THE EFFECTS OF CHRONIC INTERMITTENT ETHANOL EXPOSURE ON
WITHDRAWAL-RELATED BEHAVIORS, DOPAMINE TERMINAL
FUNCTION AND KAPPA OPIOID SYSTEM SENSITIVITY IN THE NUCLEUS
ACCUMBENS CORE

BY

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DEDICATION AND ACKNOWLEDGEMENTS

My family is nothing less than my foundation. Without the unconditional love and support of my family, I would have never left the comforts and security of home to start my educational journey and achieve all that I have thus far.

This is for them.

Mom and Dad.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<td>BEC</td>
<td>Blood ethanol concentration</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CIE/air</td>
<td>Chronic intermittent ethanol/air (exposure)</td>
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<td>CPP</td>
<td>Conditioned place preference</td>
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<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<tr>
<td>D1R</td>
<td>Dopamine D1 receptor</td>
</tr>
<tr>
<td>D2R</td>
<td>Dopamine D2 receptor</td>
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<tr>
<td>D3R</td>
<td>Dopamine D3 receptor</td>
</tr>
<tr>
<td>DOR</td>
<td>Delta opioid receptor</td>
</tr>
<tr>
<td>DSM-4</td>
<td>Diagnostic and Statistical Manual, version 4</td>
</tr>
<tr>
<td>DSM-5</td>
<td>Diagnostic and Statistical Manual, version 5</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma amino butyric acid</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein coupled receptor kinases</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'-O-[gamma-thio]triphosphate (assay)</td>
</tr>
<tr>
<td>ICSS</td>
<td>Intracranial self-stimulation</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KOR</td>
<td>Kappa opioid receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MOR</td>
<td>Mu opioid receptor</td>
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</table>
norBNI – Nor-binaltorphimine
PFC – Prefrontal cortex
PKA – Protein kinase A
PKB - Protein kinase B
RACK1 - Receptor for activated C kinase 1 scaffolding protein
SAMHSA- The Substance Abuse and Mental Health Services Administration
TrkB - Tropomyosin-related kinase B
VTA- Ventral tegmental area
ABSTRACT

THE EFFECTS OF CHRONIC INTERMITTENT ETHANOL EXPOSURE ON WITHDRAWAL-RELATED BEHAVIORS, DOPAMINE TERMINAL FUNCTION AND KAPP A OPIOID SYSTEM SENSITIVITY IN THE NUCLEUS ACCUMBENS CORE

Jamie H. Rose

Dissertation under the direction of Sara R. Jones, Ph.D., Tenured Professor of Physiology and Pharmacology
Alcohol use disorders are a rampant economic and health concern in the United States. Chronic alcohol use and abstinence is often followed by relapse to ethanol drinking occurs in response to the withdrawal symptoms of the drug. However, the neurobiological underpinnings of this disorder are not well understood. Previous work has shown that chronic ethanol exposure and withdrawal downregulates dopamine transmission in the nucleus accumbens (NAc). Reduced dopamine terminal function may be due to upregulated presynaptic receptor proteins such as kappa opioid receptors (KOR) and dopamine D2/D3 autoreceptors (D2R/D3R), and changes in receptor-system function may, in part, drive symptoms of ethanol withdrawal and subsequent relapse drinking behavior. To this end, the present body of work identified some of the effects of five weeks of chronic intermittent ethanol (CIE) exposure on ethanol withdrawal behavioral phenotypes, including ethanol drinking parameters and anxiety/compulsive-like behavior, as well as dopamine transmission and presynaptic receptor function in the NAc of C57BL/6J mice. We found that CIE increased ethanol drinking and anxiety/compulsive-like marble burying compared to air-exposed mice, phenotypes that were reduced by KOR blockade and replicated with KOR activation. Neurobiological examination of accumbal dopamine transmission with fast scan cyclic voltammetry in brain slices showed reduced dopamine release, augmented uptake and increased sensitivity of KORs and D3Rs following five weeks of CIE exposure. A subsequent examination of interactions between KORs and dopamine autoreceptors showed that D3Rs and KORs are functionally linked. Finally, the effects nalmefene, a high-affinity KOR partial agonist used in some European countries to treat alcohol use disorders, on dopamine terminal and KOR function were examined in efforts to understand its clinical
efficacy. Nalmefene reduced dopamine uptake rates and reversed the dopamine-decreasing effects of KOR activation selectively in brain slices from CIE-exposed mice. These effects are credited to the increased affinity and distinct effects of this compound on KORs. We hope that this work supports the development of KOR-specific ligands to reduce ethanol withdrawal symptoms and relapse drinking. A discussion of the present data, future directions, clinical implications of this work and experimental caveats are considered.
PREFACE

The following dissertation contains work examining the functional consequences of chronic intermittent ethanol exposure on ethanol drinking and anxiety/compulsive behavior, kappa opioid and autoreceptor responsivity to agonist, as well as a functional interaction between receptor systems, and the effects of nalmefene on dopamine terminal and kappa opioid receptor function. This document was prepared in partial fulfillment of the requirements for the degree of doctor of philosophy in biomedical science at Wake Forest University Graduate School of Arts and Sciences. Some of this work has been, or will be, submitted for publication, and has been reformatted per the Graduate School requirements for thesis preparation; however, journal requirements dictate small stylistic variations between chapters.
CHAPTER I

THE MODULATORY ROLE OF ETHANOL EXPOSURE ON AFFECT, THE NEUROCIRCUITRY OF BEHAVIOR, ACCUMBAL DOPAMINE TRANSMISSION AND KAPPA OPIOID SYSTEM FUNCTION

Jamie H. Rose
1.0. Opening

Alcohol use disorders are a major economic and financial burden in the United States, affecting approximately 18 million Americans and costing taxpayers over $223.5 billion annually (Centers for Disease Control and Prevention, 2014; Substance Abuse and Mental Health Services Administration, 2014). The magnitude of this financial burden is linked to healthcare expenses, law enforcement, criminal justice and incarceration costs, motor vehicle accidents, and lost work productivity (Centers for Disease Control and Prevention, 2014). Despite being a significant public health issue, only three pharmacotherapies are currently approved by the United States’ Food and Drug Administration (FDA) for alcohol use disorders: acamprosate, disulfiram and naltrexone (Pettinati & Rabinowitz, 2006). Regrettably, none of these drugs are completely efficacious in reducing relapse to alcohol drinking after a period of abstinence (Latt et al., 2002; Mann et al., 2004; Jørgensen et al., 2011; Koob, 2013; Yoshimura et al., 2014; Higuchi et al., 2015). In fact, resumption of ethanol drinking in alcoholics following treatment is as high as 86% (Moos & Moos, 2006); thus, there is clinical and scientific consensus over the need for novel pharmacotherapies and receptor targets that confer greater abstinence from alcohol use in this population. The present document reviews the current literature regarding alcohol use disorders in humans and animal models and provides an investigation in to a promising pharmacotherapeutic target for this disease.

1.1. Alcohol use disorders in the United States: Diagnosis and treatment

The development of an alcohol use disorder is invariably preceded by recreational alcohol intake that is characteristically driven by the positive, euphoric effects of the drug
(Avery et al., 1982; van Hemel-Ruiter et al., 2015). Alcohol users with an inclination for substance abuse disorders, due to a combination of genetic and environmental factors (Bierut et al., 1998; Chassin et al., 2004; Cservenka et al., 2015), transition from consuming alcohol for its positive reinforcing effects to drinking in an attempt to relieve the symptoms of withdrawal (Rousseau et al., 2011; Cohn et al., 2012; Kiselica & Borders, 2013), a hallmark of alcohol use disorders. Contemporary diagnosis of an alcohol use disorder is based on criteria described in the recently published version of the Diagnostic and Statistical Manual of Mental Disorders, Version 5 (DSM-5; American Psychological Association, 2013). Unlike the previous edition, the DSM-4 (American Psychological Association, 2000), alcohol use disorders are now diagnosed based on a continuum of criteria (American Psychological Association, 2013), and the distinction between “alcohol abuse” and “alcohol dependence” no longer exists (American Psychological Association, 2000, 2013). To be diagnosed as having an alcohol use disorder per the DSM-5 (American Psychological Association, 2013), patients must meet between two and six (or more) out of eleven descriptors that identify specific elements of a mild, moderate or severe alcohol use disorder. These criteria include, but are not limited to: drinking in spite of deleterious consequences, increasing consumption over time (due to tolerance to the physiological and psychological effects of ethanol), loss of friendships, romantic and familial relationships, as well as physical or psychological withdrawal symptoms and relapse drinking behavior (American Psychological Association, 2013).

Clinical diagnosis of an alcohol use disorder is often followed by psychological and, or pharmacological therapy. For example, alcoholics may participate in cognitive and/or
behavioral therapy with a trained therapist or group-based 12-step program such as Alcoholics Anonymous (Alcoholics Anonymous World Services, Inc, 2001), in conjunction with pharmacological treatment. The pharmacotherapies currently approved by the United States’ FDA are disulfiram (Antabuse®), acamprosate (Campral®) and naltrexone (Revia®). These compounds will be reviewed in-depth later in this document (Section 6.0). Unfortunately, decades of evidence show that abstinent alcoholics relapse multiple times prior to complete abstinence, even when consistently treated with one or both methods of intervention (Ballenger & Post, 1978; Lechtenberg & Worner, 1990, 1992; Worner, 1996). These clinical observations have prompted a number of laboratory models of alcohol use disorders in monkeys and rodents that replicate alcohol exposure and withdrawal symptoms per the human condition which will be discussed later in this document (Appendix II).

Withdrawal symptoms underlie relapse-drinking behavior after a period of chronic, heavy alcohol consumption and abstinence (Zywiak et al., 1996; Wetterling et al., 2006; American Psychological Association, 2000, 2013; Meyerhoff et al., 2013). These symptoms include augmented feelings of anxiety (Wetterling & Junghanns, 2000), compulsive behaviors (Suzuki et al., 2002), sleep disturbances and sleeplessness (Perney et al., 2012; de Zambotti et al., 2014), depression (Johnson & Perry, 1986; Schoonover et al., 2015), dysphoria (Hayne & Louks, 1991; Locke & Newcomb, 2003), and in severe cases, seizures and delirium tremens (Eyer et al., 2011; Kim et al., 2015). Alleviation of the ethanol withdrawal symptoms by ethanol intake negatively reinforces continued drinking behavior; thus, relief of these symptoms with innovative pharmacotherapies and identification of novel targets is of clinical and scientific interest.
2.0. The nucleus accumbens core

The region of interest for the upcoming studies is the nucleus accumbens core, a ventral portion of the striatum that is highly involved in establishing the reinforcing effects of drugs of abuse (Alderson et al., 2001; Gill et al., 2010; du Hoffman & Nicola, 2014) and ethanol (Bull et al., 2014; Pina & Cunningham, 2014; Young et al., 2014). The primary neurotransmitter in this region is the catecholamine, dopamine. Dopaminergic transmission in this region arises primarily from ventral tegmental area (VTA) dopamine neurons, and a smaller contribution of dopamine projections from the substantia nigra pars compacta (Benivoglio et al., 1979; Phillips et al., 1992; Forster & Blaha, 2000; Owesson-White et al., 2012). VTA-area dopamine cells display tonic firing patterns that provide dopamine tone in the nucleus accumbens that is present in awake animals (Grace & Bunney, 1983). Phasic or burst firing (Grace & Bunney, 1983) or electrical stimulation of VTA dopamine neurons causes a time-locked increase in dopamine levels in the nucleus accumbens, while ablation of VTA-area dopamine neurons reduces dopamine release in this region (Phillips et al., 1992; Forster & Blaha, 2000; Owesson-White et al., 2012).

The nucleus accumbens core is a region of intense and diverse neurotransmitter activity. Figure 1 depicts a representation of accumbal neurocircuitry. In addition to dopamine projections from the VTA, other inputs include norepinephrine from the locus coeruleus (Berridge et al., 1997), serotonin from the dorsal raphe nucleus (Yoshimoto & McBride, 1992) and glutamatergic inputs from the prefrontal cortex (Del Arco & Mora,
Figure 1: A visual representation of the neuronal connections in the nucleus accumbens core.

The nucleus accumbens is a highly heterogenous and complex region. Red terminal boutons: dopaminergic inputs from the ventral tegmental area (VTA). Pink, blue and purple terminal boutons: glutamatergic afferents from the amygdala, prefrontal cortex (PFC) and hippocampus, respectively. Orange terminal bouton: serotonergic projection neuron from the dorsal raphe nucleus (DR). Periwinkle terminal bouton: noradrenergic input from the locus coeruleus (LC). Maroon interneurons: GABAergic. Orange interneurons: acetylcholinergic. Green neurons: D1/D2-containing (unspecified) GABAergic medium spiny projection neurons.
2008), hippocampus (Blaha et al., 1997) and amygdala (Floresco et al., 1998). All afferents converge on to dopamine terminals, cholinergic (Bluth et al., 1985) and gamma amino butyric acid (GABA)-ergic (Tepper & Bolam, 2004) interneurons and GABAergic medium spiny projection neurons (Huang et al., 2008; Lobo & Nestler, 2011; MacAskill et al., 2012; Gangarossa et al., 2013; Grueter et al., 2013).

Medium spiny neurons, the primary output neurons of the nucleus accumbens (Huang et al., 2008; Lobo & Nestler, 2011; MacAskill et al., 2012; Gangarossa et al., 2013; Grueter et al., 2013) comprise over 95% of the accumbal milieu (O’Donnell & Grace, 1993). These neurons are classified as either D2 receptor (D2R)- or D1 (D1R)-containing (Surmeier et al., 1996; Cepeda et al., 2008) which activate distinct signaling pathways. In fact, D2-containing medium spiny neurons project to the globus pallidus externa, through the striatopallidal indirect pathway, while D1-containing medium spiny neurons provide a feedback signal to the VTA and mediate neurotransmission by the striatonigral direct pathway (Figure 2; Surmeier et al., 1996; Cepeda et al., 2008; Perreault et al., 2013). In sum, dopaminergic projections from the ventral midbrain converge in the accumbal milieu (Figure 1), mediating the downstream signaling of GABAergic medium spiny neurons. D1-containing medium spiny neurons project to non-dopaminergic neurons in the substantia nigra pars reticulata while D2-containing medium spiny neurons project through the globus pallidus externa. Inhibitory projections from the globus pallidus externa extend to the subthalamic nucleus and substantia nigra pars reticulata. The subthalamic nucleus sends glutamatergic projections to the substantia nigra pars reticulata. The substantia nigra pars reticulata receives projections from the direct and indirect pathways, sending a net GABAergic signal to the thalamus. Excitatory
Dopaminergic projections (red arrows) from the ventral midbrain (substantia nigra pars compacta and ventral tegmental area) converge in the accumbal milieu (Figure 1), mediating the signaling of post-synaptically located GABAergic medium spiny neurons. Medium spiny neurons are classified based on the presence of excitatory D1 (+, yellow circle) or inhibitory D2 (-, purple circle) receptors. The GABAergic medium spiny neurons project through distinct motor pathways through the basal ganglia and release specific opioids. D1-containing medium spiny neurons project to non-dopaminergic neurons in the substantia nigra pars reticulata whereas D2-containing medium spiny neurons project through the globus pallidus externa. In this pathway, inhibitory projections from the globus pallidus externa extend to the subthalamic nucleus and substantia nigra pars reticulata. The subthalamic nucleus sends excitatory (glutamatergic) projections to the substantia nigra pars reticulata. The substantia nigra pars reticulata receives projections from the direct and indirect pathways, sending a GABAergic signal to the thalamus. Excitatory signals from the thalamus are sent to the nucleus accumbens and cortex. Glutamatergic signaling from the cortex extends back to the striatum.
signals from the thalamus are sent to the nucleus accumbens and cortex, and glutamatergic signaling from the cortex extends back to the striatum. Recent work, however, suggests that the direct and indirect pathways through the basal ganglia may not be as clear as traditionally considered (Kupchik et al., 2015).

Notably, D1R- and D2R-expressing medium spiny neurons release distinct opioid peptides (Shuster et al., 2000; Rutter & Tsuboi, 2004). Specifically, D2-containing medium spiny neurons release enkephalin (Surmeier et al., 1996) which activate postsynaptically located delta opioid receptors (DOR; Dilts & Kalivas, 1990), while D1-containing medium spiny neurons release dynorphin (Curran & Watson, 1995) which activates presynaptic kappa opioid receptors (KOR) and inhibit dopamine transmission (Spanagel et al., 1990, 1992). See Section 4.2 for more information regarding dynorphins.

2.1. The effects of ethanol exposure on mesolimbic dopamine function

Dopamine transmission in the nucleus accumbens drives reward-learning behavior, including those associated with the rewarding effects of ethanol. For example, dopamine receptor blockade in the nucleus accumbens disrupts the acquisition or expression ethanol conditioned place preference (Young et al., 2014), a measure of ethanol reward. Furthermore, acute ethanol reduces intra-cranial self-stimulation (ICSS) brain reward thresholds (Boutros et al., 2014), suggesting that ethanol augments dopamine transmission in the mesolimbic dopamine system. Voluntary ethanol consumption in non-ethanol dependent rats (Melendez et al., 2002), and experimenter-administered ethanol in non-dependent humans (Aalto et al., 2015) induces dopamine release that is time-locked to ethanol exposure and not present with sucrose consumption, indicating a direct
pharmacological effect of ethanol on dopamine transmission, and points to this effect as a driver of continued ethanol consumption. Although the positive reinforcing effects of ethanol are mediated by accumbal dopamine transmission, chronic ethanol exposure produces adaptations in this system that fundamentally reduces its function (Volkow et al., 1992, 2006; Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al., 2015). The effects of chronic ethanol exposure on dopamine terminal and presynaptic receptor function are of particular points of interest to the present body of work and will be discussed in Section 2.2.

Acute ethanol augments dopamine transmission through multiple mechanisms. For example, ethanol increases the hyperpolarization-activated cation current ($I_h$) of VTA dopamine neurons, thereby augmenting the classic pacemaker-firing rate of these cells and dopamine release in projection areas (Okamoto et al., 2006; Tateno & Robinson, 2011; Chen et al., 2012). Additionally, ethanol reduces GABA interneuron firing in the VTA, an effect that disinhibits dopamine cell body firing and increases in dopamine release in projection regions (Chefer et al., 2009; Niikura et al., 2010; Jalabert et al., 2011; Margolis et al., 2012).

The effects of ethanol on dopamine transmission in vivo appear to be biphasic. For example, systemic ethanol administration up to 2.0g/kg increased dopamine neuron firing, while doses at and above 4.0g/kg caused a marked reduction in dopamine neuron firing rate (Mereu et al., 1984). A loss of dopamine neuron firing at high doses of the drug may explain why voluntary ethanol consumption in non-dependent animals is rarely above 2.0g/kg (Becker & Lopez, 2004; Lopez & Becker, 2005; Griffin et al., 2009). Ethanol intake up to 2.0g/kg produces pharmacologically relevant brain concentrations of
the drug. For example, systemic administration of 2.0g/kg ethanol augmented striatal levels of ethanol to 10mM (Tang et al., 2003) while a 3.0g/kg of ethanol consumed by mice provide a brain level of ~12mM ethanol (Griffin et al., 2007). Notably, the dopamine-releasing properties of ethanol do not appear to directly modify dopamine terminal function (to be discussed below). Although physiologically relevant to an intact animal, brain concentrations of ethanol in these studies are lower than the concentrations used in ex vivo preparations when dopamine cell bodies are severed from terminal regions (20-200mM; Budygin et al., 2001, 2005; Rose et al., 2013; Yorgason et al., 2014). In fact, ethanol concentration-dependently reduced dopamine release in brain slices containing the nucleus accumbens at 100-200mM of the drug, while having no effect on dopamine release or uptake at low concentrations (Budygin et al., 2001, 2005; Rose et al., 2013; Yorgason et al., 2014). These data suggest that the excitatory effects of ethanol on dopamine signaling are limited to modulation of dopamine neuron firing (Mereu et al., 1984; Gessa et al., 1985; Brodie & Appel, 2000) rather than dopamine terminals (Budygin et al., 2001, 2005; Rose et al., 2013; Yorgason et al., 2014).

2.2. Chronic ethanol exposure reduces dopamine terminal function

Chronic ethanol exposure and withdrawal reduces dopamine system function, an effect that may (Shen & Chiodo, 1993; Shen et al., 2007) or may not (Diana et al., 1995; Brodie, 2002) be due to attenuated dopamine cell firing of ventral tegmental area-located dopamine cell bodies or deficient terminal function (Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al., 2015). In fact, recent work from our laboratory has shown augmented dopamine uptake and reduced dopamine release in mice (Karkhanis et al., 2015) and rats (Budygin et al., 2007), and positron emission tomography scanning of
human alcoholics shows reduced dopamine receptor binding in the basal ganglia (Volkow et al., 1996, 2002). Despite these consistent cross-species findings (Hietala et al., 1994; Laine et al., 1999; Budygin et al., 2007; Volkow et al., 1996, 2002; Karkhanis et al., 2015), the precise mechanism(s) underlying ethanol-induced changes in dopamine transmission are not well understood.

Of particular interest are chronic ethanol-induced changes in kappa opioid receptor (KOR) and dopamine D2 (D2R) and D3 (D3R) autoreceptor function. These receptors may be presynaptic release-inhibiting receptors (Spanagel et al., 1990, 1992; Maina & Mathews, 2010) that are hypersensitive following chronic ethanol, and chronic intermittent ethanol (CIE) exposure (Perra et al., 2011; Kissler et al., 2014; Kivell et al., 2014; Karkhanis et al., 2015; Siciliano et al., 2015). These data suggest that D2Rs and D3Rs and KORs may be driving chronic ethanol-induced reductions in dopamine transmission.

3.0. The opioid system and ethanol

The three classical opioid receptors: mu (MOR), DOR and KOR are Ga<sub>q/11</sub>-coupled G-protein coupled receptors (GPCR) with specific endogenous ligands: endorphins, enkephalins and dynorphins, respectively (Kosterlitz & Paterson, 1980; Chavkin et al., 1982; Raynor et al., 1994; Le Merrer et al., 2009). All ligands share limited affinity for non-primary opioid receptor due to a conserved enkephalin pentapeptide sequence (tyrosine-glycine-glycine-phenylalanine-methionine/leucine) on the amino terminus of each endogenous neuropeptide (Kosterlitz & Paterson, 1980; Chavkin et al., 1982; Raynor et al., 1994). The opioid receptors and their ligands are located ubiquitously throughout the brain to modulate transmission of various neurotransmitters (Werling et
al., 1987; Hjelmstad & Fields, 2003; Zhang & Pan, 2012; Le Merrer et al., 2009; Pu et al., 2012; Ponterio et al., 2013; Al-Hasani et al., 2013), including dopamine (Spanagel et al., 1992; Chefer et al., 2005; Hipólito et al., 2008). Notably, the anatomical positions of MORs within the VTA and KORs on nucleus accumbens dopamine terminals modulate dopamine transmission in opposition, whereby activation of MORs and KORs positively and negatively modulate dopamine transmission, respectively (Spanagel et al., 1992; Devine et al., 1993; Mitrović & Napie, 2002).

Based on their ability to modulate dopamine release, MORs and KORs may contribute to some of the positive, euphoric and negative withdrawal symptoms associated with abstinence following chronic ethanol exposure. For example, acute ethanol activates MORs on GABAergic interneurons in the VTA, disinhibiting dopamine neurons in this area, thereby increasing dopamine release in terminal fields (Chefer et al., 2009; Guan & Ye, 2010; Niikura et al., 2010; Jalabert et al., 2011; Margolis et al., 2012; Tseng et al., 2013) and in part, driving positive hedonic states (Figure 3, green section; Méndez et al., 2001; Hipólito et al., 2011). Conversely, activation of KORs on a subpopulation of dopamine cell bodies in the VTA reduces dopamine transmission in the prefrontal cortex (Margolis et al., 2006), and of interest to the present body of work, local activation of KORs in the nucleus accumbens attenuates dopamine transmission specifically in this region (Spanagel et al., 1992, Chefer et al., 2005; Niikura et al., 2010). Notably, these effects may engender negative affective states, particularly those experienced following ethanol exposure, during withdrawal states (Figure 3, red section).

To separate the positive and withdrawal effects of ethanol into signaling by MORs and KORs, respectively, would be an oversimplification of the system. Rather, the sum
effects of ethanol exposure are due to a balance of multiple neurotransmitter and neuromodulator systems, including MORs and KORs, and not due to increased function of only one of these receptors. In fact, it has been widely accepted that chronic ethanol exposure has distinct effects on multiple neurotransmitter and neuromodulator systems, including corticotropin releasing factor and neuropeptide Y (Thorsell et al., 2005; Criado et al., 2011), serotonin (Esteban et al., 2002; Smith et al., 2008), glutamate (Christian et al., 2012; Griffin et al., 2014, 2015), GABA (Mhatre & Ticku, 1994; Kumar et al., 2002) and norepinephrine (Smith et al., 2008; Karkhanis et al., 2015b), all of which likely influence ethanol withdrawal symptoms. With respect to KORs, specifically, the rewarding effects of ethanol can be obliterated with KOR activation (Logrip et al., 2009) and prodynorphin knock-out mice and mice treated with a KOR antagonist show higher levels of ethanol-conditioned place preference than wild-type and non-treated mice (Nguyen et al., 2012). Moreover, acute ethanol not only induces dopamine release (Chefer et al., 2009; Guan & Ye, 2010; Niikura et al., 2010; Jalabert et al., 2011; Margolis et al., 2012; Tseng et al., 2013), but also increases dynorphin release and, by extension, causes KOR activation (Lindholm et al., 2000; Marinelli et al., 2006). These data suggest that the KOR system may be involved in dampening the rewarding and reinforcing effects of ethanol on an acute timescale. Despite this complexity, the separation of the effects of ethanol into changes in MOR and KOR signaling systems in the present body of work is for example purposes only.
3.1. A theoretical framework for the development of alcohol use disorders as diseases of chronic relapse

The chronic relapsing pattern of alcohol use disorders is driven by both positive and negative reinforcement (Koob & Le Moal, 1997, 2008), a pattern that can be described with the opponent process theory of motivation (Figure 3, Solomon & Corbit, 1973, 1974). The opponent process theory posits that psychological challenges and drugs of abuse would alter the hedonic state of the subject in a predictable manner. Essentially, the positive affect associated with exposure to drugs of abuse would be countered with compensatory withdrawal symptoms and negative affect (Figure 3; Solomon & Corbit, 1973; Koob & Le Moal, 1997, 2008). The affective state of this subject would return to its normal, homeostatic set-point (Figure 3, solid black line) following drug exposure and remain stable until a subsequent drug challenge (Figure 3, yellow arrow). With chronic, repeated drug use and withdrawal, this pattern of affect trends downward: the positive hedonic state associated with drug exposure is attenuated and followed by a significant increase in the withdrawal symptoms (Figure 3; Koob & Le Moal, 1997, 2008). Further, the drug abusers’ normal affective state in between drug uses is also reduced, whereby normal processes do not return to the homeostasitic set-point, rather they settle at a new, more negative hedonic state known as an “allostatic set-point” (Figure 3, dotted lines; Solomon & Corbit, 1973; Koob & Le Moal., 2008).

Notably, this pattern is strikingly similar to dopamine system modulation by opioid receptors following acute and chronic ethanol exposure and withdrawal. In fact, chronic ethanol exposure reduces MOR activity (Turchan et al., 1999; Chen & Lawrence, 2000; Saland et al., 2005) and increases KOR/dynorphin system activity (Figure 3, red section;
Acute ethanol exposure augments affect (following first yellow arrows), in part due to increased MOR signaling. After acute ethanol exposure, affect returns to the normal homeostatic set-point (solid black line) until another there is a subsequent ethanol challenge (second yellow arrow). After repeated heavy exposure to ethanol, the euphoric effects of the compound, and associated MOR signaling, is reduced (following second yellow arrow). With regard to acute and chronic ethanol exposure, the positive reinforcing effects of ethanol exposure are followed by the withdrawal effects of ethanol, KOR activation (red section). Over time and continued exposure to ethanol, the affective state of the subject does not return to the homeostatic set-point. Instead, their affective state adjusts, known as an allostatic set-point, to a reduced level (black dotted lines). Reductions in affect and the homeostatic set-point are marked with the withdrawal effects of ethanol that drive patients to volitionally re-expose themselves to the compound (pink arrows).
rat: Kissler et al., 2014; monkey: Siciliano et al., 2015). This pattern of opioid signaling may be one of the neurobiological substrates that modulate the positive and negative motivational aspects of chronic drug exposure.

4.0. KORs and dynorphin system function

4.1. KOR pharmacology

The primary interest of the present body of work centers on the modulatory effects of accumbal KORs on dopamine transmission. KORs modulate dopamine transmission via intracellular signaling pathways and modulation of ion channels. For example, KOR activation with agonists such as its endogenous ligand, dynorphin, or (trans)-3, 4-dichloro – N – methyl – N - [2 -(1-pyrrolidinyl)-cyclohexyl] benzeneacetamide methanesulfonate (U50,488H), results in liberation of the $G_{\alpha_i/o}$-protein from the $\beta\gamma$ subunit. The $\beta\gamma$ subunits of the heterotrimeric G-protein block voltage-dependent calcium channels (Gross & Macdonald, 1987; Hjelmstad & Fields, 2003; Hassan & Ruiz-Velasco, 2013), and augments potassium efflux (Grudt & Williams, 1993; Ma et al., 1995), which reduces the probability of cell firing and exocytotic release at the level of the dopamine terminal (Figure 4.1). Downstream signaling pathways such as p38 mitogen-activated protein kinase (MAPK), protein kinase B (PKB), c-Jun N-terminal kinase (JNK) $\beta$-arrestin have all been reported to be engaged following KOR activation (Figure 4.1; Lemos et al., 2011, 2012; Cunha et al., 2012; Melief et al., 2011; Kumar & Rai, 2011). Further, KOR activation attenuates adenylyl cyclase activity. Reduced adenylyl cyclase activity precludes the metabolism of adenosine triphosphate to cyclic adenosine monophosphate (cAMP) production, reducing protein kinase A (PKA) activity and
cAMP response element-binding protein (CREB) phosphorylation in the nucleus (Figure 4.1; Konkoy & Childers, 1989; Avidor-Reiss et al., 1995). Attenuated CREB phosphorylation subsequently reduces tyrosine hydroxylase transcription (Vié et al., 1999; Lewis-Tuffin et al., 2004), the rate-limiting enzyme responsible for dopamine production thus decreasing the concentration of dopamine available for release over time, while dephosphorylation of this enzyme by phosphatases attenuates its activity, reducing tyrosine hydroxylase activity on a shorter time-scale (Arbogast & Voogt, 2002; Fehér et al., 2010; Saraf et al., 2010). Finally, prolonged KOR activation drives receptor phosphorylation by G-protein coupled receptor kinases (GRK) and β-arrestin recruitment prior to receptor internalization (Figure 4.2; Li et al., 1999; Tian et al., 2014). Interestingly, KOR blockade by commonly used long-lasting KOR antagonists, such as 17,17′-(Dicyclopropylmethyl)-6,6′,7,7′-6,6′-imino-7,7′-binorphinan-3,4′,14,14′-tetrol dihydrochloride (nor-binaltorphimine hydrochloride, norBNI) and (3R)-7-hydroxy-N-[(2S)-1-[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide dihydrochloride (JDTic) activate the JNK pathway (Figure 4.3; Bruchas et al., 2007; Bruchas & Chavkin, 2010), indicating that these compounds are inverse agonists activating intracellular signaling pathways of their own (Khilnani & Khilnani, 2011; Kenakin & Williams, 2014). It is hypothesized that this is due to a putative linker protein associated with JNK signaling (Figure 4.3; Bruchas & Chavkin, 2010). Development of novel, clinically relevant and short-acting KOR antagonists that do not modulate KORs via inverse agonism appear to be promising pharmacotherapeutics for alcohol use and mood disorders (Cerecor, 2015; reviewed in the Discussion Section).
4.1 KORs mediate Ca\(^{2+}\) and K\(^{+}\) channels and downstream signaling pathways

4.2 Receptor internalization and recycling

4.3 Downstream effects of KOR blockade

ACCUMBAL DOPAMINE TERMINAL

TRANSCRIPTION

TO THE NUCLEUS

ERK
p38MAPK
JNK

Degradation

Unknown link

KOR
G\(\alpha_{i/o}\) subunit
G\(\beta\gamma\) subunits
cAMP
Ca\(^{2+}\) channel
K\(^{+}\) channel
KOR ligand
PKA
Phosphorylation
Catalytic subunit
Dopamine transporter

Degradation

Unknown link
(4.1) KOR activation with endogenous or exogenous ligands (closed teal circles), results in liberation of the $G_{\alpha_i/o}$-protein from the $\beta\gamma$ subunit. The $\beta\gamma$ subunits of the heterotrimeric G-protein reduce influx from voltage-dependent calcium channels and increase potassium outflux, thus attenuating the probability of dopamine release. The $G\alpha$ subunit reduces adenylyl cyclase activity, thereby attenuating the conversion of adenosine triphosphate to cyclic adenosine monophosphate (cAMP). This effect subsequently decreases protein kinase A (PKA) activity, its translocation into the nucleus, phosphorylation/activation of cAMP response element-binding protein (CREB) and the transcription of downstream gene products. Additional downstream signaling cascades of the KOR include p38 mitogen-activated protein kinase (MAPK), protein kinase B (PKB), c-Jun N-terminal kinase (JNK) and $\beta$-arrestin. (4.2) Prolonged KOR activation drives receptor phosphorylation by PKA and G-protein coupled receptor kinases (GRK) and subsequent $\beta$-arrestin recruitment prior to receptor internalization. Internalized receptors can be dephosphorylated and the $\beta$-arrestin molecule removed before reinsertion into the membrane. (4.3) KOR blockade (red X) by commonly used long-lasting KOR antagonists reportedly activate the JNK pathway, which is mediated by an unknown linker protein.
Not only do KORs reduce dopamine transmission by attenuating dopamine release, some reports suggest that KORs increase the rate of dopamine uptake (Thompson et al., 2000; Kivell et al., 2014). For example, in EM4 cells, KOR activation produced a norBNI-sensitive increase in dopamine transporter activity and presence at the cell membrane (Kivell et al., 2014). One of these studies suggests that a functional interaction between KORs and dopamine transporters is mediating this effect, such as the ERK1/2 pathway (Kivell et al., 2014). Augmented dopamine uptake rates would also reduce dopamine transmission by decreasing the concentration of dopamine in the synapse available to activate postsynaptic receptors. Thus, KOR activation reduces dopamine transmission by two distinct mechanisms: reduction in release and augmented rates of uptake.

4.2. Dynorphin activity

As noted earlier, dynorphins are a class of neuropeptides that are the endogenous ligands of KORs (Chavkin et al., 1982; Merg et al., 2006; Le Merrer et al., 2009). In the nucleus accumbens, activation of receptors on D1R-containing medium spiny neurons, such as D1Rs (Engber et al., 1992; Xu et al., 1994; Hara et al., 2006; Muschamp & Carlezon, 2013) and tyrosine-related kinase B (TrkB) receptors (Strömberg & Humpel, 1995; Logrip et al., 2008) results in downstream signaling and CREB phosphorylation in the nucleus. Binding of CREB to cAMP response element (CRE) in the promotor region of the prodynorphin gene sequence transcribes prodynorphin (Muschamp & Carlezon, 2013). There are several subtypes of dynorphin derived from the prodynorphin precursor peptide (Day et al., 1998; Berman et al., 2000; Hauser et al., 2005). These include dynorphin A (1-8, 1-13, 1-17), dynorphin B, big dynorphin, leumorphin and α- and β-
neoendorphin (Goldstein et al., 1979; Vaughn & Taylor, 1989; Ukai et al., 1992; Fischli et al., 1982; Kilpatrick et al., 1982; Kakidani et al., 1982). Dynorphins are stored in dense core vesicles until release in a calcium-dependent mechanism (Pickel et al., 1995; Yakovlена et al., 2006). Once released, dynorphins activate KORs (Chavkin et al., 1982; Merg et al., 2006), and are metabolized by carboxypeptidases, aminopeptidases and endopeptidases into several metabolites within minutes of release (Müller & Hochhaus, 1995; Müller et al., 1996).

All dynorphins have selectivity for KORs over the other classical opioid receptors (MORs, DORs), although the affinity of each for KORs varies. For example, some of the dynorphin A subtypes, particularly dynorphin A 1-13, appear to have the highest affinity for KORs (Chavkin & Goldstein, 1981), followed by dynorphin B and α-neoendorphin, and lastly dynorphin A 1-8 and β-neoendorphin (Chavkin & Goldstein, 1981; James et al., 1984; Goldstein et al., 1979). The dynorphin A subtypes (Khachaturian et al., 1982; Jamensky & Gianoulakis, 1997; Marinelli et al., 2006) and dynorphin B (Lindholm et al., 2000) have been found in the nucleus accumbens, thus these compounds will be referred to as “dynorphin” for the remainder of this document.

4.3. Changes in KOR/dynorphin system function in response to acute and chronic ethanol exposure

Data regarding the effects of acute ethanol on dynorphin levels are limited, largely due to the multiple subtypes of this peptide and the disparate ethanol exposure and withdrawal procedures used across laboratories. For example, using in vivo microdialysis, extracellular levels of dynorphin A (1-8) were significantly elevated for the first 30 minutes after administration of 1.6 g/kg and 3.2 g/kg ethanol in rats, returning to baseline.
60 minutes following administration (Marinelli et al., 2006). Notably, this effect was more pronounced at the 3.2 g/kg dose, indicating that ethanol-induced changes in dynorphin levels may be dose-dependent (Marinelli et al., 2006). Similarly, a treatment regimen of twice per day ethanol (2.0 g/kg; i.p.) for 13 days showed increased levels of dynorphin B in the nucleus accumbens 30 minutes following administration compared to control animals (Lindholm et al., 2000). In this study, levels of dynorphin B were augmented for at least 21 days after the final ethanol injection (Lindholm et al., 2000), demonstrating a long-lasting effect of repeated ethanol exposure on dynorphin levels. Conversely, five days after four weeks of ethanol drinking in C57BL/6 (C57) mice, dynorphin B levels were reduced in the amygdala and substantia nigra (Ploj et al., 2000). However, in this study, levels of dynorphin B were elevated above control levels 21 days following ethanol exposure (Ploj et al., 2000). Further, recent work has shown that four weeks of CIE in rats increases dynorphin A levels in the central nucleus of the amygdala, 6 to 8 hours following the cessation of ethanol exposure (Kissler et al., 2014). The effects of ethanol on dynorphin levels are inconclusive and may depend on the rodent species, ethanol exposure procedure used and withdrawal time, and dynorphin subtype examined.

Insight into the effects of CIE exposure on KOR activity is just emerging. For example, recent work in monkeys has shown an increase in KOR sensitivity to agonist using fast scan cyclic voltammetry following chronic oral ethanol consumption (3.5-6.5 hours following the cessation of ethanol exposure; Siciliano et al., 2015). Furthermore, data from the Walker laboratory has shown that four weeks of chronic ethanol exposure augments KOR activity in the central nucleus of the amygdala as measured with GTPγS assays, although this is shortly into withdrawal (6-8 hours; Kissler et al., 2014). As noted
earlier, this study also reported increased dynorphin A levels in the central nucleus of the amygdala (Kissler et al., 2014). These data (Kissler et al., 2014) are contradictory based on the normal behavior of GPCRs, whereby increased ligand binding promotes a functional downregulation and receptor internalization (Zhang et al., 2002; Li et al., 2003). Based on previous evidence suggesting a biphasic time-course of dynorphin transmission (Ploj et al., 2000; Marinelli et al., 2006), it is plausible that the duration of increased KOR activation and augmented dynorphin levels overlap; however, based on the rapid metabolism of dynorphin (Gambús et al., 1998), it is plausible that the source of the dynorphin may be due to reduced enzymatic activity rather than increased dynorphin release. Experiments that test dynorphin levels, enzymatic activity and KOR function at different time points following chronic ethanol exposure and withdrawal will shed light on this discrepancy.

Although additional work may be necessary to fully elucidate the precise effects of chronic ethanol exposure and withdrawal on KOR/dynorphin system function, it is likely that chronic ethanol exposure and withdrawal increases the sensitivity of this system. For example, rats treated with chronic ethanol exposure demonstrate augmented anxiety/compulsive-like behavior that is reduced to control levels with KOR blockade (Schank et al., 2012; Valdez & Harshberger, 2012). Moreover, consistent and significant increases in ethanol consumption after two and four weeks of CIE exposure are attenuated with systemic and intracerebroventricular KOR blockade (Walker & Koob, 2008; Walker et al., 2011). Notably, CIE-induced increases in ethanol consumption were also reduced with intra-accumbal administration of norbinaltorphimine (norBNI), a KOR antagonist (Nealey et al., 2011), demonstrating an important role of the nucleus
accumbens in ethanol drinking behavior. In all of these experiments, control animal behavior is not altered with norBNI administration, demonstrating an upregulation in KOR system function selectively in animals following chronic ethanol exposure and withdrawal.

In summation of the evidence presented thus far, reduced accumbal dopamine transmission following chronic ethanol exposure and withdrawal (Budygin et al., 2007; Karkhanis et al., 2015) may be due, in part, to accumbal KORs (Kissler et al., 2015; Siciliano et al., 2015), which may be driving the negative symptoms associated with chronic ethanol exposure and withdrawal that drive ethanol consumption during a period of abstinence (Walker & Koob, 2008; Nealey et al., 2011; Walker et al., 2011; Kissler et al., 2014). However, changes in KOR function in the nucleus accumbens at the level of the dopamine terminal following CIE exposure have not been explored, and are the focus of Chapter II.

5.0. Functional alterations in dopamine autoreceptors following chronic ethanol exposure

In addition to functional changes in KORs following chronic ethanol exposure, it is possible that CIE also functionally alters dopamine D2 (D2R)-type autoreceptors (Karkhanis et al., 2015). Similar to KORs, the D2-family of dopamine receptors, comprising D2R, D3 (D3R) and D4 (D4R), are Gαi/o-coupled receptors that reduce dopamine release upon activation (Maina & Mathews, 2010; Karkhanis et al., 2015). D2Rs and D3Rs are encoded by DRD2 and DRD3 gene sequences, respectively, (Platania et al., 2012; NCBI Genes and Expression DRD2, 2015; NCBI Genes and Expression
DRD3, 2015) and are structurally and functionally similar, and act as autoreceptors on dopamine terminals. Unlike D2R/D3Rs, D4Rs do not act as autoreceptors and are located primarily in brain regions outside the basal ganglia (Van Tol et al., 1991; Cohen et al., 1992). With respect to D2Rs and D3Rs, anatomical differences between extracellular amino acid residues and helices I, II and VII alter the electrostatic surface of the receptors and confer specificity between D2R and D3R exogenous ligand binding, although the actual binding pockets of D2Rs and D3Rs are nearly identical (Chien et al., 2010). Additionally, D2Rs have both long (D2R-Long) and short (D2R-Short) subtypes (Itokawa et al., 1996; Khan et al., 1998). Although functionally similar, D2Rs-Long are considered primarily a post-synaptic protein that mediate signaling through the striatal indirect pathway, whereas D2Rs-Short are located presynaptically and function as autoreceptors (Khan et al., 1998; Lindgren et al., 2003). The present work focuses on presynaptic D2Rs and D3Rs.

Due to the structural and functional similarity between D2Rs and D3Rs, many experiments examining ethanol-induced changes in autoreceptor function investigate combined D2Rs/D3Rs, making an interpretation of receptor-specific alterations nearly impossible. For example, previous work from our laboratory demonstrated increased D2R/D3R system responsivity to agonist following CIE in the nucleus accumbens using ex vivo fast scan cyclic voltammetry (Karkhanis et al., 2015). Notably, chronic ethanol intake increased D2R-type expression in the mesolimbic dopamine system (Kim et al., 1997), which may underscore increased receptor sensitivity reported earlier (Karkhanis et al., 2015). Although these data (Kim et al., 1997; Karkhanis et al., 2015) suggest D2R-type upregulation following chronic ethanol exposure, some data shows chronic ethanol-
induced alters each receptor, independent of the other. For example, one study found that D2Rs in the high-affinity, functional state are elevated in ethanol-withdrawn animals (Seeman et al., 2004) while another found an upregulation of D3Rs following chronic ethanol consumption (Vengeliene et al., 2006). More evidence suggests regulation of ethanol withdrawal-associated phenotypes specifically by D2Rs or D3Rs. For example, D2R knockdown increases ethanol drinking and preference, while augmented D2R levels reduces ethanol intake (Thanos et al., 2001, 2004, 2005; but see: Silvestre et al., 1996). Similarly, over-expression of D2R-Short in mice increases the ethanol consumption compared to wild-type control animals (Bulwa et al., 2011). With regards to D3Rs, D3R overexpression increases ethanol intake and conditioned place preference while D3R antagonists and genetic knockdown reduces these measures (Bahi & Dreyer, 2014). It appears as if both D2Rs and D3Rs mediate ethanol intake and are functionally changed by chronic ethanol exposure. An examination of CIE-induced alterations in receptor function at the level of the dopamine terminal would assist in a further understanding of these changes (Chapter III).

5.1. Neurobiological links between autoreceptors and KORs

Some work suggests that D2R/D3Rs and KORs are functionally interactive. For example repeated KOR activation reduced pre- and post-synaptic D2R/D3R expression (Izenwasser et al., 1998) and pretreatment with a KOR agonist reduces the dopamine-decreasing efficacy of D2R/D3R activation (Acri et al., 2011). Notably, pharmacological co-activation of D2R/D3Rs and KORs drive anxiety/compulsive-like ethanol withdrawal phenotypes noted in rodents (Perreault et al., 2006, 2007; Dvorkin et al., 2010; Ballester-Gonzales et al., 2015). A functional interaction between these receptors, particularly at
the dopamine terminal is not known, and may provide evidence to support and novel target for pharmacotherapeutic development. As previous work from our laboratory showed augments D2R-type autoreceptor system sensitivity following CIE exposure, it is also of interest whether alterations in the sensitivity of one or both autoreceptor subtypes mediate observed changes in autoreceptor function (Karkhanis et al., 2015).

5.1a. KORs and D3Rs are upregulated via a similar pathway: Potential role of receptor for activated C kinase 1 (RACK1) and the brain derived neurotrophic factor (BDNF)/tropomysosin receptor kinase B (TrkB) system

Previous work has noted that RACK1 and BDNF/TrkB systems link the KOR/dynorphin systems and D3Rs following ethanol exposure (Yaka et al., 2003; McGough et al., 2004; Jeanblanc et al., 2006; Logrip et al., 2008). For example, acute ethanol increases protein kinase C (PKC) and RACK1 binding, and translocation of RACK1 to the nucleus of the neuron, subsequently augmenting both D3Rs and BDNF levels (Ron et al., 2000; Yaka et al., 2003; McGough et al., 2004; Jeanblanc et al., 2006). D3Rs are inserted in presynaptic dopamine neurons and function as autoreceptors while BDNF is stored and released to activate TrkB receptors, particularly those located in D1R-containing medium spiny neurons (Easton et al., 1999; Yoshii & Constantine-Paton, 2010; Koo et al., 2014). Effectors downstream of TrkB, including MAPK and phospholipase C (PLC; Logrip et al., 2008; Iwakura et al., 2008) results in augmented phosphorylation of CREB, its binding to CRE, and the transcription of prodynorphin and BDNF (Logrip et al., 2008; Tropea et al., 2011). Metabolism of prodynorphin into dynorphin (Day et al., 1998; Berman et al., 2000; Hauser et al., 2005) results in KOR activation and reductions in dopamine release. The effects of chronic ethanol exposure on
this trans-synaptic circuit are still under investigation. Hypotheses developed by Logrip and colleagues (2008) suggest that sustained high levels of BDNF, due to continued ethanol exposure, would reduce TrkB sensitivity to this endogenous ligand. Reductions in presynaptic dopamine release due to CIE exposure and withdrawal (Karkhanis et al., 2015) would also attenuate D1R activation, and, in conjunction with reduced TrkB signaling, downstream products of these receptors, such as prodynorphin. Decreases in dynorphin, through this process would augment KOR sensitivity (Logrip et al., 2008). It is therefore hypothesized that KORs may be functionally upregulated concomitantly with D3Rs (Jeanblanc et al., 2006; Vengeliene et al., 2006; Kissler et al., 2014; Siciliano et al., 2015). This proposed circuit is depicted in Figure 5. Experiments that specifically investigate ethanol-induced alterations in KOR and D3R sensitivity with a measurement of their endogenous ligands and RACK1 in the same brain region may provide insight into this hypothesis.

6.0. Pharmacotherapies indicated for alcohol use disorders

6.1. Disulfiram

Disulfiram (Antabuse®) was approved by the United States’ FDA in 1951 for the treatment of alcoholism (FDA Center for Drug Evaluation and Research, 1951). Disulfiram is an acetaldehyde dehydrogenase inhibitor that reduces the conversion of acetaldehyde in to acetate, both metabolites of ethanol (Heit et al., 2013), following ethanol consumption, thereby elevating plasma and brain levels of acetaldehyde (Gaval-Cruz & Weinshenker, 2009). The rapid increase in plasma acetaldehyde causes negative physiological responses in patients, often referred to as the “Antabuse reaction”, which
(A1) Acute ethanol augments dopamine neuron firing at the level of the soma and augments RACK1 translocation to the nucleus. (A2) These actions augment dopamine release and D3R expression in dopamine terminal boutons, respectively. (A3) Increased levels of dopamine and BDNF increases D1R and TrkB receptor signaling. D1R activation increases adenylyl cyclase activation, cyclic adenosine monophosphate production from adenosine triphosphate, augmented protein kinase A activity and CREB phosphorylation in the nucleus. Activation of TrkB receptors increases phospholipase C, which metabolizes phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol. IP3 increases calcium release from intracellular stores and when paired with diacylglycerol, activates protein kinase C (PKC). PKC phosphorylates/activates CREB after its translocation to the nucleus. (A4) CREB binding to cAMP response element (CRE) on specific promoter regions of the genome augments prodynorphin (PDYN) and BDNF transcription. Once translated into protein, prodynorphin is metabolized by proprotein convertase 2 into dynorphin and further refined via post-translational processing, and stored in dense core vesicles until release. (A5) Upon release, dynorphin and BDNF are released to act on their respective receptors (A6).
includes symptoms such as flushing and sweating, dyspnea, blurred vision, headache, cardiac arrhythmia, vertigo and physical weakness (Substance Abuse and Mental Health Services Administration, Center for Substance Abuse Treatment, 2009). Some studies suggest that ethanol drinking is reduced with disulfiram treatment; however, this effect is observed largely under strict clinical observation (Kristenson, 1992, 1995), and primarily successful in those with strong social support and mild alcohol use disorders (Schuckit, 1985). Although long-term, consistent use of disulfiram appears to be helpful in maintaining ethanol abstinence over placebo controls (Elbreder et al., 2010), the negative symptoms and potentially serious health risks associated with disulfiram treatment in conjunction with relapse drinking behavior lead to poor patient compliance (Suh et al., 2006).

6.2. Acamprosate

Acamprosate (Campral®) was FDA approved for the treatment of alcoholism in 2004 (FDA, 2004). Although the precise mechanism of action of acamprosate is unknown, it is hypothesized that this compound regulates aberrant GABAergic (Daoust et al., 1992; Dahchour & De Witte, 1999) and glutamergic (Dahchour et al., 1998; Dahchour & De Witte, 1999; Hinton et al., 2012) signaling that exist during ethanol withdrawal. Alcoholics who take acamprosate for alcohol use disorders often report severe gastrointestinal disturbances (Paille et al., 1995; Bouza et al., 2004). Acamprosate has been reported as superior in maintaining abstinence in alcoholics (18-61% of patients) compared to placebo-controlled group (4-45%; Boothby & Doering, 2005; Jonas et al., 2014; Higuchi et al., 2015). However, the variability of abstinence success in treatment groups calls into question the precise efficacy of the drug and suggest there may be
individual variability in clinical responses (Boothby & Doering, 2005; Jonas et al., 2014; Higuchi et al., 2015).

6.3. Naltrexone

Of the current pharmacotherapies indicated for alcohol use disorders, naltrexone (Revia®, Vivitrol®) is of particular interest based on its ability to pharmacologically inhibit all three classical opioid receptor subtypes, MORs, DORs and KORs (Bart et al., 2005). As reviewed earlier, clear evidence points to the opposing functional and region-specific effects of MORs and KORs on dopamine transmission, making it unclear what aspects of its pharmacology are important for clinical efficacy (Turchan et al., 1999; Chen & Lawrence, 2000; Saland et al., 2005; Kivell et al., 2014). Notably, naltrexone reduces the reinforcing potency of natural rewards, such as sucrose, a finding that has been demonstrated in monkeys (Williams et al., 1998) and humans (Fantino et al., 1986; Langleben et al., 2012) and has been credited to its antagonistic effects on MORs. Although naltrexone reduces ethanol craving in humans (Kruse et al., 2012), over one-third of all patients taking this drug relapse to alcoholism (Latt et al., 2002). It is therefore possible that a compound that differentially modulates MORs and KORs (Section 4.3) and preserves the reinforcing salience of natural rewards may be a more efficacious pharmacotherapeutic agent to combat alcohol use disorders. Notably, Chapter IV of the present body of work addresses a potential compound, nalmefene, an alcohol drinking harm reduction agent and high-affinity KOR partial agonist (Bart et al., 2005; European Medicines Agency’s Committee for Medicinal Products for Human Use, 2012) on KOR function and accumbal dopamine transmission for this indication.
7.0. Conclusion

Alcohol use disorders are a major health concern in the United States (Centers for Disease Control and Prevention, 2014; Substance Abuse and Mental Health Services Administration, 2014). Current pharmacotherapeutic treatment is relegated to three FDA-approved compounds that have limited efficacy in reducing relapse drinking (Latt et al., 2002; Mann et al., 2004; Jørgensen et al., 2011; Yoshimura et al., 2014; Higuchi et al., 2015). As such, there is a need for novel pharmacotherapeutics and targets to promote the development of agents that reduce the withdrawal effects of ethanol and subsequent relapse drinking behavior. Considerable evidence strongly suggests that increased KOR function may be driving reductions in dopamine signaling in the nucleus accumbens and some of the withdrawal symptoms of ethanol following repeated exposure to ethanol that drive ethanol consumption (Walker & Koob, 2008; Nealey et al., 2011; Walker et al., 2011; Kissler et al., 2014; Kivell et al., 2014). Additional work has suggested that CIE exposure also induces functional alterations in dopamine autoreceptors (Karkhanis et al., 2015). Notably, D3Rs and KORs may be functionally connected (Izenwasser et al., 1998; Acri et al., 2011) via similar upregulation mechanisms (Jeanblanc et al., 2006; Logrip et al., 2008), demonstrating a novel and unexplored relationship between these receptors.

In short, the present body of work aimed to identify the effects of five weeks of CIE exposure on ethanol drinking and anxiety/compulsive-like phenotypes and neurochemistry in male C57BL/6J mice. Behavioral work included an examination of behavior following CIE exposure and KOR blockade. These measures were replicated by KOR activation in naïve mice. Using ex vivo fast scan cyclic voltammetry, dopamine terminal, KOR and autoreceptor function were assessed in slices containing the nucleus
accumbens core following five weeks of CIE. Experiments investigating a functional interaction between KORs and autoreceptors were conducted in naïve mice. The final study shifted the focus of this dissertation from elucidating the functional changes of dopamine terminal and KORs following CIE to examining the effects nalmefene on dopamine terminal and KOR function in efforts to understand its clinical efficacy. In short, data showing a functional link between KORs and D3Rs deserves additional investigation and may provide a novel target for pharmacotherapeutic development. We hope that these data provide evidence to support a role for KOR antagonists and partial agonists in the clinic, specifically to ameliorate the withdrawal effects of alcohol use disorders and reduce relapse drinking in treatment-seeking alcoholics.
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CHAPTER II

SUPERSENSITIVE KAPPA OPIOID RECEPTORS PROMOTE ETHANOL WITHDRAWAL-RELATED BEHAVIORS AND REDUCE DOPAMINE SIGNALING IN THE NUCLEUS ACCUMBENS

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Abstract:

**Background:** Chronic ethanol exposure reduces dopamine transmission in the nucleus accumbens (NAc), which may contribute to the negative affective symptoms associated with ethanol withdrawal. Kappa opioid receptors (KORs) have been implicated in withdrawal-induced excessive drinking and anxiety-like behaviors, and are known to inhibit dopamine release in the NAc. The effects of chronic ethanol exposure on KOR-mediated changes in dopamine transmission at the level of the dopamine terminal and withdrawal-related behaviors were examined.

**Methods:** Five weeks of chronic intermittent ethanol (CIE) exposure in male C57BL/6 mice was used to examine the role of KORs in chronic ethanol-induced increases in ethanol intake and also marble burying, a measure of anxiety/compulsive-like behavior. Drinking and marble burying were evaluated before and after CIE exposure, with and without KOR blockade by nor-binaltorphimine (norBNI, 10mg/kg i.p.). Functional alterations in KORs were assessed using fast scan cyclic voltammetry (FSCV) in brain slices containing the NAc.

**Results:** CIE-exposed mice showed increased ethanol drinking and marble burying compared to controls, which was attenuated with KOR blockade. CIE-induced increases in behavior were replicated with KOR activation in naïve mice. FSCV revealed that CIE reduced accumbal dopamine release and increased uptake rates, promoting a hypodopaminergic state of this region. KOR activation with U50,488H concentration-dependently decreased dopamine release in both groups; however, this effect was greater in CIE-treated mice, indicating KOR supersensitivity in this group.
Conclusions: These data suggest that the CIE-induced increase in ethanol intake and anxiety/compulsive-like behaviors may be driven by greater KOR sensitivity and a hypodopaminergic state of the NAc.
Introduction

Alcoholism is a disease of chronic relapse, consisting of repeated bouts of excessive intake and abstinence, resulting in withdrawal symptoms that contribute to the resumption of heavy drinking. For example, human alcoholics report increased anxiety (Wetterling & Junghanns, 2000) and compulsive behaviors (Suzuki et al., 2002) during abstinence periods, which correlate with escalated ethanol consumption (Wetterling et al., 2006; American Psychological Association, 2013). Examination of these symptoms in a mouse model of alcohol use disorders showed that C57BL/6 (C57) mice exposed to ethanol vapor in a chronic, intermittent (chronic intermittent ethanol, CIE) pattern demonstrate escalated ethanol intake during abstinence (Griffin et al., 2009). Traditional measures of anxiety-like behavior, such as the elevated plus maze and light-dark box, have not found ethanol withdrawal-induced anxiogenic phenotypes in C57 mice (Ghozland et al., 2005; McCool & Chappell, 2015). However, data supporting ethanol withdrawal-related increases in nontraditional anxiety/compulsive-like behaviors, such as marble burying, are emerging. For example, recent work utilizing C57 mice exposed to chronic ethanol injections or an ethanol-containing liquid diet (Perez & De Biasi, 2015) showed increased marble burying during ethanol withdrawal. Marble burying is used to model compulsive-like and anxiety-like behaviors in rodents, and pharmacotherapies for anxiety and obsessive-compulsive disorder reduce this behavior (Nicolas et al., 2006). Thus, marble burying may provide a useful behavioral measure of ethanol withdrawal-associated negative affect in this mouse strain.

Dopamine transmission is markedly attenuated after chronic ethanol exposure (Karkhanis et al., 2015), which may be driving CIE-induced changes in behavior. A
previous study reported reduced dopamine terminal function in the nucleus accumbens (NAc) after three cycles of CIE and 72 hours of abstinence (Karkhanis et al., 2015), indicating a hypodopaminergic state of this region during abstinence. The time-course of these findings are similar to CIE-induced increases in ethanol drinking using a similar protocol (Griffin et al., 2009), suggesting a link between attenuated accumbal function and augmented ethanol intake. As the dopamine system has been implicated as an important regulator of ethanol drinking (Nealey et al., 2011) and anxiety/compulsive-like behaviors (Ballester-González et al., 2015), it is possible that dysregulated dopamine transmission may underlie chronic ethanol-induced increases in these behaviors.

Although dopamine terminal function is attenuated after CIE, the precise mechanism(s) underlying this change are not well understood. Dopamine transmission in the NAc is regulated by a variety of receptors, including kappa opioid receptors (KORs). Intra-accumbal pharmacological activation of KORs reduces dopamine release in this region, while blockade transiently increases extracellular levels of dopamine (Spanagel et al., 1992). Consistent with these data, administration of a KOR agonist in naïve rats (Todtenkopf et al., 2004) increases brain reward thresholds, as measured by intra-cranial self-stimulation, suggesting that KOR activation reduces mesocorticolimbic signaling and produces negative affect. Notably, KORs on mesolimbic dopamine neurons mediate place aversion to KOR agonists (Chefer et al., 2012), supporting a modulatory role of KORs on mesolimbic dopamine system function that influences hedonic state. Further, withdrawal from ethanol (Schulteis et al., 1995) and cocaine (Chartoff et al., 2012) increases brain reward thresholds, an effect which is blocked with KOR antagonists.
(Chartoff et al., 2012). Thus, KOR blockade may attenuate the withdrawal symptoms of ethanol by regulating dopamine transmission.

Of interest to this particular study are data demonstrating the utility of KORs as a potential pharmacotherapeutic target to alleviate the symptoms of ethanol withdrawal. CIE-exposed rats routinely demonstrate increased ethanol self-administration after a period of abstinence compared to air-exposed controls, an effect that is reduced to control levels with systemic (Walker et al., 2011), intracerebroventricular (Walker & Koob, 2008) or intra-NAc (Nealey et al., 2011) administration of nor-binaltorphimine (norBNI), a KOR-specific antagonist. As KOR manipulation alters dopamine system function (Todtenkopf et al., 2004; Zapata & Shippenberg, 2006; Chartoff et al., 2012), and KOR blockade reduces ethanol self-administration in dependent rats (Walker & Koob, 2008; Walker et al., 2011; Nealey et al., 2011), it is possible that changes in KOR function and dopamine transmission drive CIE-induced behavioral changes observed during ethanol abstinence. The present study used repeated cycles of CIE and air exposure to examine the role of KORs in ethanol consumption and marble burying in C57 mice. Additionally, *ex vivo* fast scan cyclic voltammetry (FSCV) in brain slices of the NAc was used to identify CIE-induced changes in dopamine terminal and KOR function.

**Methods and Materials**

**Subjects**

Male C57 mice (65-75 days old, Jackson Laboratories, Bar Harbor, ME) were used for all experiments. Mice were individually housed and maintained on a 12:12 hour light cycle (lights off at 14:00), with a red room light illuminated during the dark cycle. Mice
habituated to housing conditions for one week before experiment start and were provided with standard chow and water *ad libitum*, unless otherwise noted. The Institutional Animal Care and Use Committee at Wake Forest School of Medicine approved all experimental protocols. Animals were cared for according to National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities.

**CIE Exposure: Back-to-Back CIE and Back-to-Back CIE+Drinking Protocol**

Mice were exposed to ethanol vapor or room air for four days (16 hours of exposure + 8 hours of abstinence), followed by 72 hours of room air exposure (one cycle). Cycles were repeated five times. Mice were treated (i.p.) with a solution containing 1.6g/kg ethanol (CIE) or saline (air) and 1.0mmol pyrazole (Sigma Aldrich, St. Louis, MO), an ethanol dehydrogenase inhibitor, 30 minutes prior to inhalation treatment. Blood ethanol concentrations (BEC) were tested after the first and fourth inhalation exposure to ensure physiologically relevant BECs (Griffin *et al.*, 2009; Supplementary Figure 2). The Back-to-Back CIE Protocol (Figure 1A) was modified (Back-to-Back CIE+Drinking Protocol, Figure 1B) to examine CIE-induced changes in ethanol drinking.

**Ethanol Drinking Tests**

Animals in ethanol drinking groups underwent a two bottle choice procedure that included two weeks of sucrose fade (Samson, 1986) consisting of two days each: 10% ethanol/5% sucrose, 12%/5%, 15%/5%, 15%/2%, and 15%/1% (w/v), followed by four weeks of access to 15% (v/v) ethanol. Experimental solutions were provided for two hours per day (13:30-15:30), five days per week, and paired with an identical
Figure 1. Back-to-Back chronic intermittent ethanol (CIE) exposure protocols.

(A) Visual depiction of the Back-to-Back CIE Protocol. Mice were exposed to five cycles of ethanol vapor or room air (green bars). Each cycle consisted of four days of 16 hours of exposure (red bars), 8 hours of abstinence (dark blue bars), followed by 72 hours of room air exposure (orange bar). (B) Visual depiction of the Back-to-Back CIE + Drinking Protocol. Mice underwent a two bottle choice drinking procedure (two weeks of sucrose fade (blue bar) followed by four additional weeks of access to 15% (v/v) ethanol (left purple bar). Both CIE and air groups underwent five cycles of inhalation exposure (green bars) and ethanol drinking was examined following all CIE exposure cycles (right purple bar).
bottle of water. Bottle placement was switched daily. Mice were assigned to inhalation groups, counterbalanced for intake. Ethanol drinking was examined for five days after all cycles (Figure 1B). Separate groups of animals were injected with norBNI (10mg/kg, i.p.; NIDA, Bethesda, MD) or vehicle (injectable water) 24 hours prior to the post-inhalation ethanol drinking test. A separate group of mice was not exposed to the inhalation treatment, but during the final (fourth) week of baseline (15% vs water) drinking, they were injected with saline (0.1mL, i.p.) 30 minutes prior to drinking start time (13:30). Across the following several weeks, mice were injected with saline (0.1mL) on Mondays, Wednesdays and Fridays. In a latin-square design, mice were injected i.p. with 1.0, 3.0, 6.0, and 10mg/kg U50,488 on Tuesdays and Thursdays. Drinking data were collapsed across saline and each dose of U50,488.

**Marble Burying**

Pre-inhalation marble burying tests occurred on the morning of the first day of CIE cycle 1. Mice were placed in a standard polycarbonate cage lined with 4cm of cob bedding (The Andersons, Inc., Maumee, OH) for 120 minutes before the addition of 20 clean, black glass marbles (14mm, Rainbow Turtle, Portland, OR). A template was used to ensure marble placement (Figure 4A, left panel). After the 30-minute testing session, the number of marbles over 75% buried was counted. Water was provided during habituation, but both food and water were unavailable during testing. Animals were placed into inhalation groups, counterbalanced for marbles buried during the pre-test. CIE-induced changes in marble burying were assessed 72 hours after the fifth cycle. Separate groups of mice were systemically injected with norBNI (10mg/kg) or vehicle 24 hours prior to post-inhalation testing. A separate group of mice was injected with saline
(0.1mL) or 3.0mg/kg U50,488, 30 minutes prior to testing, approximately 90 minutes into their habituation session.

**Ex Vivo FSCV**

*Ex vivo* FSCV was used to characterize dopamine kinetics in the NAc core (Karkhanis *et al.*, 2015). Mice were sacrificed 72 hours after the fifth inhalation exposure to coincide with behavioral testing. Mice were anesthetized with isoflurane, rapidly decapitated and brains removed. Brain slices containing the NAc (300µm thick) were prepared with a vibrating tissue slicer and incubated in oxygenated artificial cerebrospinal fluid (aCSF, 32°C). A carbon fiber recording electrode (∼50-100µm length, 7µM radius; Goodfellow Corporation, Berwyn, PA), and a bipolar stimulating electrode (Plastics One, Roanoke, VA) were placed (∼100µm apart) on the surface of the slice. Dopamine efflux was induced with a single, rectangular, 4.0ms duration electrical pulse (350µA, monophasic, inter-stimulus interval: 180sec). To detect dopamine release, a triangular waveform (-0.4 to +1.2 to -0.4V vs. silver/silver chloride, 400V/sec) was applied every 100ms to the recording electrode. To assess the effects of inhalation treatment on KOR function, the KOR agonist, U50,488 (0.01-1.0µM, Tocris Bioscience, Minneapolis, MN), was added cumulatively to aCSF after baseline collections were stable. To obtain clear current versus time plots, background current subtraction methods were utilized. Electrode calibration was performed following each experiment using a flow-injection system.
Data Analysis

Ethanol consumption was calculated by weighing ethanol and water bottles before and after drinking tests. Preference was calculated as the volume of ethanol consumed divided by total intake (ethanol+water) consumed in each session. Marble burying was scored by visual inspection of marbles.

Demon Voltammetry and Analysis software (Yorgason et al., 2011) was used to collect and analyze all voltammetry data. Representative signals were analyzed before drug application to determine dopamine release per electrical stimulation (μM) and uptake (V_{max}, μM/sec). Representative traces after drug application were similarly analyzed. The effects of U50,488 on dopamine release across the concentration response curve are presented as a percent of pre-drug dopamine release.

Graphs were created and statistical tests were applied using GraphPad Prism, Version 5 (La Jolla, CA). Two-way repeated measures (RM) analysis of variance (ANOVA) were used to compare the effects of CIE exposure and drug treatment (norBNI vs. vehicle) on ethanol drinking, preference and marble burying as well as the U50,488 concentration response curve, with drug concentration and inhalation treatment as factors. All drinking data are collapsed across the five-day exposure or drug dose (saline or U50,488). When a significant main effect was found, Bonferroni post hoc analysis was performed. A one-way ANOVA was used to analyze the effects of acute U50,488 on ethanol drinking and preference. When a significant main effect was detected, Tukey’s post-hoc analysis was applied. Two-tailed Student’s t-tests were used to reveal pre-inhalation ethanol drinking and preference differences between inhalation groups, the effects of CIE exposure on
baseline dopamine kinetics, the effect of 3.0mg/kg U50,488 on marble burying and U50,488 IC50.

Results

CIE increased ethanol intake and preference without inter-cycle ethanol drinking.

To confirm that the CIE exposure protocol used here increased ethanol drinking similar to previously used protocols, separate groups of mice underwent a CIE+Drinking protocol, (Griffin et al.,2009; Supplementary Figure 1). BECs were collapsed across CIE exposure protocols and were within the behaviorally relevant range (Griffin et al., 2009; Supplementary Figure 2, 204.9±58.47mg/dl). Two-way ANOVA revealed a main effect of inhalation exposure (Figure 2A, F_{1,23}=6.599, p<0.05) and time (pre vs. post inhalation exposure, F_{1,23}=11.16, p<0.01). Post hoc analysis confirmed similar pre-inhalation ethanol consumption between groups (Air: 2.53±0.33, CIE: 2.60±0.28g/kg, p>0.05), but an increase in ethanol intake in CIE-exposed mice was observed (p<0.001). An interaction between inhalation exposure and time was also detected (F_{1,23}=5.543, p<0.05). As hypothesized, CIE-exposed mice exhibited increased ethanol intake, as a percent of pre-exposure intake levels, compared to controls (Figure 2B: Air: 112.00±14.30%, CIE: 180.30±13.15%, t_{10}=4.49, p<0.01). The amount of ethanol consumed on each day is depicted in Supplementary Figure 3.

Analysis of ethanol preference revealed a main effect of inhalation treatment (Figure 2C, F_{1,22}=9.38, p<0.05) and time (pre vs. post inhalation, F_{1,22}=7.03, p<0.05). Augmented ethanol preference in CIE-exposed mice compared to pre-CIE (p<0.01) and air-exposed animals (p<0.01) were observed with post hoc analysis levels (Air: 0.81±0.02, CIE: 0.83±0.02). A two-tailed Student’s t-test revealed increased ethanol preference in CIE-
Figure 2: CIE-exposed mice demonstrated increased ethanol consumption and preference without inter-cycle ethanol drinking tests.

(A) Pre-inhalation ethanol intake was similar between groups. CIE significantly augmented ethanol drinking in CIE-exposed mice compared to air-exposed control animals and pre-CIE exposure ethanol intake. (B) Ethanol intake was significantly greater in CIE-exposed compared to air-exposed mice when expressed as a percent of pre-inhalation intake. (C) CIE increased ethanol preference in CIE-exposed mice compared to air-exposed control mice and pre-inhalation ethanol preference. (D) Ethanol preference was significantly greater in CIE-exposed mice compared to air-exposed animals. n(air)=6; n(CIE)=7. *$p<0.05$; **$p<0.01$; ***$p<0.001$. 
exposed mice compared to controls when expressed as a percent of pre-inhalation preference (Figure 2D: Air: 103.00±4.69%, CIE: 113.30±2.30%, t\(_{11}\)=3.51, p<0.05).

*KOR blockade reduced ethanol consumption and preference in CIE-treated mice.*

To examine the role of KORs in CIE-induced changes in ethanol drinking, norBNI or vehicle was systemically administered 24 hours prior to the five-day drinking test (Broadbear et al., 1994). Two-way ANOVA revealed a main effect of inhalation (Figure 3A; F\(_{1,28}\)=9.20, p<0.01) and drug treatment (norBNI vs. vehicle) on ethanol intake (F\(_{1,28}\)=28.60, p<0.001). An interaction between inhalation and drug treatment was also found (F\(_{1,28}\)=10.01, p<0.01). Post-hoc analysis confirmed a significant increase in ethanol drinking in CIE-exposed mice treated with vehicle (4.57±0.40g/kg, p<0.001) compared to CIE-exposed mice treated with norBNI (2.23±0.19g/kg). Ethanol drinking in norBNI treated/CIE-exposed mice was similar to intake of norBNI treated/air-exposed (2.27±0.19g/kg, p>0.05).

Examination of ethanol preference after CIE/air exposure revealed a main effect of norBNI treatment (Figure 3B; F\(_{1,27}\)=26.21, p<0.001). Although no main effect of inhalation treatment was detected (F\(_{1,27}\)=1.22, p=0.28), potentially reflecting a ceiling effect due to high baseline preference, an interaction between inhalation and drug treatment (norBNI vs. vehicle) was observed (F\(_{1,27}\)=11.25, p<0.01). Reduced ethanol preference in CIE-exposed mice treated with norBNI (preference ratio: 0.70±0.03) compared to CIE-exposed vehicle-treated animals (0.92±0.015, p<0.001) was found using post hoc analysis, but norBNI treated/CIE exposed mice had similar preferences to air-exposed mice treated with vehicle (0.79±0.02, p>0.05) or norBNI (0.76±0.02,
The effects of inhalation and drug treatment were maintained over the five-day drinking test (Supplementary Figure 3).

**U50,488 increased ethanol consumption and preference**

NorBNI reduced ethanol intake in CIE-exposed animals (Figure 3A, 3B), suggesting that KORs positively modulate drinking. Thus, we hypothesized that KOR activation would augment ethanol consumption and preference in naïve mice. A one-way ANOVA revealed a significant effect of U50,488 on ethanol intake (Figure 3C, $F_{5,46}=18.86$, $p<0.0001$) and preference (Figure 3D, $F_{5,46}=5.903$, $p<0.001$). Tukey’s *post-hoc* analysis revealed a significant difference increase at the 1.0 ($p<0.001$), 3.0 ($p<0.001$) and 6.0 ($p<0.5$) mg/kg doses of U50,488 in ethanol drinking compared to saline drinking. Further, Tukey’s *post hoc* analysis revealed a significant increase in ethanol preference at the 1.0 ($p<0.01$), 3.0 ($p<0.05$) and 6.0 ($p<0.5$) mg/kg doses of U50,488 compared to control (saline). In both datasets (Figure 3C, 3D), *post hoc* analysis showed that drinking in mice given saline or the 10mg/kg dose of U50,488 was similar ($p>0.05$).

**CIE increased marble burying behavior.**

Marble burying was used to examine the effects of CIE or air exposure on anxiety/compulsive-like behaviors. Representative pictures of testing chambers are shown in Figure 4A: before testing (left panel), after five cycles of air exposure (middle panel), and CIE exposure (right panel). A two-way RM ANOVA revealed a main effect of time (pre vs. post inhalation; Figure 4B; $F_{1,14}=4.69$, $p<0.05$). *Post-hoc* analysis showed an increase in marble burying behavior between pre- (9.44±2.32 marbles buried) and post (16.67±0.73 marbles buried) CIE exposure ($p<0.01$). A significant increase in marble burying between post-CIE and post-air exposure inhalation groups (8.86±1.82 marbles
Figure 3: Systemic norBNI administration reduced ethanol intake and preference of CIE-treated mice which was replicated with KOR activation.

(A) CIE significantly augmented ethanol drinking compared to air-exposed control mice, which was reduced with norBNI treatment. (B) Ethanol preference of CIE-exposed mice was significantly greater than air-exposed controls, which was reduced to control levels with norBNI treatment. A+B: n(air/vehicle)=6; n(air/norBNI)=9; n(CIE/vehicle)=7; n(CIE/norBNI)=10. (C) U50,488 increased ethanol intake and (D) preference, which returned to baseline at 10mg/kg. C+D: n(saline)=14; n(1.0mg/kg U50,488)=9; n(3.0mg/kg U50,488)=10; n(6.0mg/kg U50,488)=9; n(10.0mg/kg U50,488)=9. *p<0.05; **p<0.1 ***p<0.001
buried, \( p<0.05 \) and an interaction between inhalation exposure and time (\( F_{1,28}=5.42, p<0.05 \)) were observed.

**KOR blockade reduced CIE-induced increases in marble burying.**

Mice were systemically injected with norBNI or vehicle 24 hours prior to the post-inhalation marble burying test. Two-way ANOVA revealed a main effect of inhalation condition (Figure 4C, \( F_{1,26}=7.35, p<0.05 \)) and drug treatment (norBNI vs. vehicle, \( F_{1,26}=21.74, p<0.001 \)) on marble burying. *Post-hoc* analysis confirmed an increase in marble burying in vehicle treated CIE- vs. air-exposed mice (\( p<0.01 \)). Additionally, marble burying behavior of vehicle treated/CIE-exposed mice was greater than that of CIE-exposed mice treated with norBNI (\( p<0.001 \)). An interaction between CIE exposure and drug treatment was detected (\( F_{1,16}=8.59, p<0.001 \)). NorBNI reduced marble burying in CIE-exposed mice (6.13±1.22 marbles buried) to norBNI-treated air (6.43±1.60 marbles buried) and pre-CIE levels (Figure 4C; \( F_{1,26}=0.01, p=0.93 \)). Additional experiments in naïve mice show that the effects of norBNI on ethanol drinking and marble burying are likely not attributable to changes in locomotion (Supplementary Figure 5).

To confirm that KOR activation would also augment marble burying, naive mice were injected with 3.0mg/kg U50,488, 30 minutes prior to testing. A two-tailed Student’s t-test revealed augmented marbles buried in response to U50,488 administration (Figure 4E, Saline: 12.57±0.649 marbles buried; 3.0mg/kg U50, 488: 16.00±0.723 marbles buried; \( t_{12}=3.526, p<0.01 \)).
Figure 4: CIE exposure increased marble burying behavior which was norBNI-sensitive and was replicated with KOR agonist administration.

(A) Representative pictures of marble configuration: before testing (left panel), after five cycles of air (middle panel) and CIE (right panel) exposure. (B) CIE exposure significantly augmented marble burying compared to air-exposed mice and pre-inhalation marble burying behavior.  (C) CIE augmented marble burying in CIE-exposed compared to air-exposed mice, which was reduced with norBNI treatment. (D) Marble burying following norBNI treatment is similar to pre-inhalation exposure marble burying behavior.  A-D: n(air)=7; n(CIE)=9  (E) KOR activation with a 3.0mg/kg dose of U50,488 significantly augmented marble burying behavior in naïve mice compared to mice treated with a saline challenge. n(saline)=7; n(3.0mg/kg U50,488)=7.  **p<0.01; ***p<0.001.
CIE attenuated dopamine terminal function via reduced dopamine release and increased uptake.

To examine changes in dopamine terminal function after CIE exposure, \textit{ex vivo} FSCV was used. Figure 5A shows representative dopamine release and uptake traces with false color plots (Air: left panel; CIE: right panel). A two-tailed Student’s t-test revealed decreased dopamine release (Figure 5B; Air: 1.15±0.12µM; CIE: 0.80±0.07µM, \(t_{18}=2.687, p<0.05\)) and augmented uptake rate (Figure 5C; Air: 2.02±0.07µM/sec; CIE: 2.42±0.17µM/sec, \(t_{21}=2.20, p<0.05\)) in CIE-exposed mice vs. controls.

CIE increases KOR sensitivity in the NAc core.

\textit{Ex vivo} FSCV was used to examine CIE-induced changes in KOR sensitivity. Two-way RM ANOVA revealed a main effect of CIE treatment on KOR sensitivity, suggesting increased KOR function in CIE-exposed mice compared to controls (Figure 5D; \(F_{1,11}=5.00, p<0.05\)). A main effect of U50,488 on dopamine release (\(F_{4,11}=80.87, p<0.001\)) and an interaction between inhalation treatment and U50,488 concentration were detected (\(F_{4,11}=3.22, p<0.05\)). A reduction in the U50,488 IC\(_{50}\) in CIE- compared to air-exposed mice was found (Figure 5D inset: Air: 3.38±0.27µM, CIE: 2.10±0.22µM, \(t_{7}=3.72, p<0.01\)).
Figure 5: CIE exposure attenuated accumbal dopamine terminal function and increased KOR sensitivity.

(A) Representative voltammetric traces after electrical stimulation of dopamine and associated false color plots (air, left, green; CIE, right, purple). False color plots demonstrate changes in current over time and in response to electrically evoked dopamine release (X-axis: time, Y-axis: command voltage (~0.4 to +1.2 to -0.4V), Z-axis: current). Cyclic voltammograms (insets) identify the peak oxidation (~+0.6V) and reduction voltages (~−0.2V) of dopamine. (B) CIE reduced electrically evoked dopamine release compared to air-exposed mice. (C) CIE augmented dopamine uptake in brain compared to controls. B,C n(air)=9; n(CIE)=11. (D) U50,488 dose-dependently reduced dopamine release across increasing concentrations, an effect was more pronounced in CIE-treated brain slices, reducing its IC50 in CIE-exposed mice (inset). n(air)=6; n(CIE)=7. *p<0.05; **p<0.01.
Discussion

The present work showed that CIE-induced behavioral changes in C57 mice were associated with augmented sensitivity of KORs on dopamine terminals and reduced dopamine transmission in the NAc. Ethanol drinking and preference were increased following a five-week CIE protocol which also increased anxiety/compulsive-like marble burying behavior. FSCV in the NAc showed that CIE reduced dopamine release, increased rates of dopamine uptake, and augmented the inhibitory effects of KORs on dopamine release, promoting a hypodopaminergic state of the NAc. These data suggest that accumbal hypodopaminergia may be partially driven by increased KOR function, leading, at least in part, to the potentiation of ethanol intake and anxiety/compulsive-like behaviors during ethanol abstinence.

CIE exposure increased ethanol consumption, preference and marble burying behavior.

To examine the effects of CIE exposure on ethanol intake, both ethanol consumption and preference were examined using two distinct CIE exposure protocols. Experiments that included five days of ethanol drinking between each CIE cycle (Supplementary Figure 1) replicated previous findings that showed escalated ethanol consumption with sequential bouts of CIE and abstinence (Griffin et al., 2009). These data were similar to the abbreviated Back-to-Back CIE+Drinking protocol, suggesting that CIE exposure alone is sufficient to increase ethanol drinking and preference. Thus, the Back-to-Back CIE exposure protocol was utilized for the remainder of the experiments.

Escalation of ethanol intake is a hallmark of chronic alcohol exposure that has been documented in humans (Wetterling et al., 2006; American Psychological Association,
2013), as well as in monkey (Grant et al., 2008), rat (Walker & Koob, 2008) and C57 mouse (Griffin et al., 2009; present work) models of alcoholism. Because abstinent alcoholics also report increased anxiety (Wetterling & Junghanns, 2000) and compulsive behaviors (Suzuki et al., 2002), a marble burying assay was used to examine CIE-induced changes in anxiety/compulsive-like behaviors in C57 mice. This task is a nontraditional test for anxiety-like behavior, and appears to measure some of the negative affective components of ethanol withdrawal, including anxiety-like and compulsive-like behaviors (Nicolas et al., 2006; Perez & De Biasi, 2015). During ethanol abstinence, CIE-exposed mice exhibited increased marble burying compared to air-exposed controls. Since this CIE procedure also augmented ethanol drinking and preference, it is possible that CIE-exposed mice increase their ethanol intake to reduce the negative reinforcing effects of CIE exposure and withdrawal.

**KOR blockade attenuated CIE-induced increases in ethanol consumption, preference and marble burying.**

After finding CIE-induced elevations in ethanol intake and marble burying, the role of KORs in both behaviors was tested. A single systemic administration of norBNI ameliorated CIE-induced increases in ethanol consumption and preference. This effect was maintained over the five-day test, consistent with reports documenting a long-lasting effect of norBNI (Kishioka et al., 2013). Further, norBNI treatment attenuated CIE-induced increases in marble burying. Additional work in the present study showed that KOR activation with U50,488 increased ethanol drinking and preference in CIE-naïve mice. Moreover, a single dose of U50,488 increased the number of marbles buried, showing increased anxiety/compulsive-like behavior following KOR activation.
Together, these data suggest that KORs play a role in ethanol withdrawal-associated negative affective symptoms and augmented drinking, and suggest that pharmacological blockade of KORs could potentially serve a therapeutic role in withdrawn alcoholics.

Increased anxiety/compulsive-like behavior, opioid receptor signaling and dopamine transmission following prolonged ethanol exposure are strikingly similar to studies examining different sources of negative affective states. In particular, negative affective states engendered by chronic stress or drug exposure have been tied to elevated KOR activity. For example, symptoms of withdrawal from ethanol (Walker et al., 2011; Nealey et al., 2011) or cocaine (Chartoff et al., 2012), social isolation rearing (Karkhanis et al., submitted), social defeat stress (McLaughlin et al., 2006) and separation of pair-bonded prairie voles (Resendez et al., 2012) are rescued with KOR blockade. It is likely that KOR activity may be a ubiquitous mediator of negative affect and stress, although elevated KOR activity is a part of an extensive stress response system that also includes elevated levels of corticotropin releasing factor (CRF, Britton et al., 2000). In fact, the CRF and KOR systems often play similar roles in stress responses (Britton et al., 2000; Lu et al., 2003), which has led to the hypothesis that the CRF- and KOR-systems are functionally linked (Van’t Veer et al., 2012).

**Ethanol and opioids**

Acute ethanol administration increases both endorphin (Olive et al., 2001) and dynorphin levels (Marinelli et al., 2006; Lindholm et al., 2000), activating both mu opioid receptors (MOR) and KORs simultaneously. This would result in opposing effects on the mesolimbic dopamine system, as MOR activation increases, and KOR activation reduces, accumbal dopamine transmission (Spanagel et al., 1992, current study).
Augmented accumbal dopamine levels in response to acute ethanol are due, in part, to increased inhibitory MOR activity on ventral tegmental area GABAergic interneurons, which causes a disinhibition of dopaminergic neurons in this region (Bergevin et al., 2002). Conversely, chronic ethanol exposure reduces MOR activity (Chen & Lawrence, 2000) while augmenting KOR signaling (Kissler et al., 2014, present work), promoting an overall deficiency in reward system function (Koob et al., 2013). The neurochemical changes associated with chronic ethanol exposure may produce negative affective symptoms specifically by increasing KOR sensitivity and reducing dopamine system function. The present data support this hypothesis, as reduced dopamine transmission and increased KOR signaling are associated with augmented anxiety/compulsive-like behavior and ethanol intake after a period of abstinence, and further because KOR blockade reduces CIE-induced changes in behavior to control levels.

Currently, only one opioid receptor antagonist, naltrexone, is approved for alcohol use disorders by the Food and Drug Administration (Center for Substance Abuse Treatment, 2009). Naltrexone is an antagonist of all three classical opioid receptors (MOR, KOR, DOR). Because of the opposing functional effects of MORs, DORs and KORs, the usefulness of a pan-opioid antagonist in treating alcoholics may be reduced, as inhibition of all opioid receptors may decrease the positive reinforcing effects of natural rewards as well as ethanol, thus decreasing drug-taking compliance. For example, in humans, naltrexone decreased reward-related brain activation in response to palatable foods (Murray et al., 2014) as well as overall food (Yeomans & Wright, 1991) and sucrose (Fantino et al., 1986) intake. Additionally, naltrexone produces an aversive state in monkeys (Williams & Woods, 1999), healthy humans and alcoholics (Swift et al.,
Since the present data show that KOR blockade is sufficient to reduce anxiety/compulsive-like behavior and ethanol drinking in CIE-exposed mice to non-dependent levels, a KOR-specific antagonist may be fully efficacious at reducing relapse drinking in the human population while sparing the positive reinforcing salience of natural rewards (Fantino et al., 1986; Murray et al., 2014).

**The effects of CIE exposure on accumbal dopamine transmission**

Chronic ethanol exposure consistently reduces dopamine system function. For example, previous work from our laboratory showed that CIE exposure of mice and rats attenuates dopamine transmission by reducing dopamine release and increasing the rate of dopamine uptake (Budygin et al., 2007; Karkhanis et al., 2015; current work), which is congruent with findings of reduced limbic system function in human alcoholics compared to control subjects (Volkow et al., 2002). These functional changes in dopamine transmission may reflect changes in the expression levels of proteins such as dopamine transporters, tyrosine hydroxylase, vesicular monoamine transporters or other components of the machinery regulating dopamine release and reuptake. Further studies will be needed to define the underlying mechanisms of the functional changes documented here.

**Chronic ethanol exposure and dynorphin levels**

Despite consistent results across laboratories showing increased KOR-system function after chronic ethanol exposure, and the present work demonstrating augmented KOR function at the level of the dopamine terminal, there is controversy regarding the status of dynorphin levels after ethanol exposure. Some studies suggest that acute ethanol
increases dynorphin in the NAc (Marinelli et al., 2006; Lindholm et al., 2000) and that these effects may be long-lasting (Lindholm et al., 2000). Consistent with reduced dopamine system function following CIE, it has been hypothesized that extended exposure to ethanol may desensitize TrkB receptors on D1-containing accumbal GABAergic medium spiny neurons (Logrip et al., 2008), one of the postsynaptic receptors that regulate dynorphin release. This would result in reduced dynorphin levels and supersensitive KORs, as reported here. However, one manuscript demonstrated a transient elevation of prodynorphin levels in the NAc after chronic ethanol exposure (Przewlocka et al., 1997) and another suggested that CIE exposure increased KOR sensitivity and dynorphin levels simultaneously in the central nucleus of the amygdala (Kissler et al., 2014). These data show that the effects of ethanol exposure and withdrawal on dynorphin levels are time-dependent, as Kissler and colleagues (2014) examined dynorphin and KOR levels six hours into withdrawal in rats while Przewlocka et al (1997) found elevated prodynorphin levels 24-48 hours after the final ethanol exposure that returned to control levels by 96 hours. We report increased KOR function 72 hours into ethanol withdrawal in mice, which may or may not coincide with augmented dynorphin levels. These findings suggest that the temporal profile of dynorphin and KOR changes may be important in understanding how chronic ethanol alters KOR signaling.

**Conclusions**

Human alcoholics report relapse to alcohol drinking in an effort to reduce withdrawal symptoms experienced during abstinence (Wetterling et al., 2006). As mounting evidence supports the involvement of accumbal KORs in the development of anxiety/compulsive-
like behaviors and dependence-induced ethanol drinking (Nealey et al., 2011; Ballester-Gonzáles et al., 2015), chronic ethanol-induced reductions in dopamine transmission and increased KOR sensitivity in the NAc may promote the development of negative affective behavioral phenotypes associated with ethanol withdrawal. As such, our work supports a growing body of literature suggesting the potential utility of a KOR-antagonist to rescue chronic ethanol-induced changes in behavior and neurobiology.

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**The Statement of Interest**

None.

**Author Contributions**

JHR, SRJ, BAM, RC, HCB and MFL developed experiments. JHR and DG executed experiments. JHR analyzed and graphed all data. JHR, ANK and SRJ wrote the paper. All authors listed sufficiently contributed to and edited the manuscript before submission.
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CHAPTER II: SUPPLEMENTARY MATERIALS

Methods:

*Ethanol Drinking Tests*

Baseline drinking procedures were established prior to chronic intermittent ethanol (CIE) exposure followed that described in-text. Ethanol drinking was examined for five days between each CIE cycle (“CIE/Air+Drinking Protocol”; Figure S1A). Blood samples for blood ethanol concentration (BEC) determination were collected twice per CIE cycle (see below).

*Blood collection and analysis for BEC determination*

Blood samples of CIE-exposed mice were obtained immediately after removal from the vapor chamber on the mornings after the first and final exposure per cycle. A submandibular venipuncture was performed to collect each blood sample. Samples were collected in microtainer tubes lined with lithium heparin (Becton Dickinson & Company, Franklin Lakes, NJ) before processing. Blood collection volumes did not reach the maximum set forth by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine. For BEC measurement, standards and samples were prepared using a commercially available alcohol dehydrogenase assay (Carolina Liquid Chemistries Corporation, Brea, CA). Five microliters of each blood collection was placed in a clean eppendorf tube with 45μl of trichlorocetic acid solution (Sigma Aldrich, St. Louis, MO), and centrifuged at room temperature for ten minutes (10,000 rev/min). Thirty microliters of the supernatant from each sample was removed and placed into a
separate eppendorf tube with the buffer and enzymatic reagent provided in the alcohol dehydrogenase assay. Each standard and sample was loaded in triplicate into a 96 well pre-read plate before 15-minute incubation at 37\(^{\circ}\)C. Immediately following incubation, the plate was analyzed with SoftMax Pro Software, Version 5 (Molecular Devices Corporation, Sunnyvale, CA).

**Locomotor Analysis**

Changes in locomotor activity after systemic norBNI administration was examined as an additional control to ensure the pharmacological effects of norBNI did not inhibit locomotion. Locomotor activity was assessed via infrared beam breaks in automated locomotor activity monitors (20 cm × 20 cm × 20 cm; Med Associates, St. Albans, VT). 

*(Day 1)* To allow for chamber habituation, naïve mice were placed into activity chambers and monitored for 120 minutes. To desensitize mice to injection stress, mice were injected with saline (0.1 mL) twice, and activity was recorded for 60 minutes after each. Mice were injected with norBNI (10mg/kg) immediately after the second 60 minute recording session. *(Day 2)* Twenty-four hours following, mice were placed in the same chambers for 120 minutes followed by two saline injections. Locomotor activity was measured as distance traveled (in centimeters) and as a percent of each animal’s pre-norBNI second saline injection. The second saline injection on Day 2 (open circles) was plotted relative to the second saline injection on Day 1 (closed circles).

**Data analysis**

Graph Pad Prism (version 5, La Jolla, CA) was used to create figures and analyze all data. Column statistics were run on group BEC data. Two-way repeated measures (RM)
analysis of variance (ANOVA) was used to examine ethanol drinking and preference between inhalation groups over time, and analyze pre- versus post- norBNI effects on locomotion. One way ANOVA was used to detect any effect of time on ethanol drinking in inhalation and drug treatment groups. Student’s t-tests were used to analyze percent pre-norBNI distance traveled.

Results:

CIE Exposure: CIE/Air+Drinking Protocol

CIE exposure followed the in-text description with the addition of a two-bottle choice procedure (15% ethanol and water) for five days after each cycle. Each CIE/Air exposure cycle was followed by one-five-day drinking test (1 cycle). Each cycle was repeated four times. An additional (fifth) inhalation exposure and 72-hour withdrawal period, without the following drinking test, was added for neurobiological testing (Figure S1A). Blood samples were collected twice per inhalation cycle to examine BECs (BEC=200.4 ± 62.76 mg/dL, Figure S1B).

Two-tailed Student’s t-tests confirmed similar pre-inhalation ethanol consumption between groups (Figure S1C: \( t_{14} = 0.4556, \ p > 0.05 \)). RM two-way ANOVA revealed increased ethanol consumption across drinking tests (Figure S1D: \( F_{4, 13} = 11.90, \ p < 0.001 \)), and as a consequence of inhalation treatment (\( F_{1, 13} = 18.64, \ p < 0.001 \)). An interaction between inhalation treatment and drinking test was also detected (\( F_{4, 13} = 2.981, \ p < 0.05 \)). Bonferroni post-hoc analysis revealed increased ethanol intake in CIE-treated mice at the fourth ethanol drinking test (\( p < 0.001 \)).
Mice separated into inhalation groups, matched for ethanol drinking.
Supplementary Figure 1: CIE/Air+Drinking Protocol

(1A) Mice underwent a two bottle choice drinking procedure: two weeks of sucrose fade (Samson, 1986; [blue bar] two days each: 10% ethanol/5% sucrose, 12% ethanol/5% sucrose, 15% ethanol/5% sucrose, 15% ethanol/2%, and 15% ethanol/1% sucrose [w/v]), followed by four additional weeks of access to 15% (v/v) ethanol [left purple bar]. Mice were exposed to ethanol vapor or room air (green bars) for four days (16 hours of exposure [red bars]/8 hours of abstinence [dark blue bars]), followed by 72 hours of room air exposure [orange bar]. Following each inhalation cycle, animals were given two-bottle choice (15% ethanol and water) for five days [purple bars]. The ethanol and air inhalation exposure and five-day drinking test schedule was repeated four times, with a fifth inhalation exposure and 72 hour withdrawal period added for neurobiological testing. (1B) BECs of CIE-treated mice. (1C) Pre-inhalation ethanol consumption was similar between groups, but CIE exposure (1D) increased ethanol consumption across drinking tests, and as a consequence of inhalation treatment. Post-hoc analysis revealed increased ethanol intake in CIE-treated mice at the fourth ethanol drinking test. (S1E) Ethanol preference was similar between inhalation groups, but (1F) increased over weeks, particularly at the fourth drinking test.
Analysis of ethanol preference revealed similarities between inhalation groups prior to treatment (Figure S1E, t\textsubscript{14}=1.574, p>0.05). RM two-way ANOVA showed increased preference over weeks in CIE-treated compared to air-treated mice (Figure S1F: F\textsubscript{4,14}=10.19, p<0.0001) and a main effect of inhalation treatment (F\textsubscript{1,14}=14.67, p<0.01). Bonferroni Post-hoc analysis revealed increased ethanol preference between inhalation groups at the fourth drinking test (p<0.001).

**Blood ethanol concentrations reached physiologically relevant levels**

Column statistics revealed physiologically relevant BECs in CIE-exposed mice in the Back-to-back CIE/Air exposure protocols (204.9 ± 58.47 mg/dL).

![Bar graph showing blood ethanol concentrations](image)

**Supplementary Figure 2: Back-to-back CIE protocol blood ethanol concentrations**

BECs in mice CIE-exposed were physiologically and behaviorally relevant.
Supplementary Figure 3: CIE-induced increases in ethanol drinking and post-norBNI ethanol drinking was stable over the five day drinking test.

To ensure a consistent effect of inhalation exposure and norBNI administration over the five day drinking test, drinking levels from animals systemically administered with norBNI were analyzed. Two-way RM ANOVA revealed a main effect of inhalation and drug treatment ($F_{3,28}=20.92$, $p<0.0001$), as well as an effect of time ($F_{4,28}=3.591$, $p<0.01$) on ethanol consumption over the five day test. An interaction between inhalation/drug treatment and time was also detected ($F_{12,28}=2.007$, $p<0.05$). Bonferroni post hoc analysis revealed a significant difference in ethanol intake between CIE-exposed mice treated with vehicle and CIE-exposed mice treated with nor-BNI ([denoted with asterisks (*)] Day 1: $p<0.01$; Day 2: $p<0.001$; Day 3: $p<0.001$; Day 4: $p<0.01$). A significant difference between CIE-exposed mice treated with vehicle and air-exposed animals treated with vehicle ([denoted with ampersands (&)] Days 2 and 3: $p<0.001$; Day 4: $p<0.01$), and air-exposed mice treated with norBNI ([denoted with carrots (^)] Days 1-3: $p<0.001$; Day 4: $p<0.01$; Day 5: $p<0.05$) were also detected. Using one-way ANOVAs, the effects of time within each treatment group were examined. Data showed that ethanol consumption did not escalate over the five day drinking test in air ($F_{4,25}=0.064$, $p>0.05$) or CIE ($F_{4,30}=2.680$, $p>0.05$) exposed, vehicle treated animals. Similarly, no effect of time was detected in norBNI treated air ($F_{4,40}=0.0212$, $p>0.05$) or CIE ($F_{4,45}=0.0617$, $p>0.05$) exposed animals.
Supplementary Figure 3: CIE-induced increases in ethanol drinking and post-norBNI ethanol drinking was stable over the five day drinking test.

Analysis of inhalation and drug treatment over time revealed increased ethanol intake following CIE-exposure and saline treatment compared to and CIE-exposed mice treated with norBNI (*), differences between CIE-exposed mice treated with saline and aired-exposed animals treated with saline (&), and differences between CIE-exposed mice treated with saline and aired-exposed mice treated with norBNI (^). Ethanol consumption did not escalate over the five day drinking test. ^&p<0.05; **,^^ p<0.01; ***,&&& ^^^ p<0.001.
**NorBNI does not alter locomotor behavior.**

To ensure the effect of norBNI on ethanol drinking and marble burying was not due to absolute changes in locomotion, mice were systemically injected with norBNI (10.0mg/kg) and tested 24 hours for changes in locomotion to a systemic saline injection. Raw data are presented after the second saline injection (see above). Two-way RM ANOVA revealed no difference between pre and post nor-BNI locomotor behavior in response to a saline injection ($F_{1,13}=0.7964$, $p>0.05$). However, an effect of time was detected ($F_{5,13}=37.63$, $p<0.0001$), indicating a habituation of the mice to the locomotor chambers after injection on both days. Data were transformed to analyze within-subject changes in locomotion, and a two-tailed Student’s t-test detected no change in locomotor behavior (inset: $t_{13}=0.6525$, $p>0.05$).

![Graph showing locomotor behavior](image)

**Supplementary Figure 4: norBNI does not affect locomotor behavior.**

Raw data are presented after the second saline injection on Day 1 (closed circles) and Day 2 (open circles). There was no difference between pre- and post- norBNI locomotion to a saline injection, although an effect of time was detected. (Inset) Histogram demonstrating similarities between pre- and post-norBNI injection.
CHAPTER III

FUNCTIONAL INTERACTION BETWEEN DOPAMINE D3 AND KAPPA OPIOID RECEPTORS ON DOPAMINE TERMINALS AND PARALLEL INCREASES IN RECEPTOR SENSITIVITY FOLLOWING CHRONIC ETHANOL EXPOSURE

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Abstract

**Background:** Chronic intermittent ethanol (CIE) exposure reduces dopamine transmission in the nucleus accumbens (NAc) core. D2-type dopamine autoreceptors (D2R, D3R) and kappa opioid receptors (KOR), located on dopamine terminals, inhibit dopamine release, and are supersensitive following CIE, likely contributing to low dopamine activity. D2Rs and D3Rs are differentially modulated by chronic ethanol exposure, although differences in autoreceptor function are not well understood. Further, dopamine autoreceptors have been suggested to functionally associate with KORs, although the location of this link is unknown. Thus, CIE-induced alterations in autoreceptor and KOR system sensitivity, and interactions between these receptor systems were explored.

**Methods:** Male C57BL/6 mice were sacrificed following five weeks of CIE- or air-exposure for NAc slice voltammetry experiments. Concentration response curves (CRC) of the agonists quinpirole (D2R/D3R), sumanirole (D2R) and (+)-PD128907 (D3R) were used to assess CIE-induced alterations in autoreceptor function. KOR sensitivity was measured using U50,488. To probe D2R/D3R and KOR interactions, brain slices from naïve mice were pretreated with the antagonists raclopride (D2R/D3R), L-741,626 (D2R), U99-194 (D3R), SB-02771A (D3R) prior to CRCs of U50,488. Nornalbinaltorphimine was used to block KORs before a (+)-PD128907 CRC.

**Results:** CIE reduced dopamine neurotransmission and augmented KOR and D2R/D3R function. Increased autoreceptor sensitivity was driven selectively by D3Rs. Investigation into autoreceptor-KOR interactions revealed that D3R blockade occluded the dopamine-decreasing effects of KOR activation, without reciprocal effects of the KOR antagonist.
**Interpretation:** As CIE-induced increases in D3R function and D3R blockade reduced the dopamine-decreasing effects of KOR activation, D3Rs may be a promising pharmacotherapeutic target for alcohol use disorders.
Introduction

Considerable evidence points to a marked disruption in dopamine neurotransmission following chronic ethanol exposure, particularly in the nucleus accumbens (Zapata et al., 2006; Zapata & Shippenberg, 2006; Budygin et al., 2007; Karkhanis et al., 2015; Rose et al., 2015). Chronic ethanol-induced reductions in dopamine transmission in this region may be due to attenuated dopamine cell firing (Shen & Chiodo, 1993; Shen et al., 2007, but see: Diana et al., 1995; Brodie, 2002) or deficient terminal function (Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al., 2015; Rose et al., 2015). Several inhibitory receptors found on presynaptic dopamine nerve terminals have been shown to have increased activity following chronic ethanol exposure, including D2-type autoreceptors (D2Rs and D3Rs) and kappa opioid receptors (KOR; Perra et al., 2011; Kivell et al., 2014; Karkhanis et al., 2015; Rose et al., 2015; Siciliano et al., 2015). These data suggest that presynaptic dopamine autoreceptors and, or KORs may be driving chronic ethanol-induced reductions in dopamine transmission following chronic ethanol exposure and withdrawal.

Although CIE exposure functionally augments D2R-type autoreceptor activity (Perra et al., 2011; Karkhanis et al., 2015), it is not known whether alterations in the sensitivity of one or both autoreceptor subtypes mediate this change. For example, one autoradiography study found that D2Rs in the high-affinity, functional state were elevated for at least eight days following cessation of 10 days of twice-daily injections of ethanol in Sprague-Dawley rats (2.0 g/kg; Seeman et al., 2004); however, this study used sulpiride, a D2R/D3R antagonist, for binding studies, indicating that D3Rs may also be upregulated during ethanol withdrawal. Some evidence points to a specific involvement
of D3Rs in alcohol use disorders. For example, upregulated D3R mRNA levels following chronic ethanol consumption following one year of oral ethanol consumption in Wistar rats (Vengeliene et al., 2006), and increased D3Rs have been observed in abstinent alcoholics (Erritzoe et al., 2014). As these studies are variable in their ethanol exposure paradigms and receptor measures, an examination of CIE-induced alterations in both D2R and D3R function at the level of the dopamine terminal using a single protocol would assist in a further understanding of these changes.

Similar to chronic ethanol-induced alterations in D3R levels (Vengeliene et al., 2006; Erritzoe et al., 2014), some studies suggest augmented KOR sensitivity following chronic ethanol exposure and withdrawal (Kivell et al., 2014; Rose et al., 2015; Siciliano et al., 2015), and some evidence suggests a functional relationship between dopamine autoreceptors and KORs. In fact, repeated KOR activation reduced pre- and post-synaptic D2R/D3R expression (Izenwasser et al., 1998) and function (Acri et al., 2011). However, it is possible that this effect is a post-synaptic phenomenon or mediated by D2Rs or D3Rs. Notably, pharmacological co-activation of D2R/D3Rs and KORs drive anxiety/compulsive-like ethanol withdrawal phenotypes noted in rodents (Perreault et al., 2006, 2007; Dvorkin et al., 2010; Ballester-Gonzales et al., 2015). In sum, these findings suggest that simultaneous activation of autoreceptors and KORs may produce unique alterations in dopamine signaling via a functional connection. However, it is not known which of the dopamine autoreceptors, D2Rs or D3Rs, may be involved in KOR interactions.

The present study used ex vivo fast scan cyclic voltammetry (FSCV) to identify specific changes in D2R, D3R and KOR sensitivity following five weeks of CIE or air
exposure in C57BL/6J (C57) mice. To identify potential functional interactions between D2-type dopamine autoreceptors and KORs, experiments using blockade of one receptor and activation of others were executed in naïve mice. Overall, these investigations revealed that D3Rs, but not D2Rs, and KORs are functionally upregulated following CIE, and that D3Rs and KORs on presynaptic dopamine terminals are functionally linked in the nucleus accumbens core of C57 mice.

Methods

Subjects

Male C57 mice (65-75 days old; Jackson Laboratories, Bar Harbor, ME) were used for all experiments. Mice were individually housed and maintained on a 12:12 hour light cycle (lights on: 02:00) with a red room light illuminated during the dark hours. Animals were allowed at least one week to acclimate to housing conditions before experiment start. Mice were provided with standard rodent chow and water ad libitum. Chow from CIE-exposed cages was replaced the morning following exposure. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine. Mice were cared for according to the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care.

CIE exposure protocol

CIE exposure procedures followed those published previously (Rose et al., 2015). Briefly, C57 mice were exposed to five cycles of ethanol vapor (CIE) or air, each
consisting of four days (16 hours of exposure/8 hours of abstinence) and 72 hours of room air (one cycle). Cycles were repeated five times. All CIE-exposed animals were treated with 1.6g/kg ethanol (saline in air-exposed mice) and 1.0mmol pyrazole (Sigma Aldrich, St. Louis, MO), an ethanol dehydrogenase inhibitor, 30 minutes prior to chamber start time (17:00). Blood ethanol concentrations (BEC) were tested the mornings after the first and fourth night of ethanol exposure per cycle, at chamber stop time (09:00).

**Blood collection and BEC analysis**

A submandibular vein blood draw used to collect no more than 15µL of blood from one to two mice per BEC collection day. BECs were determined with a commercially available alcohol dehydrogenase assay, derived from a standard calibration curve that was made fresh from reagents provided in the kit (Carolina Liquid Chemistries Corporation, Brea, CA).

**Ex vivo FSCV concentration response curves**

The use of *ex vivo* FSCV allowed for examination of dopamine terminal function and receptor sensitivity on a sub-second timescale (Ferris *et al*., 2013). Using this technique, pre-drug dopamine release and uptake kinetics, as well as effects of D2R, D3R and KOR activation and blockade on dopamine transmission in the nucleus accumbens, were examined 72 hours after the cessation of five cycles of CIE or air exposure. Mice were anesthetized with isoflurane until unresponsive and rapidly decapitated. Brains were removed and placed into ice-cold oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (126), KCl (2.5), NaH₂PO₄ (1.2), CaCl₂ (2.4), MgCl₂ (1.2),
NaHCO₃ (25), glucose (11), L-ascorbic acid (0.4) and pH was adjusted to 7.4. Brain slices containing the nucleus accumbens core were prepared (300µm thick) with a vibrating tissue slicer and incubated in oxygenated aCSF, heated to 32°C, until experiment start. A carbon fiber recording electrode (≈30-50µm length, 7µm radius; Goodfellow Corporation, Berwyn, PA), and a bipolar stimulating electrode (Plastics One, Roanoke, VA) were placed in close proximity (≤100µm) on the surface of the slice. A single, rectangular, 4.0ms long electrical pulse (350µA, monophasic; inter-stimulus interval: 180sec) was used to elicit endogenous dopamine release. The concentration of dopamine released was detected by applying a triangular waveform (-0.4 to +1.2 to -0.4V vs. silver/silver chloride, 400 V/sec) every 100ms to the recording electrode. Background current subtraction methods were utilized to obtain clear current versus time plots. Calibration of recording electrodes was performed following each experiment using a flow-injection system and a known concentration of dopamine (3.0µM).

When pre-drug collections were stable for three consecutive stimulations, quinpirole (0.01-0.3µM), a D2R/D3R agonist, sumanirole (0.01-1.0µM), a D2R-specific agonist, (+)-PD 128907 (0.01-1.0µM), a D3R-specific agonist or U50,488 (0.01-1.0µM), a KOR agonist was added cumulatively to bath solution (aCSF). To explore the possibility of a functional interaction between D2R/D3Rs and KORs, accumbal brain slices from naïve mice were incubated in raclopride (100nM), a D2R/D3R antagonist, L-741,626 (30nM), a D2R-specific antagonist, U99-194 (2,3-Dihydro-5,6-dimethoxy-N, N-dipropyl-1H-inden-2-amine maleate, 1.0µM) or SB-02771A (N-[trans-4-[2-(6-Cyano-3,4-dihydro-2(1H)-isoquinolinyl)ethyl]cyclohexyl]-4-quinolinecarboxamide dihydrochloride, 300nM), both D3R antagonists, or nor-binaltorphimine (norBNI, generously provided by NIDA,
Bethesda, MD 1.0µM), a KOR antagonist, for 60 minutes prior to the addition of cumulative concentrations of either U50,488 (0.01-1.0µM), on D2R or DR3 antagonist-treated slices or (+)-PD 128907 (0.01-1.0µM), on norBNI pretreated slices. All compounds were obtained at the highest quality from Tocris Bioscience (Bristol, UK), unless otherwise noted.

**Data Analysis**

Collection and analysis of all FSCV data was completed with Demon Voltammetry and Analysis software (Yorgason *et al.*, 2011). To determine CIE exposure-induced changes in dopamine release and uptake, representative signals were analyzed before drug application. Dopamine release (µM) was quantitated as the amount of dopamine released per electrical stimulation and dopamine clearance was calculated as the maximal rate of uptake at the dopamine transporter ($V_{\text{max}}; \mu\text{M/sec}$). The effect of each receptor on dopamine release was calculated as a percent pre-drug dopamine release. In experiments where brain slices were pretreated with an antagonist, effects of subsequent agonist treatment were analyzed as a percent of dopamine release after antagonist, and the effects of antagonist on dopamine release were quantitated and compared to pre-drug dopamine release.

All graphs were created and statistical tests were applied using GraphPad Prism, Version 5 (La Jolla, CA). Effects of CIE exposure on dopamine release, U50,488 IC$_{50}$ and antagonist-induced alterations in dopamine release were analyzed with two-tailed Student’s t-tests. All concentration response curves were examined with repeated measures (RM) two-way analysis of variance (ANOVA), with inhalation exposure or
antagonist pre-treatment and drug concentration as factors. When a significant main effect was found, Bonferroni post-hoc analysis was applied.

Results

**CIE exposure promotes a hypodopaminergic state of the nucleus accumbens core.**

*Ex vivo* FSCV was used to examine the effects of CIE and air exposure on dopamine release and uptake in the nucleus accumbens core. Analysis of group data with two-tailed Student’s t-test replicated previous work from our lab (Karkhanis et al., 2015; Rose et al., 2015), showing attenuated dopamine release ($t_9=3.487, p<0.01$) and increased uptake rates ($t_9=3.252, p<0.01$) in brain slices collected from CIE-exposed mice compared to those from air-treated controls (data not shown).

**CIE exposure selectively increased the sensitivity of D3Rs to agonist**

Functional changes in D2R and D3R autoreceptors following five cycles of CIE exposure were first investigated with quinpirole, a D2R/D3R agonist. A two-way RM ANOVA, with inhalation treatment and quinpirole concentration as factors, revealed a significant effect of quinpirole ($F_{3,8}=45.63, p<0.0001$) and inhalation treatment ($F_{1,8}=7.377, p<0.05$) on dopamine release, although no interaction between these factors was detected ($F_{3,8}=0.596 p>0.05$).

Sumanirole, a D2R-specific agonist, and (+)-PD 128907, a D3R-specific agonist, were used to examine the function of each receptor after CIE exposure. A two-way RM
Figure 1: Increased accumbal D2R/D3R sensitivity in CIE-exposed mice was driven by D3Rs.

(A) Autoreceptor sensitivity is greater in CIE exposed mice compared to air-treated controls. Receptor-specific ligands were used to discern the receptor(s) driving this effect. (B) Increased autoreceptor sensitivity was not driven by D2Rs, but (C) likely driven by D3Rs, as evidenced by augmented sensitivity of this autoreceptor subtype following CIE. *p<0.05
ANOVA examining the effects of D2R activation on dopamine release showed that sumanireole concentration-dependently reduced dopamine release (Figure 1B, $F_{4,8}=54.74$, $p<0.0001$), although this effect was similar between inhalation groups ($F_{1,9}=0.078$, $p>0.05$). Similarly, a two-way RM ANOVA revealed significant dopamine-decreasing effects of D3R activation (Figure 1C, $F_{4,9}=34.51$, $p<0.0001$), although this effect was greater in brain slices from CIE exposed mice ($F_{1,9}=16.20$, $p<0.01$). An interaction between these factors was not detected ($F_{4,9}=0.57$, $p>0.05$). Bonferroni post hoc analysis revealed a significant difference between CIE and air-treated mice at the 0.03µM concentration of (+)-PD 128907 ($p<0.05$).

**CIE increased accumbal KOR sensitivity to agonist treatment compared to air-exposed controls**

To examine the effects of CIE exposure on KOR function, increasing concentrations of U50,488, a KOR-specific agonist, were added to brain slices containing the nucleus accumbens core. A two-way RM ANOVA, with drug concentration and inhalation treatment as factors, revealed the concentration-dependent reduction of dopamine release by U50,488 in brain slices from both groups (Figure 2A, $F_{4,10}=76.56$, $p<0.0001$), which was significantly greater in the CIE exposed mice ($F_{1,10}=75.89$, $p<0.0001$). An interaction between inhalation treatment and U50,488 concentration was also detected ($F_{4,10}=3.910$, $p<0.01$). Bonferroni post hoc analysis revealed a significant difference in dopamine release between inhalation groups at the 0.03µM ($p<0.05$), 0.1µM, 0.3µM and 1.0µM ($p<0.0001$) U50,488 concentrations. Two-tailed Student’s t-test of the IC$_{50}$ of U50,488 revealed a significant reduction in the IC$_{50}$ of CIE-treated mice (Figure 2B, $t_9=3.065$, $p<0.05$).
Figure 2: Augmented KOR function in the NAc of CIE-exposed mice compared to air-exposed controls.

(A) KOR activation more effectively reduces dopamine release in brain slices harvested from CIE-exposed mice compared to air-exposed controls. (B) IC\(_{50}\) analysis of the effects of KOR activation on dopamine release showed an increased potency of U50,488 in brain slices from CIE-exposed animals, underscoring the augmented efficacy of KOR activation on dopamine release in this group. *\(p<0.05\); ***\(p<0.001\).
\(p<0.05\), underscoring the increased potency of the drug in this group observed in Figure 2A.

**D2R/D3R blockade attenuated the dopamine-decreasing effects of KOR activation**

To investigate the possibility of a functional interaction between autoreceptors and KORs, a separate group of brain slices from naïve mice were incubated in raclopride, D2R/D3R antagonist, for 60 minutes prior to probing KOR function with increasing concentrations of U50,488. Raclopride had no effect on baseline dopamine release, as confirmed by a Student’s t-test between these two points (\(t_9=0.474, p>0.05\), data not shown). A two-way RM ANOVA revealed the dopamine-decreasing effects of U50,488 (Figure 3A, \(F_{5,9}=8.730, p<0.0001\)) which were blocked in slices pretreated with raclopride (\(F_{1,9}=8.368, p<0.05\)). An interaction between pretreatment (raclopride vs aCSF) and U50,488 concentration was also detected (\(F_{5,9}=4.060, p<0.01\)). Bonferroni post hoc analysis revealed a significant difference between brain slices pretreated with raclopride and non-pretreated brain slices at the 0.3µM (\(p<0.05\)) and 1.0µM (\(p<0.001\)) U50,488 concentrations. These data suggest a functional interaction between autoreceptors and KORs.

**Reduced effect of KOR activation by D2R/D3R blockade was not mediated by D2Rs.**

To identify whether the raclopride-induced attenuation of U50,488 effects on dopamine release were specifically driven by D2Rs, additional brain slices were incubated in L-741,626, a D2R-specific antagonist, for 60 minutes prior to the U50,488
Figure 3: D2R/D3R blockade attenuated the dopamine-decreasing effects of KOR activation

To investigate a functional relationship between KORs and autoreceptors, occlusion experiments targeting each receptor were executed. (A) Raclopride attenuated the dopamine-decreasing effects of U50,488. (B) L-741,626 did not influence the effects of U50,488 on dopamine release. *p<0.05; ***p<0.001.
concentration response curve. Per a Student’s t-test, L-741,626 had no effect on pre-drug dopamine release ($t_{10}$=0.338, $p$>0.05, data not shown). U50,488 significantly decreased dopamine release across the concentration response curve in both groups (Figure 3B, $F_{5,10}$=49.11, $p$<0.0001), which was not influenced by L-741,626 pretreatment ($F_{1,10}$=0.023, $p$>0.05), suggesting that the attenuation of KOR-activation on dopamine release observed following raclopride pretreatment was not mediated via D2Rs.

**D3 blockade reduced the dopamine-decreasing effects of KOR activation**

To investigate a functional interaction between KORs and D3Rs, U99-194, a D3R-specific antagonist, was applied to slices for 60 minutes prior to the U50,488 concentration response curve. U99-194 did not alter baseline dopamine release ($t_{10}$=1.440, $p$>0.05, data not shown). A two way RM ANOVA showed that U50,488 reduced dopamine release in both pretreatment groups (Figure 4A, $F_{5,10}$=40.99, $p$<0.0001), although this effect was significantly attenuated in brain slices pretreated with U99-194 ($F_{1,10}$=36.92, $p$<0.0001). A significant interaction between these factors was also detected ($F_{5,10}$=3.152, $p$<0.05). Bonferroni post hoc analysis revealed significant differences between U99-194 pretreated and non-pretreated brain slices at the 0.03µM, 0.1µM, 0.3µM and 1.0µM ($p$<0.001) concentrations.

Data shown in Figure 4A suggest a functional interaction between D3Rs and KORs. In confirmation, a separate group of brain slices were incubated in SB-02771A, a molecularly dissimilar D3R-specific antagonist from U99-194. SB-02771A had no effect on baseline dopamine release ($t_{10}$=0.887, $p$>0.05, data not shown). A two-way RM ANOVA revealed U50,488 concentration-dependent reductions in dopamine release
Figure 4: D3 blockade reduced the dopamine-decreasing effects of KOR activation

To identify the effects of D3R blockade on KOR function, two molecularly dissimilar D3R antagonists were bath applied to brain slices prior to KOR probe. (A) U99-194 reduced the dopamine-decreasing effects of U50,488. (B) SB-02771A also reduced the dopamine-decreasing effects of KOR activation. (C) KOR blockade also did not alter the dopamine-decreasing effects of D3R activation. *p<0.05; ***p<0.001.
(Figure 4B, $F_{5,10}=71.35, p<0.0001$), which was significantly attenuated by SB-02771A pretreatment ($F_{1,10}=7.174, p<0.05$). An interaction between drug pretreatment and U50,488 concentration was also detected ($F_{5,10}=3.839, p<0.01$). Bonferroni post-hoc analysis revealed significant differences in dopamine release between pretreatment groups at the 0.03µM and 1.0µM ($p<0.05$) U50,488 concentrations.

*KOR blockade did not attenuate the effects of D3R activation on dopamine release.*

As D3R blockade attenuated the dopamine-decreasing effects of KOR activation, we next blocked KORs across a D3R agonist concentration response curve. KOR blockade had no effect on baseline dopamine release ($t_{7}=1.092, p>0.05$, data not shown). Although (+)-PD 128907 reduced dopamine release in both groups (Figure 4C, $F_{5,7}=49.75, p<0.0001$), there was no effect of norBNI pretreatment on D3R activation ($F_{1,7}=4.951, p>0.05$).

**Discussion**

Although chronic ethanol exposure and withdrawal functionally downregulate the mesolimbic dopamine system in mice (Zapata et al., 2006; Karkhanis et al., 2015), rats (Carroll et al., 2006; Budygin et al., 2007) and humans (Volkow et al., 1996, 2007), the underlying causes that produce this change are not fully understood. The present series of experiments sought to identify specific changes in D2R, D3R and KOR sensitivity to agonist following five weeks of CIE exposure in C57 mice and explore a functional relationship between these receptors. We showed that D3Rs, but not D2Rs, and KORs were more sensitive in brain slices from CIE exposed animals compared to control mice.
An investigation into whether autoreceptors and KORs were functionally associated revealed that D3R blockade attenuated the dopamine-decreasing effects of KOR activation, however the converse, blocking KORs, did not reduce the inhibitory effects of D3R agonists on dopamine release. As D3Rs are functionally hypersensitive to agonist following CIE, and D3R blockade obliterates the dopamine-decreasing effects of KOR activation, it is plausible that D3Rs may be a promising target in alcohol use disorders.

**CIE-induced increases in D3R and KOR sensitivity**

Recently, we showed that quinpirole, a non-selective D2/D3 dopamine receptor agonist, reduced dopamine release to a greater extent in brain slices from CIE-exposed mice than their air-exposed counterparts (Karkhanis et al., 2015). To ascertain whether this effect was driven by D2Rs or D3Rs, we probed each receptor with a more selective agonist. We found augmented sensitivity of D3Rs, but not D2Rs, after CIE exposure, suggesting that CIE exposure-induced increases in autoreceptor function (Karkhanis et al., 2015; present work) are driven exclusively by D3Rs. Increased D3R sensitivity may reflect an increase in the number of receptors on dopamine terminals. Consistent with this hypothesis, chronic ethanol consumption produced an increase in D3R mRNA (Vengeliene et al., 2006) and receptor (Jeanblanc et al., 2006) levels in the striatum of mice. Notably, systemic treatment with a D3R antagonist reduced ethanol intake in a model of alcohol relapse (Vengeliene et al., 2006), and genetic D3R deletion (Bahi & Dreyer, 2014; Leggio et al., 2014) or pharmacological blockade (Leggio et al., 2014) reduced ethanol intake in naïve mice. Together, these data suggest that increased D3R activity may contribute to excessive ethanol drinking, and indicate that D3R antagonists
may provide a promising pharmacotherapeutic target to treat alcohol use disorders, although the influence of D3R ligands specifically on alcohol use disorders in the human condition has not been directly explored in humans (Takaki & Ujike, 2013).

**A functional association between D3Rs and KORs**

Some evidence suggests a functional relationship between autoreceptors and KORs. In fact, repeated KOR activation reduces expression (Izenwasser et al., 1998) and function (Acri et al., 2011) of D2R/D3Rs. Further, pharmacological co-activation of D2R/D3Rs and KORs promotes the development of anxiety/compulsive-like checking behavior in rats (Perreault et al., 2006, 2007; Dvorkin et al., 2010; Ballester-Gonzales et al., 2015). Therefore, a functional connection between receptors may exist and alter accumbal dopamine signaling to drive this behavioral phenotype (Dvorkin et al., 2010; Ballester-Gonzales et al., 2015). To explore a functional interaction between D3Rs and KORs, we used two pharmacologically distinct D3R antagonists, and found that D3R blockade occluded the dopamine-decreasing effects of KOR activation. We hypothesize that KOR blockade did not reduce D3R sensitivity to agonist for two primary reasons: First, the D3R/KOR interaction may be an ordered effect, such that pharmacological manipulation of D3Rs must occur before there is a measurable effect on KOR function. This hypothesis posits that D3Rs and KORs may be physically interacting, and an allosteric change in D3Rs due to antagonist binding (Lane et al., 2013) may cause a subsequent conformational shift in the associated KORs that could occlude proper KOR agonist binding. Second, it is possible that D3Rs and KORs do not physically interact, but their signaling cascades may interact. As both receptors are Ga<sub>16</sub>-coupled, D3Rs and KORs share similar intracellular signaling molecules (Ahlgren-Beckendorf & Levant,
Ligands that target specific downstream pathways of G-protein coupled receptors are informative experimental tools and may be helpful in additional investigation of this hypothesis (Zhou et al., 2013; Lovell et al., 2015).

**D3R-KORs may be simultaneously upregulated via receptor for activated C

kinase 1 (RACK1)**

Our voltammetric data show augmented sensitivity of both D3Rs and KORs to agonist following CIE exposure. It is possible that this parallel increase in receptor sensitivity is due to an intracellular protein kinase C (PKC) scaffolding protein, RACK1. In an elegant series of papers, Dorit Ron’s laboratory has described the activating effects of ethanol administration on RACK1 and the results of this activation on D3R regulation and brain derived neurotrophic factor (BDNF)/tyrosine receptor kinase B (TrkB) signaling (McGough et al., 2004; Jeanblanc et al., 2006; Logrip et al., 2008). Briefly, acute ethanol exposure increases RACK1 levels and translocation to the nucleus (Ron et al., 2000; McGough et al., 2004). In the nucleus, RACK1 phosphorylates cAMP response element-binding protein (CREB; Wan et al., 2009) and increases the transcription of downstream genetic products. One of the results of this cascade is augmented D3R expression and BDNF levels in the striatum (Jeanblanc et al., 2006; Logrip et al., 2008). Activation of TrkB receptors by BDNF, particularly on postsynaptic dopamine D1 receptor-containing medium spiny neurons (Strömberg & Humpel, 1995; Koo et al., 2014), drives increased prodynorphin production. Further, while acute ethanol exposure increases dynorphin levels (Lindholm et al., 2000; Marinelli et al., 2006), Logrip and colleagues (2008) suggested that a sustained increase in BDNF levels, due repeated increases in RACK1 over continued ethanol exposures, would reduce TrkB sensitivity. In
conjunction with reduced D1R activation from attenuated dopamine transmission following CIE exposure (Karkhanis et al., 2015; Rose et al., 2015), this would attenuate subsequent downstream signaling and dynorphin release (Logrip et al., 2008), resulting in a compensatory increase in KOR sensitivity as seen here (present work).

To evaluate one aspect of this hypothesis, we measured the levels of dynorphin in CIE treated mice, our laboratory utilized a dynorphin enzyme-linked immunosorbent assay (ELISA) of accumbal tissues from C57 mice exposed to five weeks of CIE. Levels of dynorphin in nearly all CIE-exposed animals were below the detectable range of the kit (>1.0 picogram), whereas air-exposed samples were largely within range (data not shown). As such, a statistical assessment of CIE-induced alterations in dynorphin was not possible, although we anecdotally suggest that five weeks of CIE exposure reduces dynorphin levels in the nucleus accumbens of C57 mice. Taken together, we hypothesize that the changes shown here may be due to ethanol-induced upregulation of RACK1 signaling that drives increased D3R expression and BDNF production, and ultimately reduces dynorphin levels and increases the sensitivity of KORs on dopamine terminals (McGough et al., 2004; Jeanblanc et al., 2006; Logrip et al., 2008).

**Summary and Conclusions**

Converging cross-species evidence has shown that chronic ethanol exposure and withdrawal attenuates dopamine terminal function (Volkow et al., 1996, 2007; Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al., 2015; present work), and increased D2-type receptor (Karkhanis et al., 2015; present work) and KOR sensitivity to agonist (Kissler et al., 2004; Rose et al., 2015; Siciliano et al., 2015; present work). Evidence presented here has identified CIE-induced increases in D3R function, in addition to
augmented KOR sensitivity, furthering our current understanding of autoreceptor changes following chronic ethanol and withdrawal. Moreover, we have identified a functional interaction between these receptors, which may be due to a physical coupling between receptors or connection between downstream signaling pathways. Based on the present observation that D3Rs are upregulated following CIE exposure and blockade of these receptors obliterates the dopamine-decreasing effects of KOR agonists, pharmacotherapeutics targeting these receptors, namely D3Rs, may restore appropriate dopamine transmission and alleviate some of the negative reinforcing effects of chronic ethanol use.

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**Conflict of Interest**

The authors declare no conflict of interest.
References


CHAPTER IV

DISTINCT EFFECTS OF NALMEFENE ON DOPAMINE TERMINAL FUNCTION AND KAPPA OPIOID RECEPTOR ACTIVITY IN THE NUCLEUS ACCUMBENS FOLLOWING CHRONIC INTERMITTENT ETHANOL EXPOSURE

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Abstract

The development of pharmacotherapies aimed at reducing relapse to ethanol drinking in alcoholics is of considerable research interest. Preclinical data support a role for nucleus accumbens (NAc) kappa opioid receptors (KOR) in chronic intermittent ethanol (CIE) exposure-induced increases in ethanol intake. Nalmefene, a high-affinity KOR partial agonist reduces heavy alcohol drinking in at-risk patients, potentially due to its effects on accumbal KORs; however, the effects of nalmefene on dopamine transmission and KOR function in the NAc are not well understood. The present study investigated the effects of nalmefene on dopamine transmission and KOR function in accumbal brain slices from male C57BL/6J mice following five weeks of CIE or air exposure using fast scan cyclic voltammetry. Nalmefene concentration-dependently reduced dopamine release similarly in both inhalation groups, but attenuated dopamine uptake rates to a greater extent in slices from CIE-exposed mice compared to controls. Similarities in nalmefene-induced reductions in dopamine release suggest that dyno7rphin tone is not present in brain slices, and the distinct effects of nalmefene on uptake rates suggest that dopamine transporter interactions with KORs may be fundamentally altered following CIE. Further, we found that nalmefene reversed the dopamine-decreasing effects of a maximal concentration of a KOR agonist selectively in brain slices of CIE-exposed mice, suggesting that CIE-exposure may alter the number or activity of KORs in the presence of an agonist. Modulation of dopamine transmission via KORs by nalmefene may attenuate CIE-induced reductions in dopamine transmission in vivo, and attenuate withdrawal-induced increases in ethanol consumption.
1.0 Introduction

Chronic alcohol (ethanol) use disorders are an enormous economic and financial burden in the United States (Substance Abuse and Mental Health Services Administration, 2014). A large body of literature across many species has shown that chronic alcohol exposure downregulates dopamine transmission in the nucleus accumbens (mice: Karkhanis et al., 2015; rats: Carroll et al., 2006; Budygin et al., 2007; humans: Volkow et al., 1992, 2006), potentially leading to the negative affective states experienced during withdrawal (Schulteis et al., 1995; Boutros et al., 2014). One of the potential candidates that underlies attenuated dopamine neurotransmission following chronic ethanol exposure and withdrawal is increased function of inhibitory receptors on dopamine terminals in the nucleus accumbens (Karkhanis et al., 2015; Rose et al., 2015; Siciliano et al., 2015), namely kappa opioid receptors (KOR; Kissler et al., 2014; Rose et al., 2015; Siciliano et al., 2015). Augmented accumbal KOR function may mediate CIE-induced reductions in dopamine terminal function and upregulated ethanol consumption following ethanol exposure and withdrawal (Walker & Koob, 2008; Nealey et al., 2011). In fact, intra-accumbal KOR blockade reduces relapse-drinking behavior in rodents (Nealey et al., 2011). These data suggest that KORs may be a promising pharmacotherapeutic target to reduce relapse drinking in human alcoholics. With no highly efficacious pharmacotherapeutic agent for alcoholism (Jørgensen et al., 2011; Yoshimura et al., 2014; Mann et al., 2004; Higuchi et al., 2015; Latt et al., 2002; O’Malley et al., 2015; Garbutt et al., 2005), considerable research efforts have been put forth to gather evidence to support novel pharmacotherapies for this indication,
particularly those that target KORs (Walker & Koob, 2008; Nealey et al., 2011; Walker et al., 2014).

Nalmefene (6-methylene naltrexone) is a pharmacotherapeutic agent recently approved (March, 2013) in some European countries to combat heavy alcohol drinking in at-risk patients (European Medicines Agency’s Committee for Medicinal Products for Human Use, 2012). Preclinical work using intra-accumbal infusions of nalmefene showed that this compound reduced ethanol intake at lower doses in ethanol-dependent ethanol self-administering rats than non-dependent animals (Nealey et al., 2011), an effect that was credited to the partial agonist activity and high affinity of nalmefene at KORs (Bart et al., 2005; Nealey et al., 2011). Despite this behavioral evidence, the effects of nalmefene on dopamine terminal and accumbal KOR function following chronic ethanol exposure are poorly understood, and elucidation of its effects may aid in understanding the basis of its clinical efficacy. To this end, ex vivo fast scan cyclic voltammetry (FSCV) was used to evaluate the effects of nalmefene on dopamine transmission and KOR function in the nucleus accumbens core, 72 hours following five weeks of chronic intermittent ethanol (CIE) exposure in male C57BL/6 (C57) mice.

2.0 Experimental Procedures

2.1 Subjects

Male C57 mice (6-8 weeks old, Jackson Labs, Bar Harbor, ME) were used for all experiments. Animals were allowed at least one week of habituation to the housing environment before CIE procedures began. All mice were individually housed and maintained on a 12-hr light-dark cycle (lights off at 14:00), with a red-room light
illuminated during the animals’ dark cycle. Standard rodent chow and water were available *ad libitum*, and replaced daily in the CIE exposed group. Animals were cared for according to the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care, and all experimental protocols were approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine.

2.2 CIE exposure

Mice were exposed to ethanol vapor (CIE) or room air for 16 hours/day, followed by 8 hours of room air. This procedure was repeated four times before a 72-hour abstinence period (one cycle). Cycles were repeated five times. Approximately 30 minutes prior to chamber start time (17:00), mice in both groups were systemically administered with 1.0mmol/kg pyrazole (Sigma-Aldrich, St. Louis, MO), an alcohol dehydrogenase inhibitor, mixed with 1.6g/kg ethanol (CIE exposure group) or saline (air inhalation group). Blood ethanol concentrations (BEC) were verified the mornings after the first and final inhalation exposure per each cycle.

2.3 BEC measurement

To ensure proper ethanol chamber function and physiologically relevant BECs, a submandibular vein blood draw was performed. Less than 15µL of blood was collected in BD microtainer tubes lined with lithium heparin (Becton Dickinson & Company, Franklin Lakes, NJ). For BEC measurement, standards and samples were prepared with a commercially available alcohol dehydrogenase assay (Carolina Liquid Chemistries
Corporation, Brea, CA), carefully pipetted into a 96 well plate and analyzed with SoftMax Pro Software version 5 (Molecular Devices Corporation, Sunnyvale, CA).

### 2.4 Ex vivo FSCV

FSCV was used to detect CIE-induced changes in dopamine release and uptake, as well as the effects of nalmefene, U50,488, and nor-binaltorphimine (norBNI), a KOR-specific agonist and antagonist, respectively, on these measures. Briefly, 300µm thick coronal brain slices containing the nucleus accumbens core were prepared using a vibrating tissue slicer. Slices were incubated in oxygenated artificial cerebrospinal fluid (aCSF), heated to 32°C for approximately 60 minutes prior to experiment start. A bipolar stimulating electrode (Plastics One, Roanoke, VA) and carbon fiber microelectrode (∼50µm length, 7µm radius (Goodfellow Corporation, Berwyn, PA) were placed within 100µm of each other on the surface of the slice. Dopamine efflux was induced by a single, rectangular, electrical pulse (4.0ms; 350µA, monophasic; interstimulus interval: 180sec), and detected by applying a triangular waveform every 100ms to the recording electrode (-0.4 to +1.2 to -0.4 V vs. Ag/AgCl, 400V/sec). When baseline collections were stable for three consecutive stimulations, nalmefene (1.0-100.0µM, generously provided by H. Lundbeck A/S), norBNI (1.0µM, graciously provided by NIDA) U50,488 (0.01-0.3µM, generously provided by NIDA) were cumulatively added to the bath. Following the maximal concentration of U50,488 (0.3µM), a challenge concentration of nalmefene (10.0µM) was added to the bath solution. In a separate set of experiments, a single dose of norBNI (1.0µM) was applied to brain slices to identify the effects of KOR blockade on dopamine release and uptake measures. Clear current versus time plots were obtained using background current subtraction methods. Electrodes were calibrated immediately
after experiments by recording their response (in nA) to a known concentration of dopamine (3.0μM) using a flow-injection system.

2.5 Data analysis

Representative pre-drug traces of electrically-stimulated dopamine release were individually modeled and data were collapsed across inhalation groups to obtain baseline dopamine release and uptake measures. Dopamine release was calculated as the amount of electrically-evoked dopamine released per stimulation, and $V_{\text{max}}$ was calculated as the maximal rate of uptake at the dopamine transporter. The apparent affinity of dopamine for the dopamine transporter (apparent $K_m$) remained constant at 160nM throughout analysis (Wu et al., 2001). The effects of KOR ligands on dopamine release and uptake parameters were similarly analyzed. Demon Voltammetry and Analysis software (Yorgason et al., 2011) was used to collect and analyze all data.

Graphs were created and statistical analyses were computed with GraphPad Prism (version 5, La Jolla, CA). Student’s t-tests were used to analyze the effects of inhalation exposure on dopamine release and uptake rates. Repeated measures (RM) two-way analysis of variance (ANOVA) was used to determine the effects of increasing concentrations of U50,488 and nalmefene on dopamine release and uptake, with inhalation exposure and drug concentration as factors. Additionally, non-RM two-way ANOVAs, with inhalation exposure and drug concentration as factors, were used to determine the ability of nalmefene to reverse the dopamine-decreasing effects of the 0.3μM U50,488 concentration, the effects of nalmefene on the maximal rate of uptake following 0.3μM U50,488, as well as the effects of 1.0μM norBNI on dopamine release.
and uptake parameters. When a significant main effect was detected, Bonferroni post-hoc analysis was applied.

3.0 Results

3.1 CIE exposure reduced dopamine transmission in the nucleus accumbens core

CIE exposed mice had behaviorally and physiologically relevant BECs (219.50±39.31mg/dL; Griffin et al., 2009, data not shown). Representative FSCV traces are overlaid in Figure 1A (Air: blue trace; CIE: red trace). Consistent with previous work from our laboratory (Karkhanis et al., 2015), CIE reduced dopamine release (Figure 1B, t_{18}=2.38 \ p<0.05; Air: 1.06±0.26 µM; CIE: 0.71±0.40µM) and increased rates of dopamine uptake (Figure 1C, t_{17}=3.80, \ p<0.05; Air: 1.52±0.32µM/sec; CIE: 2.46±0.68µM/sec).

3.2 Nalmefene slowed dopamine uptake rates more in brain slices from CIE-exposed mice than controls

To examine the effects of nalmefene on dopamine release and uptake, increasing concentrations of this compound were bath-applied to accumbal brain slices. Two-way RM ANOVA revealed a main effect of nalmefene concentration on dopamine release (Figure 2A, F_{4,9}=48.34, \ p<0.001), which was similar between inhalation groups (F_{1,9}=7.27, \ p>0.05). Additionally, nalmefene dose-dependently reduced uptake rates in both groups (Figure 2B, F_{4,8}=5.29, \ p<0.01), although this effect was greater in CIE
Figure 1: CIE exposure reduced dopamine signaling in the nucleus accumbens core

Representative FSCV traces are overlaid in Figure 1A (Air: blue trace; CIE: red trace). (1B) CIE reduced dopamine release in accumbal slices from CIE-exposed mice compared to their air-exposed counterparts. (1C) CIE increased dopamine uptake rates ($V_{\text{max}}$) in brain slices from CIE-exposed mice compared to controls. *$p<0.05$, **$p<0.05$
Figure 2: Nalmefene attenuates the uptake rates only in brain slices from CIE-exposed mice

(2A) Nalmefene concentration-dependently decreased dopamine release, which was similar between inhalation groups. (2B) Similarly, nalmefene dose-dependently attenuated $V_{\text{max}}$ in both groups, although this effect was greater in brain slices from CIE exposed animals compared to air-exposed mice. (2C) The KOR antagonist, norbinaltorphimine (norBNI) did not alter dopamine release. (2D) NorBNI reduced uptake rates in brain slices from CIE-exposed mice compared to controls. *$p<0.05$. 
exposed animals ($F_{1,8}=7.94, p<0.05$). No interaction between these factors was detected ($F_{4,8}=5.40, p>0.05$).

A separate group of brain slices were incubated in norBNI for 60 minutes to assess the effects of KOR blockade on dopamine transmission. Two-way ANOVA revealed no effect of norBNI on dopamine release (Figure 2C, $F_{1,24}=0.12, p>0.05$); however, a main effect of inhalation treatment on uptake rates (Figure 2D, $F_{1,26}=4.40, p<0.05$) was detected. Bonferroni post-hoc analysis revealed a significant difference between the effects of norBNI on CIE-exposed mice compared to air-exposed controls ($p<0.05$).

3.3 Nalmefene reversed the dopamine-decreasing effects of U50,488 in CIE-exposed mice

Representative traces showing the effects of 0.3µM U50,488 on dopamine release and uptake (Figure 3A, blue, air; Figure 3B, red, CIE) and 10.0µM nalmefene reversal (black line, overlaid). The effects of CIE on KOR function were examined with increasing concentrations of U50,488. A RM two-way ANOVA revealed a main effect of KOR activation on dopamine release that was dose-dependent (Figure 3C, $F_{3,10}=31.69, p<0.001$), which was greater in brain slices from mice exposed to CIE ($F_{1,10}=6.26, p<0.05$). An interaction between these factors was also detected ($F_{3,10}=5.51, p<0.01$). Bonferroni post hoc analysis revealed a significant difference between inhalation groups at the 0.1µM ($p<0.01$) and 0.3µM ($p<0.05$) U50,488 concentrations.

As nalmefene is a partial KOR agonist, it competes for the ligand binding site on the receptor (Bart et al., 2005; Zhu et al., 2005). To determine the ability of nalmefene to reverse the dopamine-decreasing effects of KOR activation, 10.0µM of the compound was added to the bath solution following the 0.3µM concentration of U50,488. A two-
Figure 3: Nalmefene reversed the dopamine-decreasing effects of U50,488 in CIE-exposed mice

(3A) Representative traces of the effects of 0.3µM U50,488 on dopamine release in brain slices from air (solid blue) and (3B) CIE (solid red) exposed mice. The black line in 3A and 3B (overlaid) represents the effect of 10.0µM nalmefene on these signals. (3C) KOR activation with U50,488 dose-dependently reduced dopamine release greater in brain slices from CIE-exposed mice compared to controls. (3D) 10µM nalmefene restored dopamine release following a maximal concentration of U50,488 in brain slices from CIE-exposed mice to control levels. (3E, 3F) U50,488 did not alter dopamine uptake rates across the concentration response curve, and nalmefene had no additional effect on uptake rates in either inhalation group. *p<0.05
way ANOVA, with U50,488 and nalmefene as factors revealed a main effect of drug (U50,488 vs nalmefene; Figure 3D, \(F_{1,10}=5.67, p<0.05\)), as well as an interaction between drug and inhalation treatment (\(F_{1,10}=6.19, p<0.05\)). An effect of inhalation treatment was not detected (\(F_{1,10}=2.46, p>0.05\)). Bonferroni post hoc analysis revealed a significant increase in dopamine release due to nalmefene reversal in the CIE-exposed group compared to the 0.3\(\mu\)M U50,488 concentration (\(p<0.01\)).

As nalmefene reduced dopamine uptake rates to a greater extent in brain slices from CIE-exposed mice compared to air-exposed controls, we examined the effects of a nalmefene challenge on U50,488-induced alterations in dopamine uptake rates. Surprisingly, a two way RM ANOVA revealed no effect of U50,488 on dopamine uptake rates (Figure 3E, \(F_{3,8}=1.26, p>0.05\)) in either inhalation group (\(F_{1,8}=0.17, p>0.05\)). Further, a two-way RM ANOVA revealed that nalmefene had no effect on dopamine uptake rates following the maximal (0.3\(\mu\)M) concentration of U50,488 (Figure 3F, \(F_{1,16}=0.04, p>0.05\)).

### 4.0 Discussion

The present study aimed to discern the pharmacological effects of nalmefene on dopamine terminal and KOR function following CIE exposure in mice. Congruent with previous work (Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al., 2015; Rose et al., 2015), CIE exposure reduced dopamine release, augmented rates of dopamine uptake and KOR sensitivity to agonist, which is consistent with a hypodopaminergic state of the nucleus accumbens. Nalmefene concentration-dependently reduced dopamine release similarly between inhalation conditions, but attenuated uptake rates more in brain slices from CIE-exposed mice compared to controls. Additionally, we found that a single
concentration of nalmefene reversed the dopamine-decreasing effects of a maximal concentration of U50,488 selectively in brain slices from CIE-exposed mice. These data are the first to demonstrate dopamine terminal modulation by nalmefene and point to mechanisms that may underlie nalmefene-induced reductions in ethanol intake following CIE exposure (Walker & Koob, 2008; Nealey et al., 2011).

### 4.1 Nalmefene reduced dopamine release equally in both inhalation groups, but attenuated dopamine uptake rates more in brain slices of CIE-exposed mice

Our data show similar dopamine-decreasing effects of nalmefene between inhalation groups, suggesting two interpretations. First, these data indicate that dynorphin tone may not be present in striatal brain slices. If dynorphin tone were present, nalmefene would compete with the endogenous ligand for receptor occupancy (Calvey & Williams, 2009), resulting in antagonist-like effects. This effect would be measured voltammetrically as an increase in stimulated dopamine release (Spanagel et al., 1992). Notably, control experiments using norBNI in this study showed no effect of KOR blockade on dopamine release in either inhalation group, further suggesting that dynorphin tone is not measurable in brain slices. As such, it is possible that nalmefene was acting as an agonist at KORs to reduce dopamine release (Bart et al., 2005).

In addition to being a partial KOR agonist, nalmefene is also a MOR and DOR antagonist (Bart et al., 2005), making definitive designation of its effects to any one opioid receptor difficult. However, DORs are thought to specifically regulate postsynaptic signaling the nucleus accumbens (Dilts & Kalivas, 1990), thus DOR-modulation of dopamine transmission is unlikely. MORs are primarily localized to GABAergic interneurons that feed onto dopamine terminals (Svingos et al., 1997).
Therefore, it is also unlikely that MOR blockade drove the dopamine-decreasing effects of nalmefene observed here, as disinhibition of dopamine terminals by GABAergic interneurons (Svingos et al., 1997) would most likely result in an increase in stimulated dopamine release. Even so the influence of nalmefene modulation of DORs/MORs on dopamine transmission is not known in this context and some contribution of these receptors to the present findings cannot be entirely ruled out.

Although the effects of nalmefene on dopamine release were similar between inhalation groups, this compound concentration-dependently reduced dopamine uptake rates more in brain slices from CIE-exposed mice compared to controls. In this instance, it appears as if nalmefene is acting to reduce dopamine release and uptake through KORs. Notably, dopamine transporters have been consistently reported to be functionally upregulated following CIE exposure (Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al., 2015; present work). Here, we found that KOR blockade slowed uptake rates in brain slices from CIE-exposed mice compared to air-exposed controls using a single concentration of norBNI, which may be due to its effects on KOR signaling cascades (Bruchas & Chavkin, 2010). A recent study showed that KORs exist both independently and in complex with dopamine transporters, and regulate dopamine transporter function via an ERK1/2-dependent pathway (Kivell et al., 2014). Therefore, we hypothesize that the physical or functional connection between dopamine transporters and KORs (Kivell et al., 2014) is fundamentally altered following CIE, driving KOR antagonist-mediated reductions in uptake rates in the present experiments. However, we also showed that KOR activation did not augment (Thompson et al., 2000; Kivell et al., 2014; Simonson et al., 2015) or attenuate (Das et al., 1994; Chudapongse et al., 2003)
dopamine uptake rates as reported previously, but eliminated nalmefene-induced reductions in dopamine uptake. Additional investigations into the interplay of U50,488 and nalmefene on KOR-induced alterations in dopamine uptake rates are needed to fully elucidate these findings.

4.2 Dopamine release is restored by nalmefene following KOR activation in brain slices from CIE-exposed mice

To better understand the distinct effects of nalmefene on KOR function between the inhalation groups, a middle concentration of nalmefene was applied to brain slices following a maximal concentration of U50,488. As earlier experiments suggested that dynorphin tone is undetectable in brain slices, the addition of U50,488 was necessary to examine any antagonistic effects of nalmefene on KORs. We found that nalmefene reversed the dopamine-decreasing effects of U50,488 in brain slices from CIE-exposed mice, suggesting that nalmefene competed with U50,488 for KOR occupancy. It is possible that this effect is due to CIE-induced receptor upregulation or functional supersensitivity of KORs compared to air-exposed animals. In other work, CIE exposure increased levels of KOR binding in seizure-resistant mice (Beadles-Bohling & Wiren, 2005) and augmented dynorphin-stimulated KOR activity in Wistar rats (Kissler et al., 2014) compared to controls. These data indicate that the observed reversal of the dopamine-decreasing effects of U50,488 in brain slices from CIE-exposed mice could be due to physical or functional upregulation of KORs. Experiments that examine these possibilities with the present model would be helpful in discerning this change.
4.3 Behavioral implications of the effects of nalmefene on dopamine terminal function

Intra-cerebroventricular (Walker & Koob, 2008) and intra-accumbal (Nealey et al., 2011) nalmefene reduces ethanol intake at lower doses in ethanol dependent animals, compared to non-dependent animals, in part due to its high affinity and the unique action of this compound on KORs. It is plausible that nalmefene-induced reductions in ethanol drinking may be due to increased dopamine transmission via attenuated uptake rates and increased dopamine release, as noted selectively in brain slices from CIE-exposed mice ex vivo. In fact, dopamine transporter knockout mice, with inherently reduced rates of dopamine clearance compared to wild-type mice (Jones et al., 1998) consume less ethanol than their wild-type and heterozygous counterparts (Mittleman et al., 2011), providing evidence to support this hypothesis.

The time-course of nalmefene-induced alterations in neurobiology is rapid and beneficial in the therapeutic application of this compound. In fact, ethanol intake in ethanol dependent rats at lower doses than non-dependent animals (Walker & Koob, 2008) was reduced only 30 minutes prior to ethanol self-administration testing. Similarly, alcoholics who are prescribed nalmefene take the compound orally when they predict they will encounter a high-risk situation (i.e. social environments where alcohol may be present), and consistently report reductions in overall ethanol consumption (Karhuvaara et al., 2007; Gual et al., 2013; Mann et al., 2013). Use of nalmefene on a continuous schedule dose-dependently reduces ethanol intake over time (Mason et al., 1994, 1999) and attenuates relapse to heavy drinking (Mason et al., 1999; Karhuvaara et al., 2007) in treatment-seeking alcoholics. Together, preclinical and clinical evidence strongly suggest
that nalmefene reduces ethanol intake on a rapid timescale, and data presented here suggest that this may be due to KOR modulation of dopamine transmission.

4.4 Conclusions

Due to the high rate of recidivism to alcoholism, the need for effective pharmacotherapies for this disorder is high. Modulation of KORs to restore dopamine system function during alcohol withdrawal is of interest therapeutically (Lichtigfeld & Gillman, 1996). Overwhelming preclinical behavioral evidence (Walker & Koob, 2008; Nealey et al., 2011) and clinical work with alcoholics (Mason et al., 1994, 1999; Karhuvaara et al., 2007; Gual et al., 2013; Mann et al., 2013) indicate favorable therapeutic effects of nalmefene on ethanol drinking. Notably, the effects of nalmefene on drinking are due, in part, to its high affinity and partial agonist activity of this compound on KORs, particularly in the nucleus accumbens (Nealey et al., 2011). We report that nalmefene reduced dopamine uptake rates and reversed the dopamine-decreasing effects of KOR activation, suggesting that nalmefene may augment dopamine transmission in vivo. Increased accumbal dopamine transmission by nalmefene would attenuate the hypodopaminergic state of this region, namely through reductions in KOR activity and dopamine uptake rates. These mechanisms may underlie nalmefene-induced reductions in ethanol intake in ethanol-dependent rodents (Walker & Koob, 2008; Nealey et al., 2011) and humans (Mason et al., 1994, 1999; Karhuvaara et al., 2007; Gual et al., 2013; Mann et al., 2013). These data provide an understanding of the pharmacological effects of nalmefene, and hope to promote understanding of its clinical efficacy.
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Conflict of Interest

Part of this work was funded by Lundbeck A/S. This corporation provided nalmefene that was utilized in the present work.
References


European Medicines Agency’s Committee for Medicinal Products for Human Use (2012) European Medicines Agency recommends approval of medicine for reduction of alcohol consumption. Press release,


CHAPTER V: DISCUSSION

Overview

The present set of studies aimed to discern the behavioral and neurobiological effects of five weeks of CIE or air exposure in male C57 mice. To begin, we explored the effects of CIE exposure on withdrawal-associated behaviors, dopamine terminal and presynaptic receptor-system function. These experiments revealed CIE-induced increases in ethanol drinking and anxiety/compulsive-like behavior in the marble burying task, which were reduced selectively in norBNI-treated, CIE exposed mice. Notably, KOR activation in naïve mice increased ethanol drinking, preference and marble burying behavior. Data collected with ex vivo FSCV showed reduced dopamine release, increased dopamine uptake rates and augmented KOR sensitivity to agonist following CIE exposure. These data suggest that CIE-induced increases in ethanol drinking and anxiety/compulsive-like behavior may be due to accumbal KORs.

Based on the heterogeneous nature of the nucleus accumbens, and the variety of presynaptic dopamine-regulating receptors (Yoshimoto & McBride, 1992; Berridge et al., 1997; Blaha et al., 1997; Floresco et al., 1998; Del Arco & Mora, 2008), it is likely that KORs are not the only presynaptic receptor system that is altered following chronic ethanol exposure. In fact, previous work from our laboratory showed similar reductions in dopamine release and uptake, in addition to increased autoreceptor system function following CIE exposure (Karkhanis et al., 2015). In this study, however, a D2R/D3R agonist, quinpirole, was utilized, making dissociation of CIE-induced changes in D2R and D3R function difficult. By using receptor-specific agonists, we found a selective
upregulation in D3R sensitivity following CIE exposure, with no effect of CIE on D2R sensitivity to agonist. Additional experiments in this chapter examining CIE-induced functional alterations in KORs confirmed Chapter II findings showing this receptor’s supersensitivity to agonist. These data suggest that D3Rs and KORs are both functionally hyper-responsive following CIE exposure. This effect may be due to a third-party neuromodulator system, namely RACK1 and BDNF/TrkB signaling (see Section 5.1a). Investigation into a functional relationship between KORs and autoreceptors showed a D3R-KOR, but not D2R-KOR, interaction, whereby D3R blockade reduced the dopamine-decreasing effects of KOR activation. These data are some of the first to show specific hypersensitivity of D3Rs following CIE, and a functional relationship between D3Rs and KORs.

The fourth chapter of the present body of work examined the effects of nalmefene, a partial KOR agonist and MOR/DOR antagonist (Bart et al., 2005), on accumbal dopamine transmission and KOR activity. We found that nalmefene concentration-dependently reduced dopamine release similarly in both inhalation groups, suggesting that dynorphin levels are undetectable in brain slices using fast scan cyclic voltammetry. Nalmefene also attenuated dopamine uptake rates across the concentration response curve, and therefore, was acting as an agonist in this assay, although whether or not this effect was via KORs, DATs or an off-target mechanism is unknown. Notably, nalmefene-induced reductions in dopamine uptake rates were greater in brain slices collected from CIE-exposed compared to air-exposed mice. It is possible that these findings are due to a CIE-induced fundamental alteration in the functional connection between KORs and dopamine transporters (Kivell et al., 2014). Additional experiments showed that
nalmeffene reversed the dopamine-decreasing effects of KOR activation in brain slices from CIE-exposed mice, suggesting that nalmeffene competes with the agonist for receptor occupancy to augment dopamine transmission to control levels (Calvey & Williams, 2009). The ability of nalmeffene to reverse CIE-induced attenuations in dopamine transmission via increased release and reduced rates of uptake may be mechanisms by which this compound alters ethanol consumption in CIE-exposed rodents (Walker & Koob, 2008; Nealey et al., 2011) and humans (Karhuvaara et al., 2007; Gual et al., 2013).

In short, the present body of work furthers our understanding of accumbal dopamine terminal receptor function following CIE exposure in mice, and some of the behavioral implications of these changes. We hope that this information promotes the development of new pharmacological agents to combat the withdrawal effects of ethanol exposure, particularly those that target KORs. Future work should focus on expanding the present knowledge to use a combination of whole animal studies and in vitro experiments to rigorously evaluate KORs as a target for the treatment of alcohol use disorders.

**Systemic administration of KOR ligands in ethanol drinking and marble burying experiments: The need for neuroanatomical specificity.**

Behavioral and neurobiological data in Chapter II suggest that CIE exposure augments KOR sensitivity, and that this effect is behaviorally relevant. In fact, voltammetric experiments revealed an increase in KOR sensitivity to agonist, and we found norBNI-sensitive CIE-induced increases in ethanol drinking and marble burying behaviors that were replicated with KOR activation in naïve mice. These studies also included findings that show specific changes in accumbal KOR function; however, the
systemic nature of drug administration during our behavioral studies makes identification of the precise brain location of CIE-induced alterations of KOR function, as they relate to CIE exposure-induced behaviors, difficult. Although the present work and additional evidence from monkeys chronically exposed to ethanol show increased KOR sensitivity (Siciliano et al., 2015), it is possible that CIE-upregulation of KORs in regions outside of the nucleus accumbens drive these behavioral phenotypes. In fact, recent work showed an increase in dynorphin-stimulated KOR activation in the central nucleus of the amygdala following four weeks of CIE exposure (Kissler et al., 2014). Based on CIE exposure-induced alterations in the direct and indirect motor pathways (discussed below), it is hypothesized that intra-nucleus accumbens core administration of norBNI would reduce ethanol drinking and marble burying behavior in C57 mice following five weeks of CIE, as this treatment would increase dopamine transmission and regulate downstream signaling. In fact, some studies have shown that deep brain stimulation of the nucleus accumbens (Kuhn et al., 2009, 2011) and transcranial magnetic stimulation of the mesolimbic dopamine system (Ceccanti et al., 2015) both augment dopamine transmission and reduce ethanol craving and consumption in alcoholics, supporting this hypothesis.

**Neurocircuitry associated with chronic ethanol exposure**

The nucleus accumbens is a portion of both the direct and indirect motor pathways, which are distinguished by D1R and D2R-containing medium spiny projection neurons that modulate regions within this circuit (Figure 2). Neurotransmission through the “direct” (D1R), and “indirect” (D2R), or “go” and “no-go” pathways, respectively, may be fundamentally altered following CIE exposure (Figure 1). Following chronic drugs of
abuse, our data suggest that stimulated dopamine release is reduced. Attenuated dopamine transmission attenuates the activity of D1R direct pathway while increasing downstream activity of D2R medium spiny neurons (Volkow & Morales, 2015). Increased D2R medium spiny neuron signaling following chronic alcohol exposure would reduce GABAergic signaling from the globus pallidus (Sano et al., 2013) and increase glutamatergic signaling from subthalamic nucleus, thus augmenting transmission from GABAergic projections from the ventral midbrain to thalamic nuclei (Di Chiara et al., 1979; Haegelen et al., 2009; Kase et al., 2015). As such, inhibition of thalamic signaling to the cortex would reduce glutamatergic signaling from cortical regions to the striatum. It is likely that low glutamatergic transmission during ethanol abstinence may confer increased withdrawal symptoms and dysregulated behavioral outputs. See Figure 1 for a representation of the proposed circuit.

Several pharmacotherapeutics that reduce the withdrawal effects of ethanol regulate aberrant neurotransmitter signaling in this circuit. For example, alcoholics can be treated with chlorodiazepoxide (Librium®) and lorazepam (Ativan®), both benzodiazepines, which augment GABAergic signaling though the basal ganglia (Kuman et al., 2009; Rajmohan et al., 2013) to reduce some of the symptoms of alcohol withdrawal. These compounds would reduce the anxiety (Baskin & Esdale, 1982; Meibach et al., 1987; Diaper et al., 2012) and depression (Lindemann et al., 2015) often associated with alcohol withdrawal. Similarly, the work presented here showed that behaviors altered by CIE, ethanol drinking and marble burying, returned to control levels with KOR blockade. By reducing the inhibitory signaling of KORs on dopamine terminals, dopamine release
Chronic ethanol exposure alters signaling through the direct and indirect motor pathways. Dopaminergic projections (red arrows) from the ventral midbrain (substantia nigra pars compacta and ventral tegmental area) are downregulated following chronic ethanol exposure. These signaling reduce signaling downstream of D1-containing medium spiny neurons and disinhibits D2R medium spiny neuron signaling through the globus pallidus externa. Increased GABAergic signaling to the globus pallidus externa reduces that regions’ output to the substantia nigra pars reticulata and subthalamic nucleus. An augmentation in the excitatory projection from the subthalamic nucleus increases the GABAergic output of the substantia nigra pars reticulata to the thalamus. Augmented GABA signaling to the thalamus from the ventral midbrain reduces the glutamatergic signaling that projects from this region. Attenuated glutamatergic signaling from the thalamus to the cortex reduces excitatory input into the striatum.

Figure 1: Direct and indirect motor pathways are altered following chronic ethanol exposure
is increased (Spanagel et al., 1992), thus augmenting signaling through the D1R medium spiny neurons and reducing signaling through the D2R medium spiny neurons, thus increasing the inhibition on to the ventral midbrain, reducing thalamic output and attenuating the behavioral manifestations of aberrant basal ganglia function.

In stark contrast to the above hypothetical neurocircuit, a considerable body of literature suggests that glutamatergic signaling is upregulated following chronic ethanol exposure. For example, chronic ethanol drinking increases extracellular glutamate levels and reductions in glutamate clearance in the nucleus accumbens (Dahchour & De Witte, 2003; Ding et al., 2013). Behaviorally, increasing glutamate transmission in the nucleus accumbens of naïve mice increases ethanol drinking to the levels of their CIE-exposed counterparts (Griffin et al., 2014). Notably, pharmacotherapeutics that augment GABA signaling, as noted above, also reduce glutamatergic transmission (Smith et al., 2007), thus regulating aberrant neurotransmission through the basal ganglia following chronic ethanol exposure and withdrawal.

**Chronic ethanol-induced reductions in dopamine transmission may be due to attenuated dopamine terminal function**

Chronic alcohol exposure in humans (Hietala et al., 1994; Laine et al., 1999; Volkow et al., 1996, 2002, 2007, 2013) and rodents (Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al., 2015) reduces dopamine transmission in the basal ganglia, although the neurobiological loci of this effect are unknown. Chronic ethanol-induced attenuation in dopamine transmission in these regions may be due to reduced dopamine cell firing (Shen & Chiodo, 1993; Shen et al., 2007, but see: Diana et al., 1995; Brodie, 2002) or deficient terminal function (Carroll et al., 2006; Budygin et al., 2007; Karkhanis et al.,
In support of reduced dopamine terminal function, we found increased KOR sensitivity to agonist, which may, in part, drive the reductions in dopamine transmission observed in the current project. However, dopamine-regulating presynaptic receptors are not limited to KORs. Thus, to investigate the effects of chronic ethanol exposure specifically on autoreceptor function, CIE-induced alterations in D2R and D3R sensitivity to agonist were examined in concert and individually. In support of previous work (Karkhanis et al., 2015), we found upregulated D2R-type sensitivity to agonist following CIE exposure, and furthered this finding by demonstrating that this increased autoreceptor function was driven by selectivity by D3Rs. As both dopamine D3Rs (Maina & Mathews, 2006) and KORs (Spanagel et al., 1992) reduce dopamine transmission following activation, it is plausible that a CIE-induced increase in the sensitivity of these receptors could reduce dopamine transmission with low concentrations of the endogenous ligand. Experiments that expand upon the mechanism of this change would provide insight CIE-induced alterations in presynaptic receptor sensitivity.

**D3Rs and KORs are hypersensitive following CIE: Hypothetical upregulation through a third-party neuromodulator**

As reviewed in Section 5.1a, ethanol exposure increases the translocation of RACK1 to the nucleus (Yaka et al., 2003; McGough et al., 2004). This movement drives an increase in D3R gene transcription and membrane expression (Vengeliene et al., 2006) in addition to an upregulation in BDNF levels (McGough et al., 2004). Release of BDNF
Figure 2: Effects of chronic ethanol exposure on dopamine terminal function

(A1) Ethanol-induced increases in RACK1 augments D3Rs in presynaptic terminal boutons and levels of BDNF in the nucleus accumbens. (A2) Increased D3R sensitivity reduces dopamine release. (A3) Consistently high levels of BDNF reduces the sensitivity of TrkB receptors and downstream signaling from these receptors. Further, reduced dopamine release attenuates D1R activation. Together, postsynaptic neuron signaling and gene transcription is attenuated. (A4) Reductions in CREB signaling due to downregulated receptor signaling attenuates the transcription of gene products. (A5) Reduced dynorphin levels drive an upregulation in KOR sensitivity which then reduces dopamine release.
into the extrasynaptic space activates TrkB on D1R-containing medium spiny neurons (Strömberg & Humpel, 1995; Logrip et al., 2008). Activation of TrkB results in the movement of PKA into the nucleus and the phosphorylation of CREB, driving the transcription of prodynorphin and other gene products (Muschamp & Carlezon, 2013). Metabolism of prodynorphin to dynorphin, and subsequent release of the endogenous KOR ligand results in presynaptic KOR activation (Day et al., 1998; Berman et al., 2000; Hauser et al., 2005). In this way, both D3Rs and KORs on presynaptic dopaminergic terminals would be upregulated and mediating dopamine transmission. See figure 2 for a hypothetical schematic of the effects of chronic ethanol on dopamine terminals.

A hypothetical scenario developed by scientists of Dorit Ron’s laboratory is supported by some of our data. Logrip and colleagues (2008) suggest that repeated bouts of ethanol exposure and withdrawal drives consistently high levels of BDNF, subsequently reducing TrKB sensitivity to its endogenous ligand and downstream signaling, including dynorphin production (Figure 2; Logrip et al., 2008). In an effort to examine this possibility, we utilized a dynorphin enzyme-linked immunosorbent assay (ELISA) to measure levels of this peptide in accumbal tissues from C57 mice following five weeks of CIE or air exposure. This experiment revealed that levels of dynorphin in nearly all CIE-exposed animals were lower than air-exposed samples; however, dynorphin levels in brain samples from CIE-exposed mice were largely below the detectable range of the kit (>1.0 picogram), whereas nearly all the air-exposed samples were within range. As such, a statistical assessment of CIE-induced alterations in accumbal dynorphin was not possible (data not shown). It is suggested that this particular experiment is repeated in order to draw more conclusive findings. Recent work from the Walker laboratory (Kissler
et al., 2014) showed a concomitant increase in KOR sensitivity and dynorphin levels in the central nucleus of the amygdala following CIE exposure in rats. It is possible that differences in species (rat vs mouse), ethanol exposure (four weeks vs five weeks) or length of abstinence before ex vivo experimentation (6 to 8 hours vs 72 hours) underlies the potential disparate findings in dynorphin levels (Kissler et al., 2014; present study).

**The effects of nalmefene on dopamine transmission: Modulation of accumbal KORs and its clinical utility**

To promote the clinical use of nalmefene in the United States and understand the clinical efficacy of this compound, the fourth chapter of the present document examined effects of nalmefene on dopamine transmission and KORs. We found that nalmefene reduced dopamine release similarly between inhalation groups across increasing concentrations of the compound, while reducing dopamine uptake rates differentially between inhalation groups. It is hypothesized that differential effects of nalmefene on maximal rates of uptake between inhalation groups are mediated by a CIE-induced alteration in the fundamental relationship between KORs and dopamine transporters (Kivell et al., 2014). Additional work examining the effects of nalmefene following KOR activation showed that a single concentration of this compound reversed the dopamine decreasing effects of U50,488 in brain slices from CIE exposed mice to control levels. These data suggest that nalmefene competed against the ligand (U50,488) for receptor occupancy, thus reversing dopamine release to control levels. It is possible that this reversal may be due to upregulated KOR expression or receptor function, although the precise mechanism cannot be determined by *ex vivo* voltammetry. Using a GTPγS assays, one study reported increased dynorphin-stimulated KOR activity in the central nucleus of
the amygdala following four weeks of CIE exposure in rats (Kissler et al., 2014). However, extension of those findings to the present body of work must be done with caution based on the distinct species, region and ethanol exposure protocols used between studies.

Nalmefene reduces ethanol drinking in chronic ethanol exposed rodents (Walker & Koob, 2008; Nealey et al., 2011) and humans (Mason et al., 1999; Karhuvaara et al., 2007). The rapid timescale by which nalmefene reduces ethanol consumption is consistent with the effects of pharmacological KOR manipulation on extracellular dopamine levels (Spanagel et al., 1992). In fact, administration of nalmefene shortly (30 minutes) before an ethanol drinking experiment reduced intake in rats (Walker & Koob, 2008; Nealey et al., 2011) and humans, who often take the compound shortly before encountering a situation where alcohol may be present, show attenuated ethanol intake compared to control subjects (Karhuvaara et al., 2007; Gual et al., 2013; Mann et al., 2013). Our findings show that nalmefene reduced dopamine uptake and reversed the dopamine-decreasing effects of KOR activation in the presence of an agonist, suggesting that this compound may also increase dopamine transmission in vivo. It is hypothesized that augmented dopamine transmission by nalmefene may underlie reductions in ethanol intake in rodents (Walker & Koob, 2008; Nealey et al., 2011), and humans (Karhuvaara et al., 2007; Gual et al., 2013; Mann et al., 2013). To this end, experiments that examine the effects of nalmefene on dopamine transmission in an in-tact animal during ethanol drinking experiments following chronic ethanol exposure would be informative.
The pharmacology of nalmefene versus naltrexone

As reviewed earlier (Section 6.3), naltrexone is a pan-opioid receptor (Bart et al., 2005), FDA approved antagonist used to reduce alcohol craving and consumption in alcoholics (Pettinati & Rabinowitz, 2006) following chronic ethanol exposure. However, naltrexone has been shown to induce negative affective state (Swift et al., 1994; Williams & Woods, 1999), and decrease the reinforcing efficacy of natural rewards such as palatable food (Fantino et al., 1986; Yeomans & Wright, 1991; Murray et al., 2014), in addition to reducing ethanol consumption. These effects may be due to MOR blockade. As some work suggests that KOR-specific blockade reduces ethanol intake (Walker & Koob, 2008; Nealey et al., 2011; Walker et al., 2011; present work) and anxiety/compulsive-like behavior (Valdez & Harshberger, 2012; present work) specifically in ethanol-dependent animals, without alterations in control animal behavior, it is plausible that a compound with a greater affinity for KORs would reduce ethanol intake and withdrawal symptoms more efficaciously than naltrexone.

Nalmefene, a structural congener of naltrexone, has augmented affinity and partial agonist activity at KORs and reduced affinity and antagonistic activity at MORs and DORs (Bart et al., 2005). Nalmefene reduced ethanol consumption in ethanol dependent rodents at lower doses of nalmefene than naltrexone (Walker & Koob, 2008; Nealey et al., 2011), and has been observed as a direct result of intra-accumbal administration of the compound (Nealey et al., 2011). The increased ability of nalmefene to reduce ethanol intake over naltrexone was hypothesized to be due to its high affinity and partial agonist activity at KORs (Walker & Koob, 2008). Despite the distinct pharmacology of naltrexone and nalmefene, clinical studies comparing these compounds showed similar
effects of on ethanol drinking in human subjects (Drobes et al., 2003, 2004). These data suggest that the pharmacological differences between nalmefene and naltrexone may have similar collective effects on neurotransmission in humans. Although the high affinity and partial agonist effects of nalmefene on KORs are not required to reduce ethanol intake in humans, nalmefene may reduce the symptoms of ethanol withdrawal and intake in a subset of alcoholics that do not positively respond to naltrexone.

**CERC-501: A novel KOR antagonist in clinical trials for mood and substance abuse disorders**

Preclinical work, including some of the findings shown here, is bolstering the development of KOR ligands for clinical use. In fact, all rights to a short-acting (Zheng et al., 2013), orally bioavailable (Rorick-Khan et al., 2014; Jackson et al., 2015) compound developed by Eli Lilly and Company has been recently sold to Cerecor (February, 2015), a clinical-stage pharmaceutical company, to develop and commercialize LY2456302 (CERC-501), a KOR antagonist, to combat substance abuse and alcohol use and mood disorders (Cerecor, 2015). As opposed to the long-lasting effects of common KOR antagonists used in laboratories today and in the present set of studies, such as norBNI (Endoh et al., 1992; Broadbear et al., 1994, Kishioka et al., 2013), CERC-501 pharmacologically blocks KORs for less than seven days in rats (Rorick-Kehn et al., 2014). One study examining the effects of CERC-501 on nicotine withdrawal revealed a reduction in anxiety-like behavior, hyperalgesia and conditioned place aversion to withdrawal compared to vehicle treated mice (Jackson et al., 2015), although additional work is needed to examine the effects of this compound on other drugs of abuse, including ethanol. Unlike naltrexone and nalmefene, CERC-501 does not display
significant activity at MORs and DORs (Lowe et al., 2014; Rorick-Khen et al., 2014), suggesting that it may not reduce the reinforcing salience of natural rewards (Fantino et al., 1986; Yeomans & Wright, 1991; Murray et al., 2014) or cause an aversive state in humans (Swift et al., 1994; Williams & Woods, 1999). In fact, in healthy humans, this compound appears to be well tolerated, without significant adverse events (Lowe et al., 2014). Additional work is needed in healthy and diseased populations to fully understand the pharmacokinetics and pharmacodynamics of this compound in and out of the presence of ethanol before a large-scale public release is executed.

**Ex vivo examination of accumbal dopamine transmission with optogenetic techniques may improve dopamine release specificity**

*Ex vivo* FSCV in accumbal brain slices allows for presynaptic measurement of dopamine transmission and investigation of receptor function (Ferris et al., 2013). However, the nucleus accumbens core is a heterogeneous region with multiple inputs that converge on medium spiny output neurons, interneurons and dopamine terminals (Cachope et al., 2012; Laplante et al., 2012; Steinberg et al., 2014), suggesting that neurotransmitters such as glutamate (Stuber et al., 2010; Fortin et al., 2012), adenosine (Quarta et al., 2004; Adamah-Biassi et al., 2015) acetylcholine (Bluth et al., 1985) and GABA (Tepper & Bolam, 2004) may be influencing dopamine transmission and the dopamine release parameters measured with FSCV.

The effects of electrically-induced neurotransmitter release on dopamine terminal function are not completely understood. Recent work from our laboratory showed that selective optogenetic activation of dopamine terminals in the nucleus accumbens released a greater concentration of dopamine compared to electrical stimulation of the same
location (Melchior et al., 2015). It was hypothesized that augmented dopamine release with optogenetic stimulation may be due to a removal of the inhibitory feedback of GABAergic interneurons that synapse onto dopamine terminals (Melchior et al., 2015). If this is the case, it is plausible that selective stimulation of dopamine terminals may reveal a dopamine signal that is larger than those recorded using electrical stimulation. Other work has shown that optogenetically-activated acetylcholine interneurons in this region induce stimulation-independent dopamine release (Cachope et al., 2012). Electrical stimulation likely induces release of these and other neurotransmitters and neuromodulators, which may also be clouding the dopamine signals measured with the present techniques.

Although it is possible that relative differences between electrically and optically evoked dopamine release may be equal across treatment groups, it is more likely that CIE also alters interneuron function, thereby altering feedback on dopamine terminals and dopamine transmission. For example, some work suggests that MORs are physically and functionally downregulated following CIE exposure (Turchan et al., 1999; Chen & Lawrence, 2000; Saland et al., 2005). As MORs are expressed in GABAergic interneurons (Svingos et al., 1997) that typically attenuate dopamine terminal function, reductions in MOR function may augment GABAergic interneuron firing, thus further reducing dopamine terminal function. In the case of acetylcholine interneurons, one study showed reductions in the varicosities of these interneurons in the nucleus accumbens following chronic ethanol exposure and withdrawal (Pereira et al., 2014). If this change is functionally relevant, electrical stimulation of the nucleus accumbens may not evoke a concentration of acetylcholine that influences dopamine transmission. Replication of
these studies using voltammetric techniques and negative optogenetic modulators, such as halorhodopsin in GABAergic and acetycholinergic interneurons, following CIE exposure would provide insight into the changes associated with each component of the accumbal milieu and how it affects dopamine terminal function.

**Future directions**

*Molecular underpinnings of CIE-induced alterations in KOR and D3R function are poorly understood*

Our studies clearly demonstrate that CIE reduced dopamine transmission and increased KOR- and D3R-system sensitivity to agonist. We surmise that reduced dopamine terminal function may be due to upregulated KOR and D3R function, although the link between CIE-induced alterations in these receptors and reductions in dopamine terminal function following chronic ethanol exposure and withdrawal has yet to be conclusively demonstrated. Examination of CIE-induced changes in each receptor, their downstream pathways and modulation of calcium and potassium channels may provide valuable information regarding the effects of CIE on dopamine release. With respect to CIE-induced alterations in dopamine uptake rates, experiments that examine the relationship between KORs or D3Rs and dopamine transporters following CIE exposure are warranted. As one study found that KORs regulate dopamine transporter function via an ERK1/2-dependent mechanism, and also proposed a physical link between KORs and dopamine transporters (Kivell et al., 2014), it is plausible that CIE alters downstream signaling or physical interactions between receptors and dopamine transporters. These
experiments may also inform on the effects that nalmefene has on this system and may shed light on the mechanism underlying nalmefene-induced reductions in uptake rates.

Tandem functional upregulation of KORs and D3Rs following CIE exposure

Similar functional upregulation in KORs and D3Rs following CIE exposure is hypothesized to be through a third-party neuromodulator, namely RACK1 and the BDNF/TrkB signaling pathway (Section 5.1a). However, it should be noted that a considerable portion of this hypothesis is yet to be tested. For example, although it is hypothesized that high levels of BDNF, due to continued intermittent ethanol exposure, would reduce TrkB sensitivity to this endogenous ligand, it is unknown whether or not CIE drives augmented levels of BDNF and reductions in TrkB sensitivity. Furthermore, attenuated presynaptic dopamine release, as measured presently, should attenuate D1R activation, which, together with reduced TrkB signaling, would reduce the downstream genetic products of these receptors, such as prodynorphin. However, it is unknown whether or not dynorphin levels are reduced following CIE exposure. In an attempt to discern levels of the KOR ligand, dynorphin, we utilized an ELISA for this neuropeptide following five weeks of CIE exposure. Regrettably, results from this experiment remain inconclusive. We suggest that this particular experiment should be repeated with higher concentrations of protein per sample, likely by combining tissue within treatment groups and increasing the total number of mice used in the study. Additional examination of the effects of CIE on RACK1, TrkB and KOR, as well as their endogenous ligands, would assist in the understanding of the mechanistic link between these neuromodulators.
The functional association between D3Rs and KORs requires more in-depth investigation

We report that D3R blockade reduces the dopamine-decreasing effects of KOR activation, indicating that these receptors interact. As these data were collected with FSCV, it is unknown whether or not there is a physical or functional interaction between D3Rs and KORs. To examine the possibility of a physical interaction between receptors, seminal experiments should include those that examine the likelihood of a physical connection, as well as similarities in receptor-attached candidate linker proteins. Alternatively, it is possible that D3Rs and KORs are associated by interactions within these receptors’ respective signaling cascades or the same population of calcium and potassium channels. Identification of the specific downstream pathway that links these two receptors may be difficult to determine, as bath (ex vivo) or site-specific (in vivo)-application of pharmacological manipulators of downstream effectors involved in receptor signaling would likely disrupt the signaling cascades of other receptors, in both pre- and post-synaptic neurons. As considerable efforts are being made to develop ligands that target specific downstream pathways of these receptors (Zhou et al., 2013; Lovell et al., 2015), development of receptor- and signaling-specific ligands may aid in the understanding of the D3R-KOR connection.

Caveats

Questions regarding the construct validity of the marble burying assay remain

Identification of anxiety-like behaviors in C57 mice is a topic of controversy in the ethanol field. Many traditional measures of anxiety-like behaviors are augmented in mice that exhibit high levels of anxiety-like behaviors, such as DBA/2J mice compared to
C57BL/6 mice, including elevated plus maze, light-dark box (Kulesskaya & Voikar, 2014; McCool & Chappell, 2015) and elevated zero maze (Flanigan & Cook, 2011). This is not to say that C57 mice do not show anxiety-like behavior, but that specific measures, such as marble burying, are needed for its demonstration (Nicolas et al., 2006; Perez & De Biasi, 2015; Pleil et al., 2015).

Measurement of phenotypic changes in rodent models is based on the construct validity of this measure. For example, the use of a forced swim assay to measure depressive-like behavior in rodents is due to the ability of antidepressants to reduce that behavior (Ushijima et al., 2005; Chung et al., 2014; Olivares-Nazario et al., 2015), just as the use of an elevated plus assay measures anxiety-like behaviors in rodents because benzodiazepines increase time spent in the open arms (Lister, 1987; File & Zangrossi, 1993; Kilic et al., 2014), an anxiogenic environment for rodents.

Rodents are known to bury aversive and anxiogenic objects, such as electrified probes, a behavior known as defensive burying (Korte et al., 1992, 1994; Dringenberg et al., 2008). As C57 mice avoid the marble-containing side in a two-compartment chamber, it is likely that marbles are aversive to these animals (Nicolas et al., 2006). Some work has shown that defensive burying is done largely with the forepaws, while hind limb burying indicates a normal digging behavior (Deacon, 2009). Anecdotally, mice in the present set of studies performed both actions, indicating that CIE-induced increases in marble burying were due, at least in part, to augmented defensive burying (Chapter II).

Marble burying is used to measure anxiety/compulsive-like behaviors based on the ability of pharmacotherapies for this indication to reduce this behavior. For example, benzodiazepines such as alprazolam, diazepam, fenobam and chlorodiazepoxide reduce
marble burying without affecting locomotor behavior at low doses (Nicholas et al., 2006), suggesting that marble burying is a valid measure of anxiety/compulsive-like behavior. However, miscellaneous compounds such as atropine, d-amphetamine and morphine also reduce marble burying while preserving locomotor activity (Nicholas et al., 2006), calling into question the construct validity of marble burying as a strict measure of anxiety/compulsive-like behavior. Additionally, antidepressants also reduce the number of marbles buried compared to control animals, suggesting a depressant-like phenotype, although this effect is largely due to the attenuating effects that these compounds have on locomotion (Nicholas et al., 2006). As marble burying behavior is reduced by compounds outside of the benzodiazepine class, an exact interpretation of its etiology is difficult. However, as marble burying has been published primarily as a measure of anxiety or compulsive-like behavior (Nicolas et al., 2006; Perez & De Biasi, 2015; Pleil et al., 2015), this claim that has been maintained through the present body of work.

The potential for D3Rs as a pharmacotherapeutic target

The behavioral relevance of D3R upregulation following CIE exposure is not known. A body of evidence showed that D3R blockade reduced ethanol intake in a model of alcohol relapse (Vengeliene et al., 2006), supporting the notion that chronic ethanol exposure and withdrawal upregulates ethanol drinking in a D3R-dependent mechanism. Further, genetic deletion of D3Rs (Bahi & Dreyer, 2014; Leggio et al., 2014) and D3R blockade (Leggio et al., 2014) reduces ethanol intake in drug-naïve mice. It has been hypothesized that compounds that reduce ethanol drinking in both dependent and non-dependent animals may also reduce the rewarding and reinforcing effects of natural
reinforcers such as food (Fantino et al., 1986; Yeomans & Wright, 1991; Murray et al., 2014). Although additional work is needed to elucidate the effects of D3R antagonists on the reinforcing efficacy of natural and drug rewards, this evidence may reduce the pharmacotherapeutic appeal of this compound.
REFERENCES


APPENDIX I

GREATER ETHANOL-INDUCED LOCOMOTOR ACTIVATION IN DBA/2J VERSUS C57BL/6J MICE IS NOT PREDICTED BY PRESYNAPTIC STRIATAL DOPAMINE DYNAMICS

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Abstract:

A large body of research has aimed to determine the neurochemical factors driving differential sensitivity to ethanol between individuals in an attempt to find predictors of ethanol abuse vulnerability. Here we find that the locomotor activating effects of ethanol are markedly greater in DBA/2J compared to C57BL/6J mice, although it is unclear as to what neurochemical differences between strains mediate this behavior. Dopamine elevations in the nucleus accumbens and caudate-putamen regulate locomotor behavior for most drugs, including ethanol; thus, we aimed to determine if differences in these regions predict strain differences in ethanol-induced locomotor activity. Previous studies suggest that ethanol interacts with the dopamine transporter, potentially mediating its locomotor activating effects; however, we found that ethanol had no effects on dopamine uptake in either strain. Ex vivo voltammetry allows for the determination of ethanol effects on presynaptic dopamine terminals, independent of drug-induced changes in firing rates of afferent inputs from either dopamine neurons or other neurotransmitter systems. However, differences in striatal dopamine dynamics did not predict the locomotor-activating effects of ethanol, since the inhibitory effects of ethanol on dopamine release were similar between strains. There were differences in presynaptic dopamine function between strains, with faster dopamine clearance in the caudate-putamen of DBA/2J mice; however, it is unclear how this difference relates to locomotor behavior. Because of the role of the dopamine system in reinforcement and reward learning, differences in dopamine signaling between the strains could have implications for addiction-related behaviors that extend beyond ethanol effects in the striatum.
Introduction

DBA/2J (DBA) and C57BL/6J (C57) mice are two inbred strains that show disparate phenotypes with respect to ethanol preference, drinking, and reward, among many other ethanol-mediated behaviors (1-6). The strains’ differential responses to ethanol exposure are often thought to model behaviors associated with alcohol abuse vulnerability, abuse and dependence. For example, C57 mice demonstrate high levels of voluntary ethanol intake, but little conditioned place preference (CPP) to ethanol, a measure of reward, while DBA mice voluntarily consume little ethanol but exhibit robust CPP for an ethanol-paired environment (1-3). Because DBA and C57 mice demonstrate differential responses to ethanol-mediated behaviors, these strains have become valuable tools for examining the individual differences that predict ethanol abuse vulnerability.

In addition to the differences in drinking behavior and ethanol reward between DBA and C57 mice, DBA mice are more sensitive to the locomotor-activating effects of ethanol (7, 8), although the neurochemical differences that are driving these behavioral disparities are unclear. Many studies have demonstrated that increases in dopamine in the ventral (nucleus accumbens, NAc) and dorsal (caudate-putamen, CPu) striatum mediate locomotor responses to drugs of abuse, including ethanol (9-12). Although it has been shown that ethanol significantly increases striatal dopamine levels, the precise mechanisms by which ethanol enhances locomotor activity is unclear. Striatal dopamine increases have been attributed to a number of factors including increases in ventral tegmental area (VTA) dopamine cell firing (13-15) and ethanol effects directly on striatal dopamine terminals (16-18). Previous work has demonstrated that the locomotor-enhancing effects of stimulant drugs such as cocaine and amphetamine are due to their
specific actions on presynaptic dopamine terminals, where they inhibit the dopamine transporter (DAT) to cause increases in synaptic dopamine levels (9, 19, 20). It has been argued previously that ethanol has direct actions on the DAT (16-18), and differences between ethanol-DAT interactions could underlie strain differences in the locomotor-activating properties of ethanol. Here, we aimed to determine if ethanol has direct effects on dopamine terminals that could in part explain the disparities in ethanol-induced locomotion between strains.

The primary focus of the current research was to assess ethanol-induced locomotor activity in DBA and C57 mice, and whether or not this activity was mediated by altered dopamine dynamics at the level of striatal terminals. DBA mice exhibited much greater locomotor activation by ethanol, an effect that was specific to ethanol, since other locomotor stimulating events elicited opposite responses. For example, DBA mice exhibit reduced locomotor activation in a novel environment compared to C57 mice, as shown here and by others (21). In order to determine whether these effects originated at the dopamine terminals, we utilized fast scan cyclic voltammetry to examine dopamine release and clearance at baseline, and in the presence of ethanol, to determine if differences in these measures predict behavioral outcomes. An advantage of ex vivo voltammetry is that it allows for the determination of the effects of ethanol on striatal terminals, independent of afferent inputs from both the dopamine system and other neurotransmitter systems. This technique is particularly useful as many pharmacological approaches to developing treatments for psychiatric disorders rely on an understanding of the region-specific effects of drugs. Because it has been demonstrated that ethanol’s ability to increase dopamine levels is due to a balance of its actions on dopamine
terminals (16) and modulation of VTA cell firing (13-15), this study determined if the actions of ethanol on dopamine terminals in the NAc core and CPu were predictive of the strain differences in the locomotor-activating effects of the drug. Our data indicate that ethanol does not change dopamine uptake, suggesting that increases in dopamine levels are via another mechanism. Further, DBA and C57 mice have similar presynaptic dopamine responses to ethanol in both striatal areas in regards to both release and dopamine uptake via the DAT, indicating that the increased sensitivity of DBA mice to the locomotor-activating effects of ethanol are likely not due to the effects of ethanol at dopamine terminals.

**Methods:**

**Subjects:**

Male DBA and C57 mice (6 weeks old; Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Animals were group housed in polycarbonate cages and maintained on a 12:12 light-dark cycle (7:00 pm lights off) with standard rodent chow and water *ad libitum*. Brain slices from both strains were obtained from naïve animals after at least one full week of habituation to the housing colony. The Institutional Animal Care and Use Committee at Wake Forest University School of Medicine approved the experimental protocol (Protocol Number: A10-177). All mice were cared for according to the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities.
**Locomotor Analysis:**

Locomotor activity was assessed via infrared beam breaks in automated locomotor activity monitors (20 cm × 20 cm × 20 cm; Med Associates, St. Albans, VT). Mice (n=9-10 per strain) were first placed into activity monitor chambers for 120 minutes to record response to novelty and to allow for habituation to the chamber. Twenty-four hours following habituation, animals were placed in the same chambers for 30 minutes before administration of two injections of saline, 60 minutes apart, to desensitize the animals to injection stress and allow for a within-subject control. Every day thereafter, animals received ethanol injections (0.125, 0.25, 0.5, 1.0, 2.0 g/kg i.p.) in the locomotor chambers after 60 minutes of habituation. Activity in response to each injection was reported for the first 30 minutes. Locomotor activity was measured as distance traveled (in centimeters) and as a percent of each animal’s second saline injection.

**In Vitro Voltammetry:**

Fast scan cyclic voltammetry in brain slices was used to characterize pre-drug striatal dopamine kinetics, as well as the effects of quinpirole and ethanol on single pulse evoked dopamine release. Briefly, a vibrating tissue slicer was used to prepare 400 µm thick coronal brain slices containing the striatum. Slices were incubated in oxygenated artificial cerebrospinal fluid and heated to 32°C. A carbon fiber microelectrode (≈150 µM length, 7 µM radius (Goodfellow Corporation, Berwyn, PA) and a bipolar stimulating electrode (Plastics One, Roanoke VA) were placed in close proximity (≈100 µM) on the surface of the slice, in either the NAc core or CPu. Endogenous dopamine efflux was induced by a single, rectangular, electrical pulse applied every five minutes for four
milliseconds (350 µA, monophasic). Dopamine released was detected by applying a triangular waveform (-0.4 to +1.2 to -0.4 V vs. silver/silver chloride, 400 V/sec) every 100 milliseconds to the recording electrode. Background current subtraction methods were applied to obtain clear current versus time plots. When baseline collections were stable for three consecutive stimulations, quinpirole (0.01, 0.03, 0.1, 0.3, 1.0 µM; n = 7 per strain) or ethanol (25, 100, 150, 200 mM; n = 9 per strain) was bath applied cumulatively to brain slices. Electrodes were calibrated immediately following experiments by recording their response (in nA) to 3µM dopamine using a flow-injection system.

To determine dopamine release and clearance, representative signals were analyzed before bath application of quinpirole or ethanol. Dopamine release (µM) was calculated as the amount of dopamine released per electrical stimulation whereas clearance was determined using the rate constant, tau. All data was collected and analyzed with Demon Voltammetry and Analysis software (22).

**Blood Collection and Analysis:**

Blood ethanol concentrations (BEC) from DBA and C57 mice were obtained at 5, 15, 30, 45, 60, 90 and 120 minutes after a 0.5 g/kg i.p. dose of ethanol (n=5-9 per strain, per time point). After injection, a submandibular vein blood draw was performed at each of the respective time points and collected in BD microtainer tubes lined with lithium heparin (Becton Dickinson & Company, Franklin Lakes, NJ). Each animal had no more than two bilateral blood draws per day and blood collection volumes did not exceed the maximum set forth by the institutional Animal Care and Use Committee.
determination of BECs, standards and samples were prepared with a commercially available alcohol dehydrogenase assay (Carolina Liquid Chemistries Corporation, Brea, CA). Briefly, five microliters of each blood collection was placed in a container with 45μl of trichlorocetic acid solution (Sigma Aldrich, St. Louis, MO), and centrifuged at 10,000 revolutions per minute at room temperature for ten minutes. 30μl of the supernatant of each sample was removed and placed into a separate container with 300μl of buffer and 112.5μl of enzymatic solution provided in the alcohol dehydrogenase assay. 100μl of each standard and sample was loaded, in triplicate, into a 96 well plate, covered and incubated at 37°-38°C for 15 minutes. Immediately following incubation, the plate was analyzed with SoftMax Pro Software version 5 (Molecular Devices Corporation, Sunnyvale, CA).

Statistical Analysis:

Statistical analyses and graphs were prepared using Graph Pad Prism (version 5, La Jolla, CA, USA). Summed data for the groups’ locomotor response to novelty and saline injection as well as baseline release and tau were compared across groups using a two-tailed Student’s t-test. Locomotor data (in time bins) and voltammetric data (quinpirole and ethanol concentration response curves) were compared using a two-way analysis of variance (ANOVA) with strain and dose as the factors. When a significant main effect was obtained (p < 0.05), Bonferroni post-hoc analysis was used to determine significant effects.
**Results**

*DBA mice exhibited enhanced locomotor responses to ethanol.*

To study the behavioral sensitivity of DBA and C57 mice to ethanol, we determined ethanol-induced locomotor activity over a range of ethanol doses (0.125-2.0 g/kg). Two-way ANOVA revealed a main effect of strain on ethanol-induced locomotor behavior ($F_{1, 19} = 14.68, p < 0.001$; Figure 1). Bonferroni post hoc analysis revealed a significantly greater locomotor response of DBA mice at the 0.25 ($p < 0.05$) and 0.5 ($p < 0.01$) g/kg doses. Analysis revealed no difference between DBA and C57 mice, with respect to their locomotor response to a saline injection, indicating that basal locomotor activity did not differ between the two strains. Because DBA and C57 do not differ in their responses to a saline injection, their differential behavioral responses to ethanol cannot be attributed to disparate baseline locomotor activity levels.

*DBA mice had reduced locomotor responses to a novel environment.*

To determine if the enhanced response to ethanol in DBA mice was specific to ethanol or was due to an enhanced response to all locomotor-activating stimuli, we examined locomotor responses to a novel environment in DBA and C57 mice. Two-way ANOVA revealed a main effect of strain on response to novelty ($F_{1, 39} = 13.45, p < 0.01$; Figure 2A). Bonferroni post hoc analysis revealed a reduced response to novelty in DBA mice, compared to C57 mice during the first 10 ($t = 6.972, p < 0.01$), 55 ($t = 3.876, p < 0.01$), 60 ($t = 3.878, p < 0.01$) 65 ($t = 3.600, p < 0.01$), 70 ($t = 3.132, p < 0.05$), 75 ($t = 3.599, p < 0.01$), 80 ($t = 3.943, p < 0.01$) and 85 ($t = 3.602, p < 0.01$) minute time points.
Figure 1. DBA mice exhibited enhanced ethanol-induced locomotor responses.

DBA mice exhibited an enhanced locomotor response over a dose response curve for ethanol, as compared to C57 mice. Data is summed over the first 30 minutes post-ethanol or saline injection. *p < 0.05; **p < 0.01
Figure 2. DBA mice exhibited reduced responses to a novel environment.

(A) DBA mice showed a reduced response to a novel environment compared to C57 mice over a 120-minute locomotor session. (B) Summed data from the 120-minute locomotor session. *, p < 0.05; **, p < 0.01; ***, p < 0.001; min, minute.
*DBA and C57 mice did not differ in blood ethanol concentrations following ethanol administration.*

Because it is possible that strain differences in blood ethanol elimination time could be driving differences in locomotor responses to ethanol, we examined the time course of BECs after a 0.5 g/kg i.p. injection in DBA and C57 mice. We found a significant effect of time on BECs (two-way ANOVA: $F_{6,20} = 32.55, p < 0.001$). However, we found no differences between strains with respect to blood ethanol elimination rate at any time point tested (Figure 3).

![Figure 3](image.png)

**Figure 3. DBA and C57 mice exhibited similar ethanol elimination time courses.**

The time course of ethanol clearance, as measured by blood ethanol concentrations (BECs) over time, was determined in C57 and DBA mice following a 0.5 g/kg ethanol challenge. There were no significant differences in ethanol clearance between the strains. Min, minute; i.p., intraperitoneal; BEC, blood ethanol concentration.
DBA and C57 mice had similar presynaptic dopamine dynamics and autoreceptor sensitivity in the NAc core.

Because we found robust differences in dopamine-mediated behaviors, we aimed to determine if there were differences in striatal dopamine system functioning between strains at baseline. To do this, we examined evoked dopamine release and tau, a measure of dopamine clearance, in the NAc core. We found no differences between strains in regards to dopamine release (Figure 4B, left) or clearance in this region (Figure 4B, right).

Additionally, we assayed D2-like autoreceptor activity in the NAc core by conducting dose-response curves for the D2/D3 agonist, quinpirole. A repeated measures two-way ANOVA revealed a significant effect of quinpirole concentration on dopamine release ($F_{4,19} = 57.81, p < 0.001$), where quinpirole dose-dependently reduced evoked dopamine release. The effects of quinpirole on dopamine release were similar between strains, demonstrating that D2-like autoreceptor function was not different (Figure 4C).

DBA mice have faster dopamine clearance in the CPu.

Next, we aimed to determine if differences between strains were present in the CPu. We found that DBA and C57 mice have similar dopamine release in the CPu (Figure 5B, left); however, a Student’s t-test revealed that DBA mice had a faster rate of dopamine clearance in this region (Figure 5B, right; $t_{13} = 9.43, p < 0.05$).

In order to determine the D2-like autoreceptor function between the strains, we ran concentration-response curves for quinpirole. A repeated measures two-way ANOVA
Figure 4. Dopamine release and clearance in the nucleus accumbens (NAc) core of DBA and C57 mice.

DBA and C57 mice have similar presynaptic dopamine dynamics in the NAc core. (A) Raw dopamine traces from the NAc core of C57 (left; red) and DBA (right; blue) mice. (B) Electrically evoked dopamine release (left) and tau (dopamine clearance, right) were similar between strains. (C) Quinpirole, a D2-like autoreceptor agonist, was applied to brain slices containing the NAc core to determine autoreceptor sensitivity. There were no differences between strains with respect to autoreceptor sensitivity. DA, dopamine; Stim, stimulation.
revealed a significant effect of quinpirole concentration on dopamine release ($F_{4,13} = 41.17, p < 0.001$), where quinpirole significantly decreased dopamine release over increasing concentrations of the compound. We found no strain differences in autoreceptor function as both strains had similar sensitivity to the effects of quinpirole (Figure 5C) in the CPu.

The effects of ethanol on evoked dopamine release striatal subregions were not different between DBA and C57 mice.

To determine if increased ethanol-induced locomotion in DBA mice is mediated by ethanol effects on striatal dopamine terminals, we examined the effects of ethanol at the terminal by bath application of increasing doses of the drug over brain slices. A two-way repeated measures ANOVA revealed a significant effect of ethanol in both the NAc core ($F_{3,15} = 14.91, p < 0.001$) and CPu ($F_{3,12} = 16.23, p < 0.001$), where increasing concentrations of ethanol significantly reduced evoked dopamine release. We found that DBA and C57 mice have similar responses to bath-applied ethanol in both the NAc core (Figure 6A) and CPu (Figure 6B).

In addition to determining the effects of ethanol on evoked dopamine release, we also determined the effects of ethanol on dopamine clearance. Dopamine clearance is mediated by the DAT, thus changes in the clearance of dopamine following bath application of ethanol can give information as to how ethanol alters DAT function. Contrary to previously published reports, we showed no effect of ethanol on dopamine clearance. Further, DBA and C57 mice have similar synaptic dopamine clearance.
Figure 5. Dopamine release and clearance in the caudate-putamen (CPu) of DBA and C57 mice.

(A) Raw dopamine traces from the CPu of C57 (left; red) and DBA (right; blue) mice. 
(B) Strains were similar in electrically evoked dopamine release (left), however DBA mice had a faster tau, indicating increased dopamine clearance (right). 
(C) The sensitivity of D2-like autoreceptors in the CPu was not different between the two strains. *, p < 0.05; DA, dopamine; Stim, stimulation.
Figure 6. The effects of ethanol on dopamine terminals in the NAc core and CPu were similar between strains.

DBA and C57 mice had similar dopamine responses to bath applied ethanol in both the NAc core (A) and CPu (B). Furthermore, brain slices from DBA and C57 mice demonstrated similar dopamine clearance rates (tau) in both the NAc core (C) and CPu (D) in the presence of increasing concentrations of ethanol. DA, dopamine.
measures in the NAc core (Figure 6C) and CPu (Figure 6D) in the presence of increasing concentrations of ethanol.

**Discussion**

Here we show that, although ethanol-induced locomotor activity is enhanced in DBA versus C57 mice, this difference is not due to ethanol’s effects on dopamine release or DAT activity. The ability of ethanol to reduce stimulated dopamine release in the NAc core and CPu was similar between the two strains of mice. Also, evoked dopamine release and uptake were comparable between the two strains, except in the CPu, where DBA mice exhibited faster clearance. Although some work has suggested that ethanol has direct effects at the DAT, here we show that ethanol does not influence dopamine uptake via the DAT in either the NAc core or CPu. Previous microdialysis work has shown that ethanol increases dopamine levels in the striatum to a greater extent in DBA mice (23), and this likely mediates the enhanced locomotor activity in this strain. Additionally, our data suggest that differences in ethanol-mediated increases in dopamine levels observed previously between the two strains may not be due to differential pharmacokinetic effects of ethanol, because elimination rates were the same. Previous work has suggested that ethanol-induced increases in dopamine levels are due to both increased dopamine cell firing in the VTA (13, 14) as well as ethanol effects on striatal dopamine terminals (16). We suggest that the elevations in dopamine levels observed in in vivo models following ethanol administration are most likely not due to the effects of ethanol on dopamine terminals.

The enhanced response to ethanol in DBA mice as compared to C57 mice is not due to an enhanced response to all locomotor-activating stimuli, as DBA mice showed a
reduced response to novelty. Responses to novelty have been shown previously to correlate with acquisition of stimulant self-administration and addiction vulnerability for these compounds (24-26). Accordingly, C57 mice, which have enhanced novelty responses, are also more sensitive to the locomotor activating effects of psychostimulants (27, 28). However, while DBA mice are less sensitive to the behavioral activating effects of stimulants, they are more sensitive to ethanol, as highlighted by enhanced ethanol-induced CPP (2, 3) and locomotor activity (7, 8). These data, combined with previous work, highlight the unique effects of ethanol, as DBA mice do not exhibit an increased sensitivity for all drugs of abuse. Additionally, these data suggest that there is not an overall hyperactivity of the dopamine system in DBA mice, but rather, drug-specific behavioral differences between strains.

Although differences in locomotor activity point to differential striatal dopamine system functioning between DBA and C57 mice, our data indicate that these effects do not occur at the level of the dopamine terminal. It has been shown previously that DBA mice have enhanced ethanol-induced dopamine overflow, an effect that is likely mediating the enhanced ethanol-induced locomotion in this strain (23). Electrophysiological reports using brain slices containing VTA dopaminergic cell bodies suggest that DBA mice have an enhanced firing rate in response to bath applied ethanol, as compared to C57 mice (13, 14). The enhanced firing could be responsible for in vivo increases in dopamine overflow, and could explain the locomotor differences between the two strains. In addition, postsynaptic dopamine receptors, which have been shown to have differential expression levels between the two strains, may play a role in the behavioral disparities (29).
Although previous research has pointed to the DAT as being altered by acute ethanol exposure (16-18), it remained uncertain as to whether ethanol increases, decreases or does not alter the function of dopamine transporters in the striatum. Here we show that ethanol does not change dopamine clearance. Voltammetric analyses of the effects of ethanol on DAT function in an in vivo preparation have found ethanol-induced decreases in dopamine uptake in the olfactory tubercle, an effect that could lead to increased dopamine levels following ethanol administration (16). However, because this work was conducted in vivo, it is possible that ethanol effects on other neurotransmitters systems are involved in modulating dopamine dynamics, including uptake. Ex vivo voltammetry allows for the isolation of dopamine terminals separate from afferent inputs, which allows for the determination of ethanol effects directly at the DAT. Here we show that ethanol does not affect dopamine clearance by direct interactions with the DAT.

Here we demonstrate that ethanol-induced modulations of dopamine release and clearance at the level of the striatum are not mediating ethanol-induced locomotor activity. We demonstrate that the dopamine release inhibiting effects of ethanol do not differ between strains, and that ethanol does not have any direct effects at the DAT. Furthermore, our data adds to a body of literature showing that the effects of ethanol on the dopamine system are a balance of its inhibitory and excitatory effects. We show here that ethanol, when applied to the dopamine terminal, results in reduced stimulated release, while previous work has shown that ethanol, when bath applied to VTA cell bodies, results in enhanced firing. It is likely that these effects converge to result in the behavioral outputs that are observed following ethanol administration.
References:


APPENDIX II

DEVELOPMENT OF THE CURRENT CHRONIC INTERMITTENT ETHANOL EXPOSURE PROCEDURE

Jamie H. Rose
Seminal work examining alcohol use disorders in humans prompted the development of alcohol exposure paradigms of laboratory animals that model this chronic relapsing disorder. Largely, these procedures are considered to have construct validity via the expression of withdrawal symptoms following a period of ethanol abstinence, namely escalated ethanol drinking compared to control animals (Becker & Lopez, 2004; Lopez & Becker, 2005; Griffin et al., 2009). Some of the more popular paradigms that induce ethanol dependence-like symptoms include an ethanol liquid diet, whereby animals are given only a nutritionally balanced ethanol-containing liquid diet to consume for an experimenter-determined length of time (Pilström et al., 1972; Umathe et al., 2008; Perez & De Biasi, 2015), chronic ethanol injections, often several times a day over a series of days or weeks (Griffin et al., 2009; Iwaniec & Turner, 2013; Perez & De Biasi, 2015), and exposure to ethanol vapor for several hours per day, commonly over several weeks (Becker & Hale, 1993; Walker & Koob, 2008; Griffin et al., 2009; Nealey et al., 2011; Walker et al., 2011; present work). Some of these procedures engender increased ethanol drinking (Walker & Koob, 2008; Griffin et al., 2009; Nealey et al., 2011; Walker et al., 2011; present work), anxiety/compulsive-like (Valdez et al., 2002, 2004; Umathe et al., 2008; Valdez & Harshberger, 2012; Perez & De Biasi, 2015; present work) and depressive-like symptoms (Schulteis et al., 1995; Boutros et al., 2014) in rodents. However, not all procedures produce the classic negative reinforcing effects of ethanol, such as those listed. For example, extended exposure to oral ethanol and chronic ethanol injections do not cause an increase in ethanol intake after a period of abstinence (Griffin et al., 2009), while CIE exposure models alcohol use disorders as a disease of chronic relapse by providing animals with prolonged bouts of intense ethanol vapor exposure and
abstinence periods, similar to the human condition (Dahlgren, 1978; Becker & Lopez, 2004; Lopez & Becker, 2005; Griffin et al., 2009). In fact, this procedure reliably increases ethanol drinking (Becker & Lopez, 2004; Lopez & Becker, 2005; Griffin et al., 2009; present work) and anxiety/compulsive-like behavior (Pleil et al., 2015; present work) over repeated cycles of vapor exposure. As such, a pattern of CIE exposure was used in the present body of work.

Both mice and rats are used to examine CIE-induced changes in behavior and neurobiology, although these alterations are examined at distinct time-points due to inherent differences in procedure. For example, mice are treated with repeated cycles (four days) of ethanol vapor or air exposure (16 hours of exposure, 8 hours of room air) followed by three complete days of abstinence (Becker & Lopez, 2004; Lopez & Becker, 2005; Griffin et al., 2009; present work) while rats undergo several weeks of ethanol or air inhalation (14 hours) with daily abstinence sessions (10 hours) for multiple weeks without a prolonged abstinence period (Walker & Koob, 2008; Walker et al., 2011; Nealey et al., 2011). As such, inhalation-induced changes in ethanol drinking in mouse models is typically examined between each CIE cycle (Becker & Lopez, 2004; Griffin et al., 2009) while the effects of CIE exposure on ethanol consumption in rats occurs after the inhalation exposure treatment (Walker et al., 2008; Nealey et al., 2011; Walker et al., 2011). The ethanol exposure protocol used in the current work is a hybrid of these procedures. Our experimental procedure used C57 mice that established ethanol drinking prior to CIE exposure, underwent five weeks of CIE or air exposure (four days inhalation treatment/three days room air exposure) followed by a five-day post-CIE exposure drinking test or neurobiological examination. Notably, we report increased ethanol
consumption, preference and anxiety/compulsive-like behavior when tested 72 hours following the final CIE or air exposure.
REFERENCES


APPENDIX III

GENERAL METHODOLOGY

Jamie H. Rose
1. Subjects

Male C57 mice (65-75 days old; Jackson Laboratories, Bar Harbor, ME) were used for all experiments. Mice were individually housed and maintained on a 12:12 hour light cycle (off 14:00), with a red room light illuminated during the animals’ dark cycle (14:00-02:00). Mice habituated to housing conditions for at least one week before experiment start and were provided with standard rodent chow and water *ad libitum*, unless otherwise noted. Chow exposed to ethanol vapor was replaced each morning. The Institutional Animal Care and Use Committee at Wake Forest University School of Medicine approved all experimental protocols. All animals were cared for according to National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities.

2. Chronic intermittent ethanol/air exposure

The basis for all ethanol exposure protocols is adapted from a well-established protocol developed by the Becker Laboratory (Becker & Hale, 1993; Becker & Lopez, 2004; Lopez & Becker, 2005; Griffin et al., 2009). In short, mice were exposed to ethanol vapor or room air for four days (16 hours of exposure/8 hours of abstinence), followed by 72 hours of room air exposure (one cycle). Cycles were repeated five times. All CIE-exposed animals were treated with 1.6 g/kg ethanol (CIE exposure) or saline (air exposure) and 1.0mM pyrazole (Sigma Aldrich, St. Louis, MO) an ethanol dehydrogenase inhibitor, approximately 30 minutes prior to chamber start time. Blood ethanol concentrations (BEC) were tested after the first and fourth inhalation exposure to ensure proper chamber function and physiologically relevant BECs (Figure 8; Griffin et al., 2009).
3. Blood collection and analysis for BEC determination

Blood samples of CIE-exposed mice were obtained immediately after removal from the vapor chamber on the mornings after the first and final exposure per cycle. A submandibular vein puncture was performed to collect no more than 15µL of blood from no more than two animals per collection day. Samples were held in microtainer tubes lined with lithium heparin (Becton Dickinson & Company, Franklin Lakes, NJ) before processing. Blood collection volumes did not reach the maximum set forth by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine. For BEC measurement, standards and samples were prepared using a commercially available alcohol dehydrogenase assay (Carolina Liquid Chemistries Corporation, Brea, CA). Briefly, five microliters of each blood collection was placed in a container with 45µl of trichlorocetic acid solution (Sigma Aldrich, St. Louis, MO), and centrifuged at 10,000 revolutions per minute at room temperature for ten minutes. 30µl of the supernatant of each sample was removed and placed into a separate container with 300µl of buffer and 112.5µl of enzymatic solution provided in the alcohol dehydrogenase assay. 100µl of each standard and sample was loaded, in triplicate, into a 96 well plate, covered and incubated at 37°-38°C for 15 minutes. Immediately following incubation, the plate was analyzed with SoftMax Pro Software version 5 (Molecular Devices Corporation, Sunnyvale, CA).
Figure 8: Ethanol vapor exposure maintains elevated BECs

Mice in CIE-exposure groups are systemically injected with 1.6g/kg ethanol + 1.0mmol/kg pyrazole at approximately 16:30, 30 minutes prior to chamber start-time (17:00). Administration of this solution raises BECs (blue dotted line), which are then maintained by ethanol vapor exposure (green dashed line) until the vapor chambers are stopped by the experimenter (09:00). Dark portion: dark cycle. Ordinate: Ethanol concentration. Abscissa: time.
4. Ex vivo fast scan cyclic voltammetry

*Ex vivo* fast scan cyclic voltammetry is a neurobiological technique that allows for spatiotemporal-specific examination of dopamine kinetics (Ferris *et al*., 2013). Using this technique, pre-drug accumbal dopamine release and uptake kinetics, as well as changes in presynaptic receptor function, were examined in the NAc core after five cycles of CIE or air-exposure. In all experiments, mice were sacrificed 72 hours after the fifth inhalation exposure to coincide with the onset of all behavioral testing. Mice were anesthetized with isoflurane, rapidly decapitated and brains removed. Brain slices containing the NAc core (300μm thick) were prepared with a vibrating tissue slicer and incubated in oxygenated artificial cerebrospinal fluid (aCSF, 32°C). A carbon fiber recording electrode (∼50-100μM length, 7μM radius; Goodfellow Corporation, Berwyn, PA), and a bipolar stimulating electrode (Plastics One, Roanoke, VA) were placed (∼100μM apart) on the surface of the slice. Dopamine efflux was induced with a single, rectangular, 4.0ms long electrical pulse (350μA, monophasic; inter-stimulus interval: 180sec). To detect dopamine release, a triangular waveform (-0.4 to +1.2 to -0.4V vs. silver/silver chloride, 400 V/sec) was applied every 100ms to the recording electrode. Proper identification of electroactive neurotransmitters in voltammetry is ensured via a species-specific cyclic voltammogram, which identifies the peak oxidation and reduction potentials of the neurotransmitter, as dopamine is oxidized at to dopamine-o-quinone at +0.6V, and reduced at -0.2V (Heien *et al*., 2003). To obtain clear current versus time plots, background current subtraction methods were utilized. To assess dopamine terminal function, all compounds were added cumulatively to aCSF when baseline collections
were stable. All compounds were obtained at the highest quality from Tocris Bioscience (Bristol, UK) unless otherwise noted.

All graphs were created and statistical tests were applied using GraphPad Prism, Version 5 (La Jolla, CA). Baseline apparent $K_m$ was set to 160nM for each slice in both regions, based on the known affinity of dopamine for the DAT (Wu et al., 2001) while pre-drug $V_{\text{max}}$ values were allowed to vary. To ensure proper detection of dopamine release, specific experimental measures, notably the cyclic voltammogram, are used. Cyclic voltammograms allows experimenters to specifically identify the electroactive species being examined, particularly dopamine. Electrode calibration was performed immediately following each experiment using a flow-injection system. Traces that did not return to baseline were minimally corrected using baseline correction techniques within the Demon software.

5. Ethanol drinking procedures

Animals in ethanol drinking groups began a two bottle choice procedure after housing habituation (two weeks of sucrose fade [Samson, 1986]; two days each: 10% ethanol/5% sucrose, 12% /5%, 15% /5%, 15% /2%, and 15% /1% (w/v), followed by four additional weeks of access to 15% [v/v] ethanol). Experimental solutions were provided for two hours per day (13:30-15:30), five days per week, and paired with an identical bottle of water. Bottle placement was switched daily. Mice were assigned to CIE or air inhalation groups immediately prior to five cycles of CIE/Air exposure (below), counterbalanced for intake. Ethanol drinking was examined for five days after all cycles (Fig. 1B). Separate groups of animals were systemically injected with norBNI (10mg/kg i.p.; NIDA, Bethesda, MD) or vehicle (saline) 24 hours prior to the ethanol drinking test.
6. Marble burying behavior

Pre-inhalation marble burying tests occurred on the morning of Cycle 1. Mice were placed in a testing chamber, a polycarbonate cage lined with 3.5 cm of cob bedding (The Andersons, Inc., Maumee, OH) for 120 minutes (Rose et al., 2013) before the addition of 20 clean, black glass marbles (14mm, Rainbow Turtle, Portland, OR). A template was used to ensure consistent placement of marbles (Fig. 4A, left panel). After the 30-minute testing session, mice were returned to their home cages. The number of marbles over 75% buried was counted in analysis. Water was provided during the habituation session while both food and water were restricted during the test session. Animals were placed into inhalation groups, counterbalanced for the number of marbles buried during the pre-test. CIE/Air-induced changes in marble burying were assessed 72 hours after the fifth cycle. Separate groups of mice were systemically injected with norBNI (10mg/kg) or saline 24 hours prior to post-inhalation treatment testing.
REFERENCES


APPENDIX IV:

CURRICULUM VITAE

JAMIE HANNAH ROSE
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PERSONAL INFORMATION

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EDUCATION

Jan 2016-Mar 2018
Elon University Physician Assistant Program
Elon, NC

Aug, 2010-Present
Wake Forest School of Medicine
Winston-Salem, NC
Advisor, Sara R. Jones, Ph.D.
Ph.D. in Neuroscience

Aug, 2006-Dec, 2010
High Point University
High Point, NC
Advisor, Dinene Crater, Ph.D.
Bachelor’s of Science Degree acquired, Biology
Graduated Summa Cum Laude

Aug, 2006-Dec, 2010
High Point University
High Point, NC
Advisor, Deborah Danzis, Ph.D.
Bachelor’s of Science Degree acquired, Psychology
Minor concentration, Chemistry
Graduated Summa Cum Laude
RESEARCH EMPLOYMENT HISTORY

Graduate
Aug, 2010-Present
Doctoral Candidate
Department of Physiology and Pharmacology
Program in Neuroscience
Wake Forest School of Medicine
Laboratory of Sara R. Jones, PhD

Dec-Aug, 2010
Clinical Research Assistant
Mendenhall Clinical Research Center
High Point, NC
Melanie Fein, MD

Undergraduate
June-Aug, 2009
Research Assistant
Behavioral Pharmacology
Emory University
Laboratory of Leonard Howell, PhD
Mentor, Heather Kimmel, PhD

HONORS AND AWARDS

Dec, 2014
Awarded 3rd place in poster and presentation during the
Wake Forest University Health Sciences Neuroscience
Poster Day

Feb, 2014
Alumni Student Travel Award to attend the Gordon
Research Conference: Alcohol and the Nervous System
Meeting in Galveston, TX

Oct, 2013
Individual Ruth L. Kirschstein National Research Service
Award (Graduate NRSA; F31 DA035558), Wake Forest
School of Medicine

Jun, 2013
Research Society on Alcoholism (RSA) Student Merit
Award from the RSA to attend the 36th Annual RSA
Scientific Meeting in Orlando, FL

Jun, 2013
Alumni Student Travel Award to attend the 36th Annual
Research Society on Alcoholism Scientific Meeting in
Orlando, FL
Aug, 2012  Ruth L. Kirschstein Institutional National Research Service Award (Graduate NRSA; T32 AA07565), Wake Forest School of Medicine

Mar, 2007-Dec, 2010  Alpha Chi – Academic Honor Society President

PROFESSIONAL MEMBERSHIPS

Mar, 2013 - Present  Research Society on Alcoholism
Aug, 2012 - Present  Western North Carolina Society for Neuroscience
Jan, 2008 - Present  Beta Beta Beta Biology Honor Society
Apr, 2007 - Present  Psi Chi Psychology Honor Society
Mar, 2007 - Present  Alpha Chi, Academic Honor Society,

Ad hoc manuscript reviewer for Neuropharmacology

SERVICE: VOLUNTEERING AND OUTREACH

Institutional

July, 2015  National Youth Leadership Forum organizer
June, 2015  SciTech Institute Outreach presenter
Feb, 2012 - Present  Authorship contributor, Western North Carolina Society for Neuroscience newsletter, The Neurotransmitter
Mar, 2011 - Present  Brain Awareness Council Chair for Brain Awareness Week
Dec, 2010 - Present  Presenter, Kernersville Cares for Kids Initiative
Nov, 2010 - Present  Presenter, Brain Awareness Council School Visits

Student Mentorship / Training

July, 2014-Present  Sarah Ewin, Wake Forest University, Integrated Physiology and Pharmacology Graduate Program
Feb, 2014 - Present  Deborah Luessen, Wake Forest University, Integrated Physiology and Pharmacology Graduate Program
Jan-Aug, 2015  Molly McGinnis, Wake Forest University, Neuroscience Graduate Program

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Aug-Dec, 2014  Josh Seideman, Wake Forest University, Neuroscience Graduate Program

Mar-Aug, 2014  Stephanie Saadeh, Vanderbilt University, Excellence in Cardiovascular Sciences Research Program

Jun-Oct, 2012  Crystal Bolden, Wake Forest School of Medicine, Post-Baccalaureate Program

PUBLICATIONS


* Editor’s choice, July, 2015 issue.

*Authors contributed equally to work

Rose JH, Calipari ES, Matthews TJ, Jones SR (2013) Greater ethanol-induced locomotor activation in DBA/2J versus C57BL/6J mice is not predicted by presynaptic striatal dopamine dynamics. PLOS One 8(12): e83852.

Published Abstracts


PRESENTATIONS

Institutional / Departmental

Rose, JR (2015, March). Increased kappa opioid receptor function may underline ethanol-induced increased compulsive behaviors and decreases in dopamine signaling. Program in Neuroscience Seminar, Wake Forest School of Medicine, Winston-Salem, NC.

Rose, JR (2013, November). Chronic Intermittent Ethanol Treatment Results in Increased Ethanol Consumption and Preference in C57BL/6J Mice. Program in Neuroscience Seminar, Wake Forest School of Medicine, Winston-Salem, NC.

Rose, JR (2013, April). Dopamine Dynamics and Psychomotor Activation in a Mouse Model of Ethanol Use Vulnerability. Program in Neuroscience Seminar, Wake Forest School of Medicine, Winston-Salem, NC.
Rose, JR (2013, February). Differential Dopamine System Dynamics and Behavioral Responses in Mouse Models of Alcohol Vulnerability. Department of Physiology and Pharmacology Seminar, Wake Forest School of Medicine, Winston-Salem, NC.

Rose, JR (2012, August). Amphetamine Reverses Cocaine-Induced Changes in the Dopamine Transporter. Neuroscience Graduate Student Summer Seminar, Wake Forest School of Medicine, Winston-Salem, NC.

Invited Presentations


DISSERTATION PROJECT

Title: The effects of chronic intermittent ethanol exposure on withdrawal phenotypes, dopamine terminal function and kappa opioid receptor sensitivity in the nucleus accumens
CURRENT RESEARCH SUPPORT
F31DA035558 Rose
Mentor: Sara. R. Jones, Ph.D.
Recurrent
NIDA
Role: Principal Investigator/Graduate Student.

Project Title: Cocaine-Induced Dopamine Transporter Changes are Reversed by Amphetamine

Project Summary/Abstract:
Cocaine dependence in the United States is a major health problem with no pharmacotherapeutic treatment. To study this condition in the laboratory, rats self-administer compounds, particularly cocaine, intravenously. We have used this model to show that extended exposure to cocaine (1.5mg/kg/infusion, 40 infusions/day, 5 days) results in a pharmacodynamic tolerance of the dopamine transporter to cocaine (Ferris et al., 2015). Notably, we also found that administration of a single amphetamine bolus (0.56 mg/kg IV) rapidly and completely reversed pharmacodynamic tolerance of the dopamine transporter to cocaine, and even augmenting the potency of this compound above control levels. The specific aims of this grant include experiments that characterize and expand on this discovery. In Specific Aim 1, we will explore the lowest effective dose of amphetamine that can cause a reversal of dopamine transporter plasticity and the time course of this effect. Although this characterization is important, we are primarily interested in discovering the structural and functional changes in the dopamine transporter that are responsible for this effect. Thus, in Specific Aim 2, we will use structurally different dopamine releasers and blockers with different dopamine transporter affinities in place of an amphetamine bolus and use slice voltammetry to explore cocaine potency and presynaptic dopamine function (release and uptake) in response to these compounds. Additionally, we are interested in the behavioral implications for our neurochemical data. In Specific Aim 3 we will test animals before and after self-administration and an amphetamine bolus with locomotor assessments while another group will be assessed on a progressive ratio schedule of cocaine self-administration. While our
interest is primarily in the basic pharmacology driving the dopamine transporter changes, this may be relevant to the literature proposing dopamine transporter releasers such as amphetamine as putative cocaine addiction pharmacotherapies, as agonist therapies are commonly used for indications such as heroin (methadone) and cigarettes (nicotine patches, gum). Although agonist therapies for cocaine addiction are controversial, we may be able to define a putative mechanism for some of their therapeutic effects which could potentially drive more rationale design of such therapies.

**COMPLETED REASEARCH SUPPORT**

T32 AA07565 Rose  
Mentor: Sara. R. Jones, Ph.D.  
2012-2013  
NIAAA  
Role: Graduate student.  

**Project Title:** Differential dopamine system dynamics in model systems for alcohol vulnerability: Comparison of C57BL/6 and DBA/2 neurochemical and behavioral measures.

**Project Summary/Abstract:**

A large body of research has aimed to determine the neurochemical factors driving differential sensitivity to ethanol between individuals, in an attempt to find predictors of ethanol abuse vulnerability. Here, we find that the locomotor activating effects of ethanol are markedly greater in DBA/2J compared to C57BL/6J mice, although it is unclear as to what neurochemical differences between strains mediate this behavior. Dopamine elevations in the nucleus accumbens and caudate-putamen regulate locomotor behavior for most drugs, including ethanol; thus, we aimed to determine if differences in these regions predict strain differences in ethanol-induced locomotor activity. Ex vivo voltammetry allows for the determination of ethanol effects on presynaptic dopamine terminals, independent of drug-induced changes in firing rates of afferent inputs from either dopamine neurons or other neurotransmitter systems. Previous studies suggest that ethanol interacts with the dopamine transporter,
potentially mediating its locomotor activating effects; however, using fast scan cyclic voltammetry, we found that ethanol had no effects on dopamine uptake in either strain. However, differences in striatal dopamine dynamics did not predict the locomotor-activating effects of ethanol, since the inhibitory effects of ethanol on dopamine release were similar between strains. There were differences in presynaptic dopamine function between strains, with faster dopamine clearance in the caudate-putamen of DBA/2J mice; however, it is unclear how this difference relates to locomotor behavior, although this finding may have implications for the augmented anxiety that this strain displays. Because of the role of the dopamine system in reinforcement and reward learning, differences in dopamine signaling between the strains could have implications for addiction-related behaviors that extend beyond ethanol effects in the striatum.

TECHNICAL PROFICIENCY

Since beginning research in neuroscience in August, 2011, I have acquired proficiency in a number of techniques used in mice and rats. Training received in the laboratories of Rong Chen (WFU), Sara Jones (WFU), and Dave Roberts (WFU) include biochemical assays for western blot hybridization, in vitro voltammetry, various behavioral assays (e.g., locomotor assessment, conditioned place preference, forced swim test, marble burying assays), ethanol drinking and vapor chamber procedures, blood ethanol concentration calculation as well as operant procedures such as rodent self-administration of psychostimulants. Data rendered by projects are graphing and analyzed using statistical packages, particularly GraphPad Prism.

CURRENT RESEARCH INTERESTS

Although alcoholism is a rampant social, economic and financial burden in the United States, there are only three Food and Drug Administration-approved pharmacotherapies for this indication, and none are completely efficacious. Previous work from our laboratory indicates that striatal dopamine system signaling decreases with chronic ethanol exposure. It has been speculated that chronic ethanol exposure-induced reductions in dopamine system function in the nucleus accumbens may drive
withdrawal phenotypes, namely increased withdrawal symptoms that lead to relapse drinking. Because opioid receptors are known to modulate dopamine neurotransmission, it is plausible that ethanol-induced changes in kappa opioid receptor function may underlie aberrant dopamine system signaling during ethanol withdrawal. Considerable research points to the utility of the kappa opioid receptor as a pharmacotherapeutic target to relieve the symptoms of alcohol withdrawal, which often drive relapse drinking in the human alcoholic. As such, I am interested in how changes in kappa opioid receptor function affects mesolimbic dopamine/reward system function and withdrawal-mediated phenotypes, namely ethanol drinking parameters and marble burying, a measure of anxiety/compulsive behavior, following five weeks of chronic ethanol exposure. Notably, kappa opioid receptors are not the only presynaptic dopamine regulating protein, thus, my interests also expand to D2 and D3 autoreceptors, and interactions between kappa opioid receptors and autoreceptors. Because the striatal dopamine system is a critical regulator of reward, restoration of proper striatal dopamine signaling may alleviate some of the withdrawal symptoms of chronic ethanol exposure and withdrawal, and curtail ethanol drinking during abstinence.