulants cocaine (Martin et al., 2006a; Thomas et al., 2001) and amphetamine (Mao et al., 2009) as well as heroin (Shen & Kalivas, 2013) and ethanol (Abrahao et al., 2013; Jeanes et al., 2014; Jeanes et al., 2011a; Renteria et al., 2017; Renteria, Maier, et al., 2017; Spiga et al., 2014). Importantly in animals that self-administer drugs such as cocaine or ethanol, only a subset of animals show persistent drug-seeking characteristics similar to those seen in human who are substance dependent (Abrahao et al., 2013; Deroche-Gamonet, 2004). Dependent animals displayed persistent changes in behavior such as sensitization to the locomotor effects of the drug of abuse. These behavioral impairments were associated with persistent impairments in LTD which suggests that long-lasting impairments of LTD may be important for the transition from drug seeking to drug dependence.

In vitro ethanol can inhibit NMDARs in the NAc (Maldve et al., 2002; Zhang et al., 2005) and blocks expression of NMDAR-LTD (Jeanes et al., 2011). Chronic intermittent *in vivo* ethanol exposure is a widely accepted binge-like model that reliably induces ethanol dependence in mice (Becker & Lopez, 2004). Animals that undergo CIE exposure exhibit a reversal in the expression of plasticity (Jeanes et al., 2011; Renteria, et al., 2017) where an induction protocol that normally produces LTD instead result in long term potentiation (LTP) in C57Bl/6J mice. Interestingly, in Swiss Webster mice exposure to CIE vapor does not elicit LTP. The Swiss Webster mice do not readily consume ethanol as compared to high consuming C57BL/6J mice. This suggests that alterations in accumbal plasticity may relate to ethanol consummatory behavior and the loss of LTD expression, or shift towards the expression of LTP following the same

induction protocol in animals, may serve as a predictor for ethanol consumption and dependence.

In the present studies we examined if a viral mediated disruption of GluA2 subunit internalization modulates CIE-enhanced ethanol intake. Two-bottle choice (2BC) drinking interspersed with bouts of CIE vapor exposure typically enhances ethanol preference and intake by approximately 50% (Renteria et al., 2017). We endeavored to determine if the loss of the ability to produce LTD by blocking GluA2 containing AMPAR internalization prevents this observed escalation in volitional ethanol intake following CIE vapor exposure. If alterations in accumbal plasticity are critical for the formation of ethanol dependence we would suspect that preventing adaptations in accumbal plasticity would disrupt CIE-enhanced ethanol intake.

In this study we also examined the predictive validity of accumbal plasticity by assessing NAc Shell plasticity in a genetic model of binge-like drinking in animals that have been selectively bred for high blood ethanol concentrations (BECs), following drinking in the dark (DID) procedures (rodent model of binge ethanol consumption). This model has been shown to produce intoxicating levels of consumption in mice (Crabbe et al., 2009; Rhodes et al., 2005). Outbred HS/NPt mice underwent DID procedures and those mice with the highest BECs were selectively bred together resulting in high drinking in the dark (HDID) mice. These HDID mice display enhanced ethanol consumption and BECs in the DID paradigm compared to progenitor HS/NPt mice (Crabbe et al., 2009). If accumbal plasticity is a predictor of ethanol consummatory behaviors and the formation of dependence we would expect to see differential alterations in HDID mice as compared to HS/NPt mice following brief ethanol exposure.

LENTIVIRAL MEDIATED DISRUPTION OF GLUA2 SUBUNIT AMPAR INTERNALIZATION MODULATES CIE-ENHANCED ETHANOL INTAKE

Stereotaxic viral injections were conducted by Daniel Kircher and Esther Maier. Electrophysiological recordings were conducted by Daniel Kircher. Behavioral experiments were conducted by Esther Maier. This work contributes to a manuscript entitled: Authors: Esther Maier, Daniel Kircher, and Richard Morrisett.

Methods

Animals

Mice were heterozygous BAC transgenic mice in which the tomato fluorophore expression was driven by dopamine D1 receptor (Drd1a-tdTomato from Jackson Laboratories) gene regulatory elements. All animals used were male. Transgenic mice were backcrossed in the C57BL/6J line. Mice were single housed after surgery. All animals were kept in a temperature and humidity controlled environment with a 12 h light/ 12 h dark cycle (lights on at 0700 h). Animals were given a minimum of 3 days of recovery from surgery prior to light reversal (lights on at 2100 h). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Stereotaxic injections

5- to 6-week-old mice. Anesthesia was induced at 3% and maintained at a range of 1.5-2% isoflurane (w/v) (Animal Health International) as determined by depth of animal breathing. The animal was placed in a stereotaxic frame (Kopf) and bilateral craniotomies were performed using stereotaxic coordinates adapted from a mouse brain atlas and then refined by sham injections (for NAc Shell: anterior-posterior = +1.345; medial-lateral = ± 0.750; dorsal-ventral (from the surface of the skull) = -5.0). Injections of virus (0.20 µl per injection site) were made using graduated pipettes (Drummond Scientific Company), broken back to a tip diameter of 10-15 µm, at an infusion rate of ≈0.05 µl per min. Animals given one week to recover prior to two bottle choice Drinking.

Two Bottle Choice

Two bottle choice (2BC) drinking is a limited access (2 hour access) paradigm used to measure changes in volitional consumption (Griffin, 2014). Thirty minutes prior to the beginning of the dark cycle (light out 0930 hours) mice are given access to two bottles, one containing a 15% ethanol and tap water solution and the other containing tap water alone. This procedure was to accustom the mice to ethanol consumption. Prior to the drinking session, bottles and mice were weighed. After the two hours access bottles were again weighed. Bottle weights prior to and after drinking were compared to give the amount consumed (g alcohol per kg animal body weight). Animals with prior 2BC and operant self-administration experience underwent 12 days of consecutive 2BC to establish baseline consumption.

Chronic Intermittent Ethanol

Mice were exposed to CIE vapor using previously established model that has been shown to lead to increases in volitional ethanol consumption in mice (Becker & Lopez,

2004). Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 3.5 liter/min. Ethanol vapor was then combined with a separate air steam to give a total flow rate of approximately 4 liters/min. Ethanol enriched air was delivered to mice in special mouse chamber units with airtight tops (Allentown Inc., Allentown NJ). Each bout of vapor consisted of 16 hours of ethanol vapor exposure followed by 8 hours of withdrawal, repeated for 4 consecutive days. Mice were injected (i.p.) with a loading dose of ethanol (20% v/v, 1.5 g/kg) and pyrazole (68.1 mg/kg) suspended in 0.1M PBS prior to vapor exposure to achieve a blood ethanol concentration (BEC) of 150-200 mg/dl (37-47 mM). Air control mice were handled the same as ethanol exposed mice but received an injection of only pyrazole suspended in PBS. Following baseline 2BC consumption, animals were exposed to a single bout of vapor followed by a 3 day withdrawal period before 2BC consumption was assessed for 5 days (2BC:1). Animals then received a 7 day abstinence period followed by a second bout of vapor exposure and withdrawal and a second consumption assessment (2BC:2). Following drinking animals underwent a 3rd and final bout of vapor exposure and withdrawal period followed by a final consumption assessment (2BC:3).

Blood ethanol concentrations

Following the completion of each 16 hour vapor exposure, tail blood samples of approximately 5 µl were collected upon removal from the vapor chambers each day prior to withdrawal. BECs were measured using gas chromatography with a Bruker 430-GC (Bruker Corporation, Fremont CA) equipped with a flame ionization detector and CombiPAL autosampler. For each mouse, two samples of tail blood were added to 10 ml vials containing 45 µl supersaturated sodium chloride solution. Samples were warmed to 65°C and the solid-phase micro extraction fiber (SPME; 75 µm CAR/PDMS, fused silica;

Supelco, Bellefonte, PA) was used to absorb ethanol vapor from each sample. The stationary phase was a capillary column (30 m x 0.53 mm x 1 µm film thickness; Agilent Technologies, Santa Clara, CA) and helium, at a flow rate of 8.5 ml/min, was used in the mobile phase. Ethanol peaks determined by the GC were analyzed using CompassCDS Workstation software (Bruker Corporation, Fremont CA). BECs of tail blood were calibrated against ethanol peaks from prepared ethanol standards (100-400 mM). Ethanol vapor flow rates and ethanol and loading injections were adjusted to maintain mice within a BEC range of 150-200 mg/dl (37-47 mM) following each 16 hour chambering period.

Brain slice preparation

Mice were at least 14 weeks of age at the time of slice preparation. Parasagittal slices (230-240 µm thick) containing the NAc were prepared using a Leica vibrating microtome. Mice were anesthetized by inhalation of isoflurane and the brains were rapidly removed and placed in 4°C oxygenated artificial cerebrospinal fluid (ACSF) containing the following in (mM): 210 sucrose, 26.2 $NaHCO_3$, $1NaH_2PO_4$, 2.5 KCl, 11 dextrose, bubbled with 95% O_2/ 5% CO_2. Slices were transferred to a non-sucrose based ACSF solution for incubation containing the following (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 2.4 $MgCl_2$, 1.8 $CaCl_2$, 10 dextrose, were continuously bubbled with 95% O_2/ 5% CO_2; pH 7.4, 32°C, and were maintained in this solution for at least 60 minutes prior to recording.

Patch clamp electrophysiology

Whole cell voltage clamp recordings were conducted in the nucleus accumbens shell. Cells were identified using a BX50 microscope (Olympus) mounted on a vibration

isolation table. Recordings were made in ACSF containing (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 0.9 $MgCl_2$, 2.0 $CaCl_2$, and 10 dextrose, bubbled with 95% O_2/ 5% CO_2. ACSF was continuously perfused at a rate of 2.0 mL/min and maintained at a temperature of 32°C. Picrotoxin (50μM) was included in the recording ACSF to block $GABA_A$ receptor-mediated synaptic currents. 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 3-6 MΩ. For LTD, Paired Pulse, and sEPSC recordings, electrodes were filled with (in mM): 120 CsMeSO4, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4 Mg-ATP, 0.3 Na-GTP, 0.1 spermine, and 5 QX-314-Cl. For all recordings input and access resistances were monitored throughout the experiments. Cells in which input and access resistance varied more than 20% were not included in analysis.

Data acquisition and analysis

Whole-cell voltage- and current- clamp recordings were conducted on NAc shell D1-MSNs that exhibited Lentiviral expression of $GluA2_{3Y}$ or $GluA2_{3A}$ as indicated by eGFP expression. D1-MSNs were identified by the presence of td-Tomato and peptide was identified by the presence of eGFP using fluorescent light. Excitatory postsynaptic currents (EPSCs) were acquired using an amplifier (Dagan) filtered at 1 kHz, and digitized at 10-20 kHz with a Digidata 1440B interface board using pClamp 10.6 (Axon Instruments). EPSCs were evoked with pulses of electrical stimulation using a stainless steel bipolar stimulating electrode (FHC Inc.) placed about 15-300μm from the cell body. In experiments for long term plasticity, EPSCs were evoked for at least 10 minutes every 15 seconds, to ensure a stable baseline. To induce LTD, a conditioning stimulus of 500 light pulses at 1 Hz was paired with continuous postsynaptic depolarization to -50 mV.

EPSCs were then monitored for 30 minutes after pairing. The magnitude of LTD was calculated by averaging normalized (to baseline) EPSC values from 20-30 minutes post-pairing and comparing that value to the average normalized EPSCs during the 10 minute baseline. If the average EPSCs between 20 to 30 minutes post-pairing was greater than 2 standard deviations away from the 10 minute baseline then, plasticity was observed. Data from each neuron within a treatment group was combined and represented as percent baseline values. Summary data are presented as mean ± SEM. Data was analyzed using a one-way ANOVA and Bonferroni post hoc test or Student's t test in which statistical significance from baseline for within each group was defined as $p < 0.05$.

Results

Lentiviral expression of a peptide mimic of the C-terminus tail of GluA2 AMPAR subunits prevented expression of NMDAR-LTD

Animals were injected with a lentiviral vector that was designed to express a peptide that mimics the intracellular c-terminus tail of GluA2 subunits. This synthetic peptide "GluA2-3Y" was derived from the rat GluA2 carboxyl tail (^{869}YKEGYNVYG^{877}) and has been shown to prevent the clathrin mediated endocytosis of GluA2 AMPARs when included in patch pipettes (Brebner et al., 2005b; Jeanes et al., 2011). Virus, labeled with GFP, was injected in the shell of the NAc and D1R-MSNs infected with the peptide virus (as indicated by GFP expression) were isolated for whole cell patch clamp recordings. Control animals with no virus expression exhibited a reduction in EPSC amplitude of around 50% as compared to baseline following our pairing protocol which mirrors previous findings from our lab.

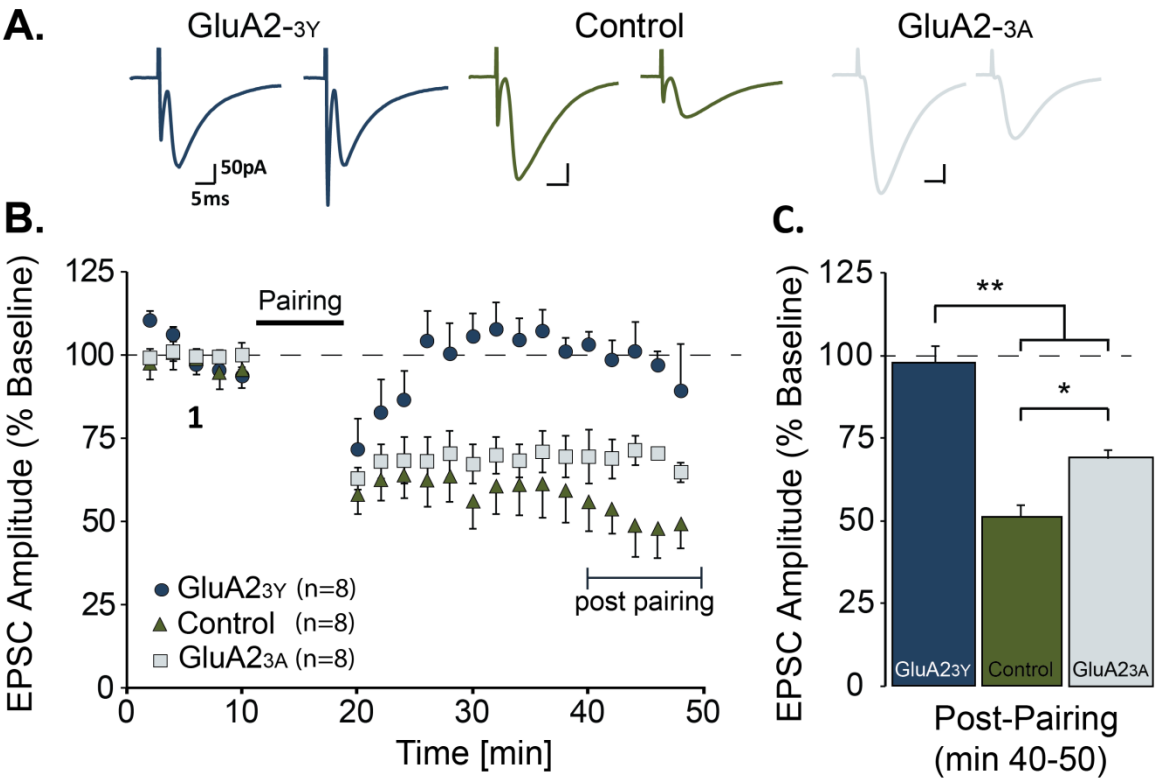
Viral mediated expression of the GluA2-3Y peptide that mimics the C-terminus tail of GluA2 subunit containing AMPARs blocked the expression of NMDAR-LTD with

no change in EPSC amplitude following our pairing protocol (**P < 0.01 GluA2-3Y vs Control, Fig 7.). We also observed a significant reduction in the magnitude of LTD in animals that were injected with a viral vector expressing the GluA2-3A (scrambled control) peptide (*P < 0.05 GluA2-3A vs Control). There was a significant difference in EPSC amplitude following pairing between the active peptide (GluA2-3Y) vs the scrambled peptide (GluA2-3A) (**P < 0.01 GluA2-3Y vs GluA2-3A, Fig 7).

Viral mediated GluA2-3Y peptide expression disrupts CIE induced escalations in ethanol consumption after a period of abstinence.

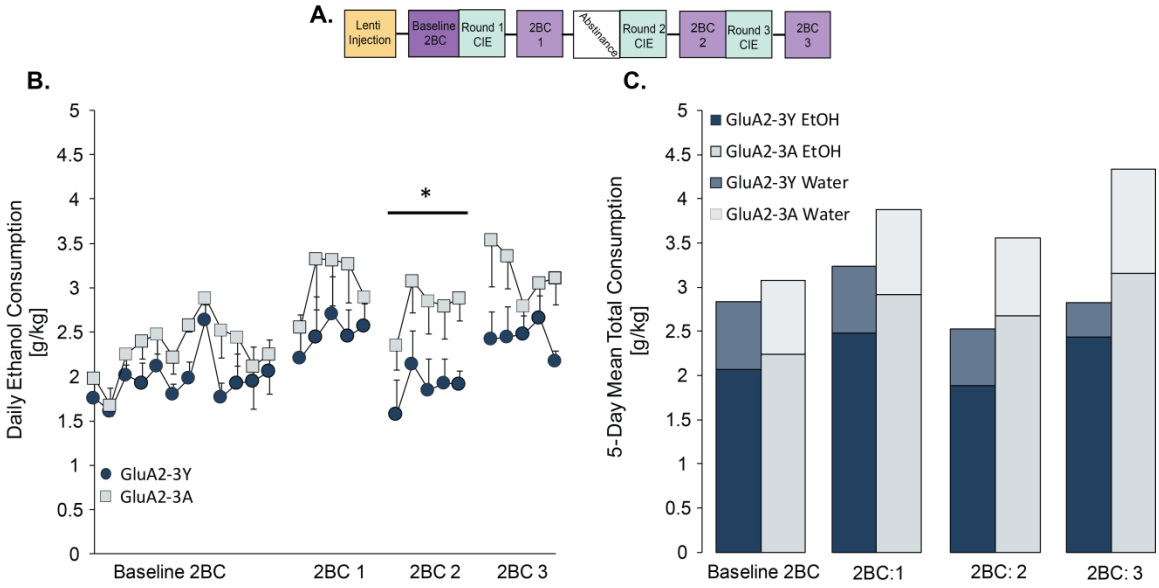
Animals injected with either the virus that expressed the active peptide (GluA2-3Y) or the scrambled peptide (GluA2-3A) exhibited similar levels of consumption in the 2BC paradigm during baseline intake (Figure 9). Animals did not exhibit a significant difference in 2BC consumption following the first bout of CIE vapor. However, following a 7 day abstinence period prior to a second bout of vapor exposure, there was a significant reduction in ethanol consumption in animals injected with the active peptide as compared to the inactive peptide (*P <0.05, GluA2-3y vs GluA2-3A). This difference in ethanol intake observed between the two groups was lost after the 3rd bout of vapor exposure.

Figure 7. Viral mediated expression of the GluA2-3Y peptide blocks NMDAR-LTD expression in D1R-MSNs of the NAc shell.



(A) Representative traces of EPSC amplitudes before and after a pairing protocol. (B). the pairing protocol resulted in LTD in control and GluA2-3A peptide groups but not in GluA2-3Y peptide group. GluA2-3A peptide animals exhibit a reduction in LTD magnitude. (C) Bar graph represents percentage change of baseline ± S.E.M. for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each treatment group. *P < 0.05 vs control, **P < 0.01 vs control and GluA2-3A. (Control n=8 cells from 6 mice; $GluA2_{3Y}$ n=8 cells from 7 animals; $GluA2_{3A}$ n=8 cells from 6 animals)

Figure 8. GluA2 c-terminus peptide mimic disrupts CIE induced enhancement in ethanol consumption after an abstinence period



(A) The CIE exposure consisted of 3 bouts of ethanol vapor (or air control) exposure separated by 3 day withdrawal and 5 day 2BC drinking (15% ethanol vs water) periods. A 7 day abstinence period occurred between the second drinking session (2BC:1) and the second bout of CIE vapor exposure. (B) Daily ethanol consumption in animals with prior drinking experience. Ethanol consumption was enhanced following the second bout of CIE vapor in the GluA2-3A but not the GluA2-3Y peptide treatment group. (C) Bar graph indicates average amount of ethanol and water consumed in each 5 day 2BC drinking period.

Discussion

Including a peptide, that mimics the intracellular tail of GluA2 AMPAR subunits, in the intracellular solution of a recording pipette during whole cell patch clamp electrophysiology, results in the loss of expression of NMDAR-LTD (Jeanes et al., 2011). See illustration 1 for a diagram of peptide action on synaptic depression in the NAc. This finding supports the hypothesis that internalization of GluA2 containing AMPARs is critical for the expression of reduced EPSC amplitude exhibited as a result of NMDAR-dependent LTD. In the current study we used a lentiviral vector to expresses the same peptide. Peptide expression in D1R-MSNs resulted in a loss of NMDAR-LTD suggesting that viral mediated delivery of the peptide worked to prevent LTD expression by preventing the internalization of GluA2 containing AMPARs. We also observed a change in the magnitude of LTD in D1R-MSNs that expressed the scrambled GluA2-3A peptide. This change in the magnitude could indicate that saturation of the postsynaptic density with the scrambled peptide may have, to some degree, interfered with the cellular machinery responsible for the internalization of GluA2 containing AMPARs which was not mediated by binding to the adaptor proteins.

This change in synaptic plasticity produced by the overexpression of the GluA2-3Y peptide did not alter ethanol drinking in animal models of moderate ethanol consumption such as operant self-administration (Esther Maier, unpublished findings) or during 2BC drinking (Figure 8). However, we did observe that CIE vapor exposure, a binge-like ethanol exposure model that leads to prolonged high BECs resulting in increases ethanol consumption (Figure 3), did not result in enhanced drinking after two separate vapor exposures. This finding supports the notion that AMPAR trafficking plays a critical role in alcohol reinforcement during intense levels of ethanol exposure. We also

observed that the animals that received injections of the active peptide did not show large enhancement in volitional consumption after the repeated bouts of ethanol vapor similar to what has been presented above or in prior work from the lab (Renteria et al., 2017).

It is important to note that while the injection of the lentiviral vectors did result in peptide expression in D1R-MSNs, this expression only occurred in a subset of these neurons within the NAc shell. It is possible, that by preventing the expression of LTD in only a subset of shell D1R-MSNs, we are not sufficiently disrupting ethanol induced adaptations within the entire shell, which may allow for a sufficient group of D1R-MSNs to adapt in response to ethanol exposure resulting in little change in ethanol consumption in low to moderate drinking paradigms. Ideally, future techniques could allow for expression of such a peptide in all D1R-MSNs which could have a more profound impact on ethanol consumption.

Our current findings do still lend some credence to the hypothesis that there is significant predictive value in accumbal plasticity as an engram of abuse potential. Using accumbal plasticity studies to screen and validate potential medications in order to treat alcohol abuse and dependence.

EXAMINATION OF ACCUMBAL PLASTICITY IN A GENETIC MOUSE MODEL OF BINGE-LIKE DRINKING

This work supported a manuscript entitled: Alcohol-Related Gene Networks in Mice and Humans: Neuronal Plasticity in Extended Amygdala

Authors: Laura B. Furguson, Lingling Zhang, Daniel M. Kircher, Shi Wang, R. Dayne Mayfield, John C. Crabbe, Richard Morrisett, R. Adron Harris, Igor Ponomarev. All electrophysiological experiments were conducted by Daniel Kircher.

Methods

Animals

Mice were HDID and HS/Npt mice, around 56 days old. Mice were single housed after surgery. All animals were kept in a temperature and humidity controlled environment with a 12 h light/ 12 h dark cycle (lights on at 0700 h). Animals were given a minimum of 3 days of recovery from surgery prior to light reversal (lights on at 2100 h). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Stereotaxic Surgery for labeling D1R-MSNs

HDID and HS/Npt mice, around 56 days old, underwent bilateral injection of the fluorescent tracer cholera toxin subunit B (CTB: Alexa Fluor 555) into the VTA (from bregma, medial: lateral = ± 0.38; anterior: posterior = -3.10). CTB is a retrograde tracer that infects neuron terminals. Resulting in labeling of MSNs that project to the VTA which are believed to be D1R-MSNs. Anesthesia was induced at 3%and maintained at 1.5% isoflurane (w/v) (Baxter AG) during the surgery. The animal was placed in a stereotaxic frame (Kopf) and craniotomies were performed using stereotaxic coordinates adapted from a mouse brain atlas (Paxinos & Watson). Animals were given 14 days of recovery prior to ethanol vapor or air exposure.

Chronic Intermittent Ethanol

Mice were exposed to CIE vapor or air (control mice) using previously established model that has been shown to lead to increases in volitional ethanol consumption in mice (Becker & Lopez, 2004). Ethanol was volatilized by bubbling air through a flask containing 95% ethanol at a rate of 3.5 liter/min. Ethanol vapor was then

combined with a separate air steam to give a total flow rate of approximately 4 liters/min. Ethanol enriched air (or air alone for the control group) was delivered to mice in special mouse chamber units with airtight tops (Allentown Inc., Allentown NJ). Ethanol vapor exposure consisted of 16 hours exposure followed by an 8 hours of withdrawal, repeated for 4 consecutive days. Prior to chambering, mice received intraperitoneal injections of a loading dose of ethanol (20% v/v, 1.5 g/kg) and pyrazole (68.1 mg/kg for HDID and 34 mg/kg for HS/Npt) suspended in PBS, in order to achieve a blood ethanol concentration (BEC) of 150-200 mg/dl. Ethanol naïve mice were handled the same but were injected with a solution of only pyrazole in PBS and were exposed to air.

Blood ethanol concentrations

Following the completion of each 16 hour vapor exposure, tail blood samples of approximately 5 µl were collected upon removal from the vapor chambers each day, prior to withdrawal. BECs were measured using gas chromatography with a Bruker 430-GC (Bruker Corporation, Fremont CA) equipped with a flame ionization detector and CombiPAL autosampler. For each mouse, two samples of tail blood were added to 10 ml vials containing 45 µl supersaturated sodium chloride solution. Samples were warmed to 65°C and the solid-phase micro extraction fiber (SPME; 75 µm CAR/PDMS, fused silica; Supelco, Bellefonte, PA) was used to absorb ethanol vapor from each sample. The stationary phase was a capillary column (30 m x 0.53 mm x 1 µm film thickness; Agilent Technologies, Santa Clara, CA) and helium, at a flow rate of 8.5 ml/min was used in the mobile phase. Ethanol peaks determined by GC were analyzed using CompassCDS Workstation software (Bruker Corporation, Fremont CA). BECs of tail blood were calibrated against ethanol peaks from prepared ethanol standards (100-400 mM). Ethanol vapor flow rates and ethanol and loading injections were adjusted to maintain mice

within a BEC range of 150-200 mg/dl (37-47 mM) following each 16 hour chambering period.

Brain slice preparation

Mice were at least 75 days old at the time of slice preparation. Parasagittal slices (230-240 µm thick) containing the NAc were prepared using a Leica vibrating microtome. Mice were anesthetized by inhalation of isoflurane and the brains were rapidly removed and placed in 4°C oxygenated artificial cerebrospinal fluid (ACSF) containing the following in (mM): 210 sucrose, 26.2 $NaHCO_3$, 1NaH_2PO_4, 2.5 KCl, 11 dextrose, bubbled with 95% O_2/ 5% CO_2. Slices were transferred to a non-sucrose based ACSF solution for incubation containing the following (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 2.4 $MgCl_2$, 1.8 $CaCl_2$, 10 dextrose, were continuously bubbled with 95% O_2/ 5% CO_2; pH 7.4, 32°C, and were maintained in this solution for at least 60 minutes prior to recording.

Patch clamp electrophysiology

Whole cell voltage clamp recordings were conducted in the nucleus accumbens shell. Cells were identified using a BX50 microscope (Olympus) mounted on a vibration isolation table. Recordings were made in ACSF containing (in mM): 120 NaCl, 25 $NaHCO_3$, 1.23 NaH_2PO_4, 3.3 KCl, 0.9 $MgCl_2$, 2.0 $CaCl_2$, and 10 dextrose, bubbled with 95% O_2/ 5% CO_2. ACSF was continuously perfused at a rate of 2.0 mL/min and maintained at a temperature of 32°C. Picrotoxin (50µM) was included in the recording ACSF to block $GABA_A$ receptor-mediated synaptic currents. 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 3-6 MΩ. For

current injection experiments, electrodes were filled with (in mM): 135 $KMeSO_4$, 12 NaCl, 0.5 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Tris-GTP, 260-270 mOsm (pH 7.3). For Plasticity, Paired Pulse Ratio, and sEPSC recordings, electrodes were filled with (in mM): 120 CsMeSO4, 15 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 10 TEA-Cl, 4 Mg-ATP, 0.3 Na-GTP, 0.1 spermine, and 5 QX-314-Cl. For all recordings input and access resistances were monitored throughout the experiments. Cells in which input and access resistance varied more than 20% were not included in analysis.

Data acquisition and analysis

Whole-cell voltage- and current- clamp recordings were conducted on NAc shell D1R-MSNs. D1R-MSNs were identified by the presence of CTB (Alexa Fluor 555) following tracer injection into the VTA that retrogradely labeled VTA-projecting D1R-MSNs. Excitatory postsynaptic currents (EPSCs) were acquired using an amplifier (Dagan) filtered at 1 kHz, and digitized at 10-20 kHz with a Digidata 1440B interface board using pClamp 10.6 (Axon Instruments). EPSCs were evoked with pulses of electrical stimulation using a stainless steel bipolar stimulating electrode (FHC Inc.) placed about 15-300μm from the cell body. In experiments for long term plasticity, EPSCs were evoked for at least 10 minutes every 15 seconds, to ensure a stable baseline. To induce LTD, a conditioning stimulus of 500 pulses at 1 Hz was paired with continuous postsynaptic depolarization to -50 mV. Light-EPSCs were then monitored for 30 minutes after pairing. The magnitude of LTD was calculated by averaging normalized (to baseline) EPSC values from 20-30 minutes post pairing and comparing that value to the average normalized EPSCs during the 10 minute baseline. If the average EPSCs between 20 to 30 minutes post-pairing was greater than 2 standard deviations away from the 10 minute baseline then plasticity was observed. Data from each neuron within a

treatment group was combined and represented as percent baseline values. Summary data is presented as mean ± SEM. Data was analyzed using a one-way ANOVA and Bonferroni post hoc test or Student's t test in which statistical significance from baseline for within each group was defined as $p < 0.05$.

Results

Ethanol naïve HDID and HS/NPt mice do not differ in the passive properties of NAc Shell D1R-MSNs or basal glutamatergic signaling in the accumbens

A subset of HDID and HS/NPt animals underwent air control exposure to our CIE procedures. These animals were handled similarly to our CIE vapor exposed animals, including injections with pyrazole, except they were ethanol naïve. Electrophysiological experiments occurred 24 hours after the last sham CIE treatment. Whole cell current clamp recordings measuring the voltage response to increasing current steps (-400 - +100 pA) revealed no strain difference in excitability as measured by the number of action potential spikes observed at the different current steps ($F_{(5, 186)} = 43.53$, P = 0.9962, Fig 7). No strain difference was observed in spontaneous (sEPSC) amplitude ($F_{(3, 80)} = 0.7308$, P = 0.5367, Fig 8) or frequency ($F_{(3, 66)} = 4.612$, P = **0.0054, P = 0.978 HDID Air vs HS/NPt Air, Fig 8) between HDID and HS/NPt mice. Paired pulse ratio recordings 50ms ISI, EPSC2/EPSC1) revealed no strain difference in presynaptic glutamate signaling ($F_{(3, 25)} = 2.258$, P = 0.1063, Fig 9). These findings suggest that there is no basal difference in MSN excitability or pre or postsynaptic glutamatergic signaling between the HDID and HS/NPt strains.

CIE vapor exposure enhances excitability of NAc Shell D1R-MSNs in both HDID and HS/NPt mice

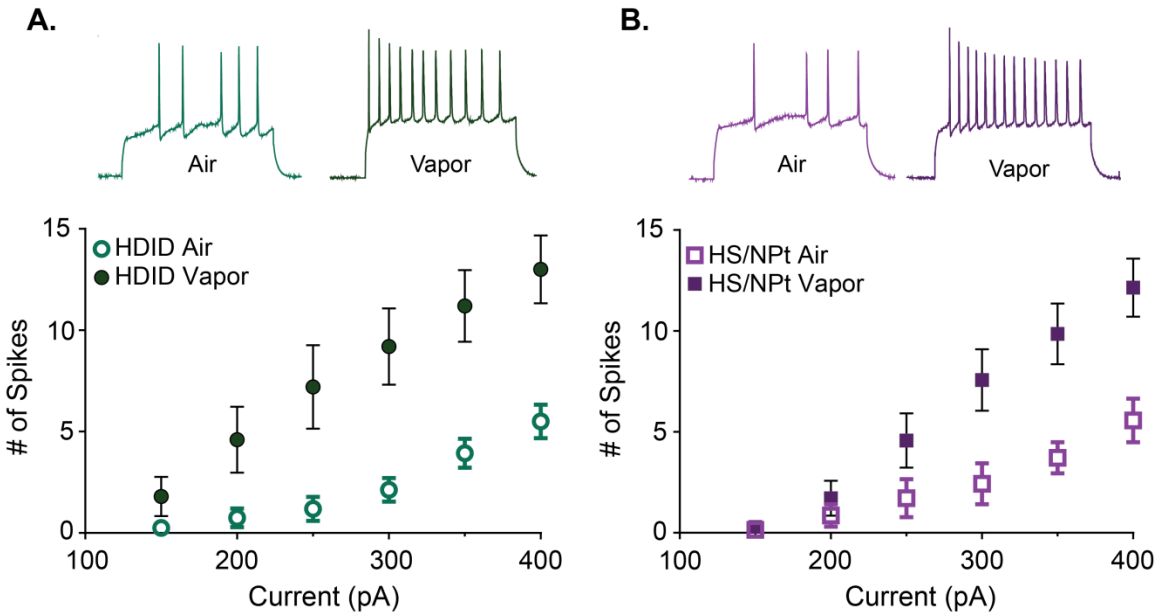
A subset of HDID and HS/NPt animals underwent at 4 day bout of CIE vapor exposure, which resulted in a daily BEC of 150-200 mg/dl, and electrophysiological recordings were conducted 24 hours after the last vapor exposure. Prior work in the lab indicates that a single 4 day bout of vapor exposure results in an enhancement in the excitability of D1R-MSNs but not in D1R-lacking MSNs (Renteria et al., 2017).Whole cell current clamp recordings measuring the voltage response to increasing current steps (-400 - +100 pA) revealed an increase in excitability following CIE vapor exposure as compared to ethanol naïve animals but no strain difference in excitability as measured by the number of action potential spikes observed at the different current steps ($F_{(15, 186)}$ = 2.353, **P = 0.0041, ****P < 0.0001 HDID Vapor vs HDID Air, ****P < 0.0001 HS/NPt Vapor vs HS/NPt Air, Fig 7). This finding recapitulates our labs prior findings using C57BL/6J mice and supports the idea that withdrawal from CIE vapor exposure increases the excitability of D1R-MSNs.

CIE vapor exposure alters accumbal glutamatergic signaling and differentially alters synaptic plasticity in HDID and HS/NPt mice

HDID and HS/NPt animals that underwent CIE vapor exposure exhibited no change in sEPSC amplitude but did exhibit an increase in sEPSC frequency 24 hours after the last vapor exposure. this finding is similar to previous work in the lab where D1R-MSNs show enhanced sEPSC Frequency, likely due to enhanced presynaptic glutamate signaling (Renteria et al., 2017). No change in the paired pulse ratio was observed in either strain following CIE vapor exposure. CIE vapor exposure differentially altered synaptic plasticity of NAc Shell D1R-MSNs 24 hours after the last exposure. HDID mice exhibited a loss of NMDAR-LTD following withdrawal from vapor exposure

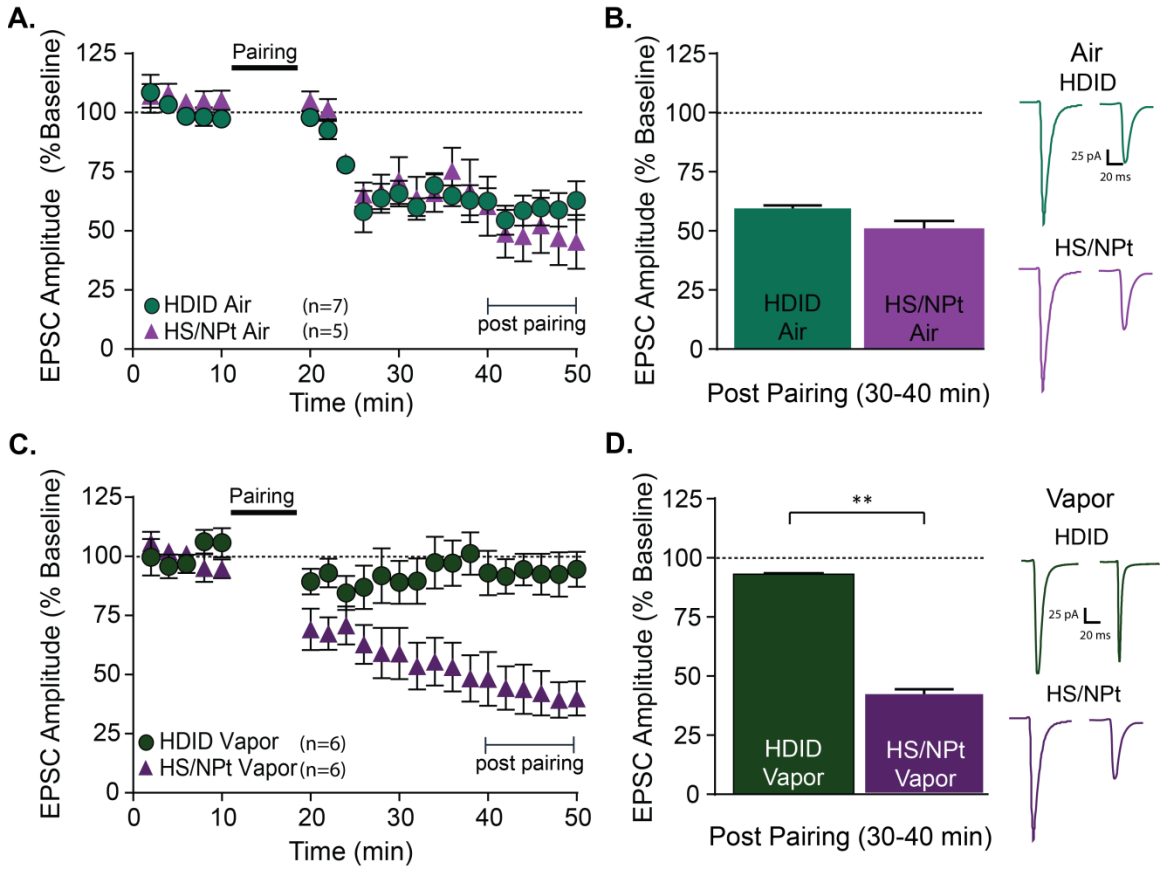
similar to what was previously reported following CIE Vapor exposure in Swiss Webster mice (Jeanes et al., 2014a). Interestingly NMDAR-LTD was still observed in D1R-MSNs isolated from HS/NPt mice 24 hours after the last vapor exposure. There was a significant difference in EPSC amplitude (% baseline) in HDID and HS/NPt mice during post pairing (40-50 min, **P < 0.01, HDID vapor vs HS/NPt vapor, Figure 10). This suggests that Accumbal plasticity in HS/NPt mice is less sensitive to the effects of ethanol exposure used in this study.

Figure 9. CIE vapor exposure enhances excitability of D1R-MSNs from HDID and HS/NPt mice.



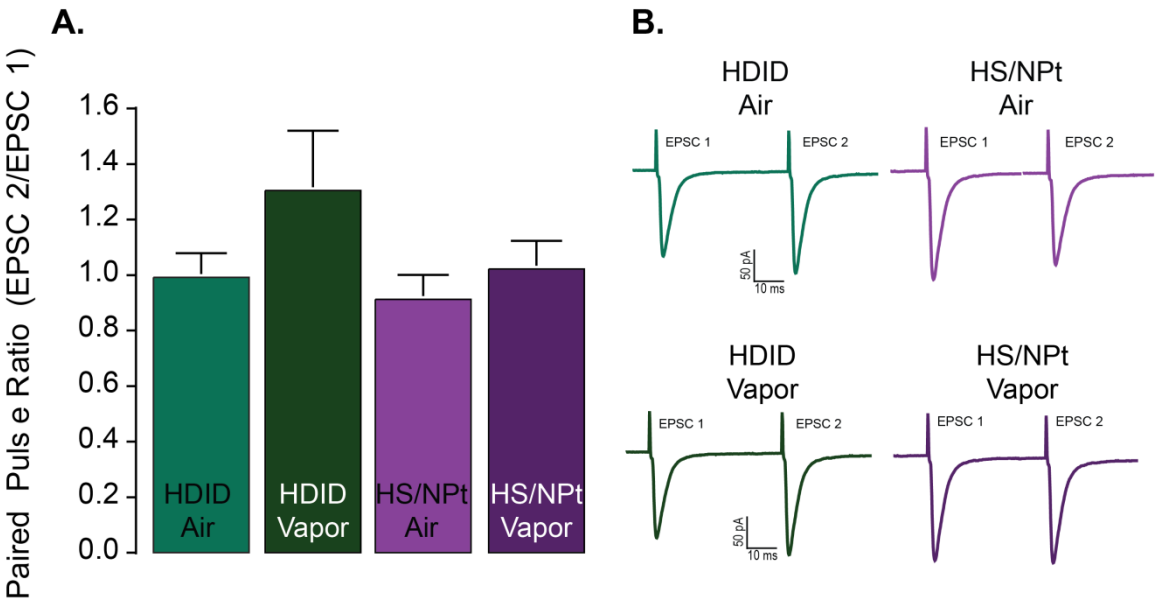
(A) D1R-MSNs from HDID mice exhibit increased firing in response to depolarizing current injections after CIE vapor exposure. Representative traces of action potential spikes and the average number of spikes observed for each current step. (B) D1R-MSNs from HS/NPt mice exhibit increased firing in response to depolarizing current injections after CIE vapor exposure. Representative traces of action potential spikes and the average number of spikes observed for each current step.

Figure 10. CIE vapor exposure results in differential expression in NMDAR-LTD in D1R-MSNs from HDID and HS/NPt mice.



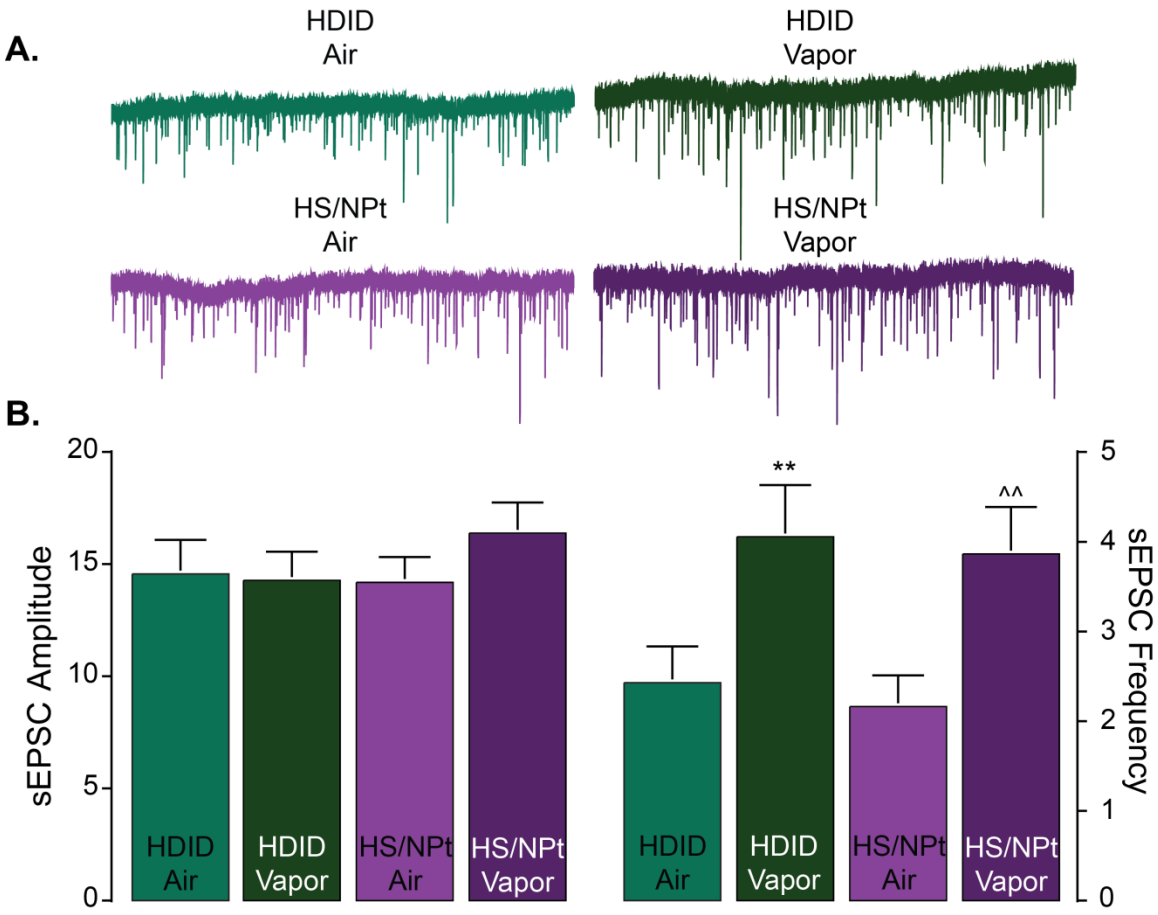
(A) The pairing protocol resulted in LTD in naïve HDID and HS/NPt animals (B) Bar graphs represent percentage change of baseline ± S.E.M. for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each ethanol exposure group. (C) Twenty four hours after CIE vapor exposure D1R-MSNs from HDID mice exhibit a loss of LTD whereas D1R-MSNs from HS/NPt mice still show LTD. (D) Bar graphs represent percentage change of baseline ± S.E.M. for average EPSC amplitude between baseline (min 0-10) and post-pairing (min 40-50) for each ethanol exposure group. **P < 0.01 HDID vs HS/NPt.

Figure 11. CIE vapor exposure does not altered paired pulse ratio of EPSCs in either HDID or HS/NPt animals.



(A) Bar graphs representing paired pulse ratio (second EPSC amplitude / first EPSC amplitude). Ethanol experience did not alter PPR in either HDID or HS/NPt mice. (B) Representative traces of paired pule (50 ms ISI) recordings.

Figure 12. CIE vapor exposure enhances sEPSC frequency in D1R-MSNs from HDID and HS/NPt mice



(A) Representative traces of sEPSCs. (B) Bar graphs represent average (± S.E.M.) amplitude and frequency of sEPSCs in both naïve and CIE vapor exposed HDID and HS/NPt mice. **P < 0.01 vs HDID air, ^^P < 0.01 vs HS/NPt air.

Discussion

In the present study we compared electrophysiological properties of D1R-MSNs between HDID and HS/NPt mouse lines. HDID mice were selectively bread for high BECs following the drinking in the dark (DID) paradigm (animals exhibiting high BECs following DID procedures were bred together with each successive generation showing

higher BECs following DID) with outbred HS/NPt mice as the progenitor line. By the eleventh generation ethanol consumption and BECs are significantly higher in HDID mice compared to HS/NPt mice (Crabbe et al., 2009). They also show increased sensitivity to ethanol and consumed modestly more ethanol in limited access 2BC paradigms compared to their progenitors(Crabbe et al., 2011; Crabbe, Harkness, et al., 2012; Crabbe, Kruse, et al., 2012). This suggests that selective breeding for high BECs selects for alleles that share common genetic control with some ethanol related responses. Our current hypothesis is that there is significant predictive value in accumbal plasticity as an engram of abuse potential. Here we aimed to use accumbal plasticity to screen the HDID and HS/NPt mouse line to determine if selectively breeding for high BECs results in alterations in accumbal plasticity.

Given the difference in ethanol consumption phenotypes between the mouse strains we expected to observe a difference in synaptic plasticity between the HDID and HS/NPt mice. We observed that a single 4 day bout of vapor exposure enhances the intrinsic excitability of D1R-MSNs from HDID and HS/NPt mice, increasing spike firing in response to depolarizing current steps, in both strains similar to what we have previously observed in C57Bl/6J mice (Renteria et al., 2017). We also overserved that vapor exposure increases the frequency of sEPSCs in both HDID and HS/NPt animals. This finding is also consistent with our prior work and suggests an enhancement in the probability of glutamate release presynaptically. This increase in observed sEPSC frequency may be the result of a rebound effect produced by the dampening of excitatory signaling during vapor exposure with the enhancement occurring during withdrawal. Similar increases in glutamate release has been observed following chronic ethanol exposure (Griffin, 2014; Roberto et al., 2004).

The major finding of the current work is that following CIE vapor exposure differential alterations in synaptic plasticity was observed between the two mouse strains. We observed no change in accumbal plasticity in the outbred HS/NPt strain following vapor exposure compared to naïve HS/NPt animals. We did observe a loss of LTD in the NAc of HDID animals that underwent CIE vapor exposure. Prior work indicates that different mouse strains with different ethanol consumption profiles display different adaptations in accumbal plasticity following vapor exposure (Jeanes et al., 2014; Renteria, Buske, et al., 2017; Renteria, Maier, et al., 2017).

High drinking C57Bl/6J mice exhibit LTP following vapor exposure whereas lower drinking Swiss Webster mice exhibit only a loss of LTD after vapor exposure. HDID mice present a change in plasticity following vapor exposure whereas; HS/NPt mice display an electrophysiological phenotype that is unique from mice that drink greater quantities of ethanol. This suggests that selective breeding for high BECs does not alter accumbal CIE-induced metaplasticity. We believe that these findings do support the hypothesis that there is significant predictive value in accumbal plasticity as an engram of abuse potential. The HDID animals that have higher behavioral sensitivity to the effects of ethanol express adaptations in accumbal plasticity after ethanol exposure whereas the HS/NPt mice which are less sensitive to the behavioral effects of ethanol do not.

Chapter 4

Concluding remarks and future direction

Due to the current lack of effective pharmacotherapies for the treatment of addiction, and in particular alcohol use disorders, a greater understanding of drug induced neuroadaptations is needed. Novel targets for intervention that reverse synaptic adaptations produced by ethanol exposure could lead to more effective treatment strategies. To that end there is a fundamental need to understand how the brain‘s reward circuitry is altered after alcohol exposure and withdrawal. The nucleus accumbens (NAc) has a critical role in both reward processing and motivated behaviors, and is a critical hub of the brain’s reward circuitry. It is vital that research identifies drug and ethanol induced neuroadaptations produced within this brain region (Lüscher & Malenka, 2011b). The findings presented here further our understanding of how repeated ethanol experience is encoded within this pivotal brain region.

The driving force of this investigation was to begin teasing apart if ethanol exposure produces input specific neuroadaptations within the nucleus accumbens. The use of state of the art tools such as optogenetics enables the isolation of individual components of the brain’s reward circuitry, in order to examine how these components adapt to repeated drug exposure. Using this approach, a growing body of evidence has emerged, indicating individual glutamatergic inputs to the NAc adapt in distinct ways to repeated drug exposure and withdrawal (Britt et al., 2012; Hearing et al., 2016a; Joffe & Grueter, 2016; Pascoli et al., 2012; Pascoli et al., 2014). In order to isolate a single glutamatergic input to the NAc shell we injected a viral vector to express the light activated channelrhodopsin (ChR2) into ventral hippocampal (vHipp) neurons. This vector resulted in the insertion of ChR2 into the membrane of glutamatergic neurons that

project from the vHipp and terminates in the shell. The use of the CaMKII promotor in our viral vector specifically targets neurons that express CaMKII, which include the primary output neurons of the ventral regions of the hippocampus (ventral CA1 and ventral subiculum). Local blue light illumination of terminals present in the shell of the NAc, activates ChR2 allowing cation flow through ChR2 channels resulting in depolarization of the terminals and subsequent glutamate release selectively from vHipp terminals.

The vHipp has dense projections to the NAc (Groenewegen et al., 1987) and provides strongest projection to the shell subregion of the accumbens (Britt et al., 2012). Hippocampal input to the accumbens plays a role in gating synaptic transmission (O'Donnell & Grace, 1995) and works to shift NAc MSNs to their "upstate". The vHipp also plays an important role in reward processing (Wassum et al., 2009b) and drug seeking behaviors (Fuchs et al., 2005; Riaz et al., 2017a). Inactivation of the vHipp impairs the consolidation of contextual memory (Zhu et al., 2014) and disrupts retrieval of cue-reward memories (Riaz et al., 2017a). Selective inactivation of the ventral subiculum, one of the primary output structures of the vHipp, results in decreased context-induced relapse in ethanol consumption following punishment-imposed abstinence in rats (Marchant et al., 2016). Exposure to other drugs of abuse such as cocaine produce persistent changes in synaptic transmission in the vHipp following extended access to cocaine self-administration (Keralapurath et al., 2017). Finally, cocaine self-administration and withdrawal produces specific adaptations within the vHipp-NAc shell circuit that are specific to D1R-MSNs (Pascoli et al., 2014). Together these findings suggest that the vHipp is a good candidate input to initially target for

isolation in order to examine input specific alterations in glutamatergic signaling and plasticity following ethanol exposure.

First, we investigated whether light evoked EPSCs would exhibit NMDAR-dependent LTD following a pairing protocol similar to one previously employed which involved electrical stimulation of all available inputs onto D1R-MSNs in the NAc shell. We confirmed that low frequency light pulses paired with membrane depolarization reliably produced depression of light evoked EPSCa (LTD). We also confirmed that this form of plasticity is NMDAR-dependent, as indicated by the loss of LTD expression when the NMDAR antagonist D-APV was present. Next, we investigated the neuroadaptive alterations in excitatory synaptic plasticity in response to acute *in vitro* ethanol exposure. Acute ethanol, within the concentration range that we use to induce ethanol dependence *in vivo*, is capable of inhibiting NMDAR-LTD of isolated vHipp-Shell D1-MSN synapses. This form of plasticity has been shown to be disrupted by a number of drugs of abuse (Abrahao et al., 2013; Brebner et al., 2005a; Jeanes et al., 2014; Jeanes et al., 2011; Shen & Kalivas, 2012; Thomas et al., 2001) and the loss of expression of this form of plasticity has been associated with drug dependent behaviors (Abrahao et al., 2013; Brebner et al., 2005a).

The loss of LTD expression in accumbens MSNs appears to be a common feature produced by repeated exposure and withdrawal from many different classes of drugs. However, the mechanism for this loss of LTD expression may differ depending on the class of drug being assessed. For example cocaine exposure causes an initial depression of EPSCs (Huang et al., 2009; Kourrich et al., 2007b; Ortinski et al., 2012; Thomas et al., 2001) followed by a potentiation of excitatory input, and loss of LTD expression following withdrawal that is accompanied by the insertion of GluA2 lacking AMPA

receptors (Britt et al., 2012; Conrad et al., 2008a; Gipson et al., 2014; Kourrich et al., 2007a; Pascoli et al., 2014; Pascoli et al., 2012). Withdrawal from nicotine self-administration produces a similar potentiation in excitatory input (Gipson et al., 2013). Similar enhancements in excitatory signaling onto nucleus accumbens neurons, and loss of LTD expression, has also been observed following exposure to opiates (Russo et al., 2010; Shen & Kalivas, 2012; Wu et al., 2012). In contrast, acute ethanol exposure appears to suppress LTD expression by inhibiting NMDA receptors (Jeanes et al., 2011). Withdrawal from chronic ethanol exposure appears to enhance excitatory signaling onto accumbens MSNs accompanied by a loss of LTD expression, insertion of CPARs, and changes in spine morphology (Abrahao et al., 2013; Jeanes et al., 2014; Renteria et al., 2017; Spiga et al., 2014). Rebound excitation of NMDA receptors has also been observed following withdrawal from some ethanol exposure paradigms (Renteria et al., 2017).

We found that *in vitro* ethanol exposure at both low to moderate (20 mM) and moderate to highly (40 mM) intoxicating concentrations results in a loss of LTD expression in D1R-MSNs. This finding is in contrast to prior work that found that only the moderate to highly intoxicating concentration abolished LTD expression (Jeanes et al., 2011). To us this finding suggests that plasticity within the vHipp-Shell circuit is highly sensitive to ethanol exposure as we observed a loss of expression of LTD at a much lower concentration then previously reported. The expression of this form of LTD requires the activation of NR2B-containing NMDA receptors (Jeanes et al., 2011) and was blocked in the presence of the NMDA receptor antagonist D-APV. There is extensive literature that demonstrates ethanol inhibition of NMDA receptors in a number of brain regions including the NAc (Lovinger et al., 1989, 1990; Maldve et al., 2002; Wang et al., 2007; Zhang et al., 2005), suggesting that the most likely mechanism for the

loss of LTD expression following acute ethanol exposure is via the inhibition of NMDA receptors. Ethanol at a low concentration similar to the one used in our study (20 mM) is capable of reducing glutamatergic EPSPs, most likely due to effects on NMDAR receptors (Nie et al., 1993); however these findings were in young animals and to our knowledge these findings have not be replicated in adult animals. As there is no evidence that indicates ethanol in the 20 mM concentration range alters the function of NMDA receptors in adult animals it is possible, but not likely, that the loss of LTD expression in response to acute ethanol exposure is not the result of NMDA receptor inhibition.

The hypothesis that there is an unmasking of adaptations at the vHipp-shell circuit at lower concentrations or levels of ethanol exposure then has been previously observed when examining all available inputs to the NAc shell, is supported by our findings following *in vivo* ethanol exposure. Previously only animals that increase their ethanol intake following CIE vapor exposure exhibited enhanced glutamatergic signaling and a loss of LTD expression (Renteria et al., 2017). In contrast, the present study shows that animals expressing ChR2 in vHipp-Shell circuit that drank ethanol but did not escalate their intake following air exposure, exhibited a decrease in the magnitude of LTD at D1R-MSNs that was correlated with the volume (g/kg) of ethanol they consumed. Those animals that consumed greater amounts of ethanol following air exposure showed a greater reduction in the magnitude of LTD (smaller reduction in EPSC amplitudes post-pairing) than animals that drank less. A similar finding has recently been reported where mice in an operant self-administration paradigm that consume greater quantities of ethanol exhibit reduced magnitudes of LTD (Mangieri et al., 2017). This change in the magnitude may be a first step leading towards the enhancement of D1R-MSN activity, shifting these neurons away from the ability to express synaptic depression and towards

synaptic potentiation. This shift may begin first in the vHipp-shell circuit in response to lower levels of ethanol exposure and may eventually lead to adaptations at other inputs and eventually the complete loss in expression of LTD as seen following CIE-induced escalation in intake. Glutamatergic input from the vHipp is important for gating of synaptic responses to other inputs (O'Donnell & Grace, 1995). The enhancements in presynaptic glutamate signaling that we observed following ethanol consumption in both air and vapor exposed animals may be working to drive these MSNs to a more depolarized state where other inputs can enhance the activation of the D1R-MSNs eventually leading to a complete loss of LTD expression. Since these neuronal recordings are conducted in brain slices, they give us a snapshot into the circuit specific adaptations occurring in response to different ethanol exposures. However, the functional implications of how these circuit specific adaptations are incorporated into rest of the brain's reward system and their impact on complex behaviors remains speculative, at the very least, the observed change in synaptic plasticity at this isolated portion of the circuit represents the fact that ethanol exposure and withdrawal can seriously augment information processing of the NAc and in particular processing of information relayed by the vHipp.

In the vHipp-Shell ChR2 animals, we also observed the presence of GluA2 subunit lacking calcium permeable AMPA receptors (CPARs) after drinking in both air and vapor exposed animals. The presence of CPARs was indicated by changes in rectification and alterations in EPSC amplitude in the presence of the CPAR antagonist NASPM. This change in AMPAR subunit expression may play a role in the observed change in synaptic plasticity after ethanol consumption. CPARs generally have greater single-channel conductance, and faster activation kinetics than GluA2 containing

AMPARs. In addition CPARs have been shown to be necessary for the incubation of cue induce drug seeking (Loweth et al., 2014). The presence of CPARs has also been observed in a circuit specific manner following self-administration of cocaine (Pascoli et al., 2014; Terrier et al., 2015; Terrier et al., 2016) or opiates (Hearing et al., 2016a).

The calcium-permeability of CPARs likely has important functional consequences on plasticity. As calcium entry into MSNs is a critical initiator for many forms of plasticity including NMDAR-LTD, it's possible that the incorporation of CPARs alters the volume of calcium entering D1R-MSNs and could lead to metaplastic shifts in the form of plasticity observed following afferent stimulation. According to the Artola-Brocher-Singer rule, induction of LTD or LTP depends on the ability of the postsynaptic membrane to reach sequential thresholds in order for each form of plasticity to be expressed (Artola & Singer, 1993). Increases in postsynaptic calcium levels, typically mediated by NMDARs, as a result of afferent stimulation can reach three potential "concentrations": low, resulting in LTD (Cho et al., 2001), intermediate, resulting in no plasticity, or high, leading to LTP. A CPAR-mediated increase in postsynaptic calcium levels may alter the basal concentration of calcium that enters the cell upon stimulation and likely would contribute towards a shift in the form of plasticity expressed. Future work using protein analysis techniques to examine AMPAR subunit composition would be useful to gain a better understanding of the degree to which AMPAR subunit expression is altered in the vHipp-Shell circuit following ethanol experience.

Here we observed differential expression in plasticity of vHipp-Shell D1R-MSNs from air and vapor exposed animals. The loss of LTD expression observed in the vapor exposed animals was accompanied by enhancements in asEPSC amplitude that significantly differed from those observed in the air treated group. We also observed an

enhancement in asEPSC frequency that was only observed in the vapor exposed group. These increases in pre and postsynaptic glutamatergic signaling that were present in the vapor exposed animals may in part account for the observed difference between the air and vapor exposed groups in the expression of plasticity. It is also possible that the observed differences in plasticity between the air and vapor exposed animals is due to either: 1) synaptic adaptations within the vHipp-Shell circuit which we were not capable of detecting using the electrophysiological techniques we employed (i.e. increased expression of silent synapses in vapor exposed animals that is not observed in AMPA/NMDA ratios due to masking by enhanced AMPA component); or more likely, 2) is a compound effect produced by the interplay between the adaptations produced within the vHipp-Shell circuit and adaptations occurring at the other glutamatergic inputs onto the same MSNs.

Anatomical studies indicate that the input and output of dorsal and ventral hippocampus (dHipp & vHipp) are distinct (Swanson & Cowan, 1977). Spatial memory appears to depend on dHipp but not the vHipp (Moser et al., 1995) whereas, the vHipp and not dHipp, appears critical for stress responses and emotional behaviors. Lesion studies of the vHipp suggest that the loss of the vHipp decreases fear and anxiety including suppressing corticosterone in response to stressful stimuli (Kjelstrup et al., 2002). The ventral CA1, ventral subiculum, and the medial band of the lateral and medial entorhinal cortex give rise to projection to the NAc shell (Groenewegen et al., 1996; Naber & Witter, 1998). In this arrangement input from the vHipp to the NAc shell may convey memory for affective aspects of reward (Wassum et al., 2009a). Our finding of increased glutamatergic activity from the vHipp and an alteration in plasticity onto D1R-MSNs of the shell may comprise an alteration in the memory of the affective aspects of

alcohol, with the larger changes following binge-like CIE exposure potentially indicating an alteration in the animal's emotional state during withdrawal from robust ethanol experience. This conclusion is merely speculation given the reported nature of information being transmitted by the vHipp. Future work should be conducted to determine a direct relationship between the vHipp to shell circuitry and the expression of ethanol consummatory behavior.

A limitation of the present study is that we did not directly tie our observed circuit specific changes in plasticity and glutamatergic signaling with behavior. Our findings show a nice correlation between the amount of ethanol consumed and the magnitude of LTD observed in the vHipp-Shell circuit but we cannot say for certain that the change in signaling is responsible for increases in ethanol intake. Future work in the lab should aim to prove a link between synaptic adaptations and consummatory behavior. One possible approach would be to conduct experiments where animals are given 2BC access and undergo ethanol vapor exposure to enhance ethanol intake. If the adaptations in accumbal glutamatergic signaling are indeed critical for the expression of enhanced ethanol intake, pharmacological or physiological manipulations during the withdrawal periods following vapor exposure should prevent the increase in ethanol intake or even reduce the amount of ethanol consumed.

One such approach would be to infuse the CPAR antagonist NASPM directly into the NAc shell during this time point. It's possible that by blocking CPAR activity we can reduce ethanol consumption. This hypothesis is supported by the finding that cocaine seeking is significantly reduced after infusing NASPM into the NAc (Schmidt et al., 2015). Another potential approach would be to use *in vivo* optogenetics to optically induce plasticity in a particular circuit. mGluR1 activation can result in the internalization

of postsynaptic CPARs. Activation of mGluRs has been shown to reduce cue induced responding for cocaine (McCutcheon et al., 2011). The involvement of mGluRs has been demonstrated in ethanol drinking behaviors (Cozzoli et al., 2012, 2014). By stimulating at 13 Hz, we could induce mGluR1 dependent LTD that can result in the insertion of CPARs, in order to examine if a CIE-induced increase in consumption is prevented by the reversal of CPAR expression. A similar approach found that *in vivo* stimulation at 13 Hz reverses cocaine-induced expression of CPARs and abolishes cue-induced cocaine seeking (Pascoli et al., 2014). If this approach were to successfully reverse the expression of CPARs and abolishes cue-induced ethanol seeking it would indicate mGluR1 as an interesting candidate for pharmacological intervention for alcohol dependence and alcohol use disorders. It could be particularly useful for future experiments to assess the ability of mGluR1 agonists to alter ethanol consummatory behavior in animals.

Additional future studies should also investigate the involvement and modulation of the other major specific inputs to the NAc in the ethanol induced modulation of plasticity including the medial prefrontal cortex, the basolateral amygdala, and the thalamus. Other studies indicate that input specific drug-induced adaptations can occur depending on which input is being assessed (Hearing et al., 2016a; Joffe & Grueter, 2016; Pascoli et al., 2014). It is critical to determine if input specific adaptations are universal for the various drugs of abuse, or if a drug like ethanol, which has a unique pharmacology, produces unique input specific adaptations following ethanol exposure. Our current findings indicate that similar overall adaptations are occurring but that the input at which they are occurring may differ depending on the drug of abuse assessed (i.e. CPAR insertion in vHipp seen here following ethanol, vs CPAR insertion in the mPFC following cocaine or opiates).

Another interesting set of future experiments could involve the use of optogenetics or chemogenetics to selectively inhibit the activity of D1R-MSNs during either 2BC drinking or operant self-administration of ethanol. These studies would further support the critical nature of accumbal excitation, and in particular the activity of D1R-MSNs, in the formation of ethanol dependent consummatory behaviors. By incorporating this approach into an operant self-administration paradigm it would be possible to examine the role of this neuronal population on ethanol seeking behavior or the drive to work for access to ethanol. By optically or chemogenetically inhibiting the different inputs onto D1R-MSNs within the shell during experiments where an increasing degree of effort is required, we could gauge if different inputs to the shell are critical for the motivational aspect of ethanol responding. If in turn, we conduct cue-induced reinstatement experiments while suppressing the different inputs, we could gain information on those inputs roles in relapse-like behavior. Gaining a better understand of the circuit specific changes will hopefully allow researchers to identify novel targets for intervention that are ideally shared between various types of drugs of abuse.

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