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Involvement and Neuroplasticity of Cholinergic Interneurons of the Nucleus Accumbens in Initiation and Excessive Alcohol Drinking

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Involvement and Neuroplasticity of Cholinergic Interneurons of the Nucleus Accumbens in Initiation and Excessive Alcohol Drinking

by

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Dedication

For my parents, Martha Lane and Ted Camp

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Involvement and Neuroplasticity of Cholinergic Interneurons of the Nucleus Accumbens in Initiation and Excessive Alcohol Drinking

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Alcoholism is a complex disease that exists as a specific set of behaviors, such as the preoccupation with obtaining alcohol and compulsive alcohol drinking. Currently, more than 18 million adults in the United States suffer from alcohol abuse or alcoholism. This disease poses serious medical and economic consequences for society. Identifying the neurobiological mechanisms that underlie alcohol drinking, specifically the transition from initiation to binge drinking is critical for improved treatments for alcoholics and the vulnerability for relapse in those recovering. Many studies have identified brain regions and molecular mechanisms that underlie various stages of alcohol abuse; however few have investigated the role of specific cell types within these areas.

The overarching hypothesis of the studies in this dissertation is that cholinergic interneurons of the nucleus accumbens (NAc) are key neural substrates that underlie alcohol drinking, and as drinking continues; neuroadaptations within these cells then

facilitate such behaviors as compulsive alcohol drinking. More specifically, these studies tested whether 1) cholinergic cell ablation in the NAc causes a decrease in alcohol drinking in C57BL/6J mice, 2) neuroadaptive changes in dopamine (DA) D2 receptor and cyclin dependent kinase 5 (Cdk5) occur within these cells following initiation alcohol drinking, and to a greater extent following binge alcohol drinking in C57BL/6J mice, and 3) neuroadaptive changes in DA D2 receptor and Cdk5 also occur in brain regions that have been implicated in the rewarding and reinforcing effects of alcohol in inbred alcohol-preferring (iP) rats. The present findings report a causal role for accumbal cholinergic neurons in binge alcohol drinking and identify DA D2 receptor and Cdk5 neuroadaptations following initiation and binge alcohol drinking.

These studies identify the involvement of cholinergic interneurons in binge drinking and reveal alcohol-induced region- and cell-specific receptor and molecular changes that occur with continued drinking. These findings contribute to the understanding of the neurobiological mechanisms that underlie alcohol drinking, and provide the basis for cholinergic targeted treatments designed to attenuate binge drinking. These data also provide the groundwork for future studies aimed to examine receptor and intracellular molecular changes that occur with compulsive alcohol drinking, craving, and relapse.

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Chapter 1: Introduction

1.1 Overview

Alcoholism is a complex disease that exists as a combination of physiological, behavioral, and cognitive impairments that lead to the preoccupation with obtaining alcohol, a loss of control over limiting intake, the development of tolerance and dependence, and impairment in social and occupational functioning (American Psychiatric Association, 1994). For an alcoholic the use of alcohol takes on a much higher priority than other behaviors that once had greater value, and for hundreds of years people considered addiction to be a problem of will power or moral failing. We now know that alcoholism is a disease much like other chronic illnesses, and like other chronic illnesses, understanding the underlying neurobiology of this disease will lead to the development of improved treatments. Numerous neurobiological studies have identified brain regions and molecular mechanisms that underlie various stages of alcohol abuse, yet the development of improved site-specific treatments will require a more in depth understanding of the role of specific cell types within those previously identified brain areas and the neuroadaptations that occur within those specific cell circuits with exposure to alcohol. The purpose of my dissertation research is to identify the involvement of a specific cell type, the cholinergic interneuron, in alcohol drinking and to examine changes in dopamine (DA) D2 receptor and Cdk5 expression that occur within this cell population following voluntary alcohol drinking. These findings may lead to improved region- and cell-specific pharmacotherapeutic and behavioral treatment programs for alcohol abuse and dependence.

The central hypothesis of these dissertation studies is that cholinergic interneurons of the nucleus accumbens (NAc) are key components that underlie alcohol drinking, and as drinking continues; neuroadaptive changes within these cells facilitate such behaviors as compulsive alcohol drinking. The studies in this dissertation focus on the involvement and ~~neuro~~plasticity of cholinergic interneurons, specifically in the NAc. This is not to say that the NAc is the only, or most important, brain region underlying alcohol drinking. This region, however, has long been identified in the reinforcing effects of alcohol and drug-seeking behavior and was therefore of interest to examine in these studies.

The first study (Chapter 2) examined a causal role of cholinergic neurons in the NAc on binge alcohol drinking. This study used a cell ablation technique to selectively destroy cholinergic interneurons of the NAc in C57BL/6J mice. The following 2 chapters discuss studies that employed immunocytochemical (ICC) procedures to examine the effects of alcohol drinking on cholinergic neuronal plasticity in C57BL/6J mice. More specifically, the study in Chapter 3 examined dopamine (DA) D2 receptor neuroadaptation in cholinergic interneurons of the NAc following a model of initiation alcohol drinking and a model of binge alcohol drinking. The following study (Chapter 4) examined cyclin dependent kinase 5 (Cdk5) neuroplasticity in cholinergic cells following the initiation and binge alcohol drinking models. The last study (Chapter 5) assessed the effects of 1 month of initiation drinking on both D2 receptor and Cdk5 neuroadaptations in the NAc of inbred alcohol-preferring (iP) rats. Chapter 6 provides a general discussion of the findings and concepts covered throughout the studies in this dissertation.

The remainder of this chapter provides background information for the studies presented in this dissertation. The first sections give an overview of alcoholism and the mechanisms of action of ethanol, and introduce microcircuits and brain regions that underlie this disease. Animal models of alcoholism used throughout the studies in this dissertation are presented in the following sections. The final sections provide cholinergic cell ablation background and give an overview of the receptor subtype and intracellular molecular mechanisms examined in Chapters 3-5.

1.2 Alcoholism

Alcoholism is a chronic relapsing disease that is characterized by a specific set of behaviors including persistent and intense alcohol-seeking, compulsive alcohol drinking, the development of tolerance and dependence, and an impairment in social and occupational functioning (American Psychiatric Association, 1994). Alcohol is the most widely used drug in the world today. In the United States alcohol use is greater than all other drugs of abuse combined (Substance Abuse and Mental Health Services Administration, 2006). Alcohol, or more specifically, ethanol, produces a wide range of effects in humans. It is a central nervous system depressant and produces characteristic mood elevation, anxiolytic, sedative and ataxic effects. In 2005 over half of Americans aged 12 or older (an estimated 126 million people) reported being current drinkers of alcohol; of these, 18.2 million were dependent on or had abused alcohol in the past year (Substance Abuse and Mental Health Services Administration, 2006). In 1998 the estimated economic costs of alcohol abuse in the US was over $184 billion (Harwood, 2000).

The overall goal of this dissertation is to identify the specific involvement of cholinergic interneurons in alcohol drinking, and to examine neuronal adaptations that occur within these cells following initiation and binge drinking. It is hypothesized that neuroadaptive changes in these cells can contribute to the regulation of such behaviors as compulsive alcohol drinking. Alcoholism is a widespread problem in today's society and poses serious health and economic consequences. It is therefore imperative that we gain a better understanding of the neurobiological factors that cause or contribute to this disease in order to develop improved pharmacotherapeutic treatments. More specifically, the findings from the studies reported in this dissertation may contribute to an improved understanding of the underlying neurobiology of alcoholism which will hopefully lead to the future development of cellular- and molecular-specific treatments for this disease.

1.3 Mechanisms of Action of Ethanol

Traditional theories concerning the actions of ethanol considered this molecule to have non-specific effects on neuronal membrane lipids, however more recent studies have revealed selectively for specific sites of action on neuronal proteins (e.g., Chandler et al., 1998; Davies, 2003; Harris, 1999; Tabakoff and Hoffman, 1996). It is currently thought that ethanol exerts its effects by altering the function of membrane-bound ligand-gated ion channels, voltage-dependent ion channels (Chandler et al., 1998; Davies, 2003; Harris, 1999; Tabakoff and Hoffman, 1996), as well as altering the function of second-messenger proteins (Macdonald, 1995; Pandey, 1998). More specifically, ethanol potentiates $GABA_A$ receptor currents, inhibits NMDA glutamate receptors, both inhibits and excites nicotinic ACh receptors, enhances activity of Ca^{2+} activated K^+ channels,

inhibits N- and P/Q-type Ca^{2+} channels, and modulates inwardly rectifying K^{+} channels (Davies, 2003; Pierce and Kumaresan, 2006).

Ethanol, therefore, produces various effects on neurotransmitter systems in the brain, which combine to produce specific behavioral actions such as mood elevation, sedation, anxiolytic, and ataxic effects. The initial rewarding effects of ethanol have been hypothesized to occur through the activation of the mesocorticolimbic DA system. The DA system is believed to play an important role not only in the initial rewarding effects of alcohol but also in habit or compulsive alcohol drinking. Brain areas such as the NAc, dorsal striatum (DS), and prefrontal cortex (PFC), which are targeted by the DA system, are of particular interest to examine in order to identify the neuroadaptive changes that underlie initial alcohol drinking, binge drinking, and the potential switch that occurs from one stage to the other.

1.4 Mesocorticolimbic Dopamine System

The mesocorticolimbic DA system (Figure 1.1) is a circuit that arises in the ventral tegmental area (VTA) and sends dopaminergic projections to limbic and cortical regions, including the NAc and PFC. This system has been implicated in the positive reinforcing effects of alcohol and other drugs of abuse (Di Chiara and Imperato, 1988, Di Chiara et al., 2004), and it has been identified as a critical component of alcohol self-administration (Pierce and Kumaresan, 2006). Most drugs of abuse, including alcohol, enhance activity in this system leading to an increase in DA release in terminal areas (Yim and Gonzales, 2000). For example, studies show that alcohol produces increased cell body firing in the VTA both in vitro (Brodie et al., 1990) and in vivo (Gessa et al.

1985). Increased activation of VTA DA neurons results in elevated extracellular DA levels in the rat NAc (Budygin et al., 2001; Di Chiara and Imperato, 1988; Gonzales et al., 2004; Imperato and Di Chiara, 1986; Yim and Gonzales, 2000), PFC (Gronier et al., 2000), and DS (Di Chiara and Imperato, 1988).

While not traditionally associated with the mesocorticolimbic DA system, the DS receives the majority of its dopaminergic afferents from the substantia nigra (SN). The pathway of dopaminergic projections from the SN to the DS is commonly referred to as the nigrostriatal pathway and has long been implicated in motor related function. Recently, however, the DS has been suggested to play a role in habit learning and long-term plasticity as casual drug use progresses to habitual drug-seeking (Berke and Hyman, 2000; Everitt and Wolf, 2002).

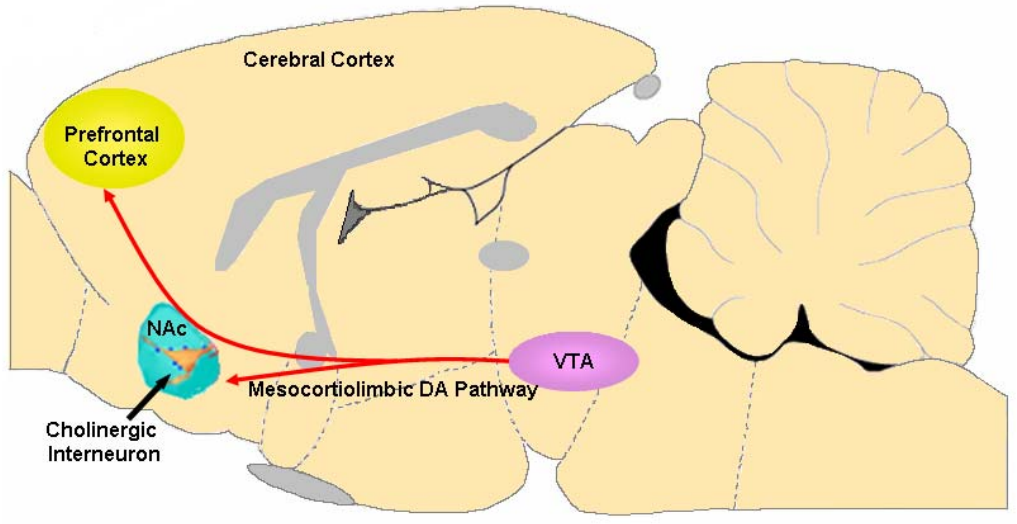

Figure 1.1. Schematic representation of the mesocorticolimbic DA pathway. In the mesocorticolimbic pathway, dopaminergic cell bodies located in the VTA send projections to the NAc and PFC. NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

1.5 Nucleus Accumbens

The NAc, the major area of the ventral striatum, is a primary brain region associated with the reinforcing effects of alcohol and other drugs of abuse (Di Chiara and Imperato, 1988; Koob, 2003; Koob and Bloom, 1988; Pontieri et al., 1995; Rassnick et al., 1992). This brain region is thought to integrate cortical and limbic information and generate goal-directed behaviors (Kalivas, 2004; Mogenson et al., 1980; Voorn et al.,

1986). The NAc is hypothesized to be a primary site of action for the initial effect of alcohol on mesolimbic DA activity (Löf et al., 2007), and microdialysis studies have recently shown that accumbal DA is increased in the first 5 minutes following ethanol availability (Doyon et al., 2003; Doyon et al., 2005). In addition, this brain region has been identified as the initial site of cellular and molecular adaptations in the brain following exposure to other drugs of abuse, such as cocaine (Macey et al., 2004). Specifically, it is thought that the more alcohol-induced DA that is released in the NAc, the greater the alcohol preference (Katner and Weiss, 2001).

The NAc is a heterogeneous structure comprised of two functionally distinct compartments, termed the shell and core (Figure 1.2), which have been characterized by histochemical, electrophysiological, in vivo neurochemical, morphological, and ultrastructural studies (Heimer and Alheid, 1991; Kelley, 2004; Zahm, 2000). The NAc contains various cell types, including GABAergic medium spiny projection neurons (MSNs), GABAergic interneurons, and cholinergic interneurons (Kawaguchi et al., 1995). The studies contained in this dissertation will focus specifically on the role of accumbal cholinergic interneurons in alcohol drinking.

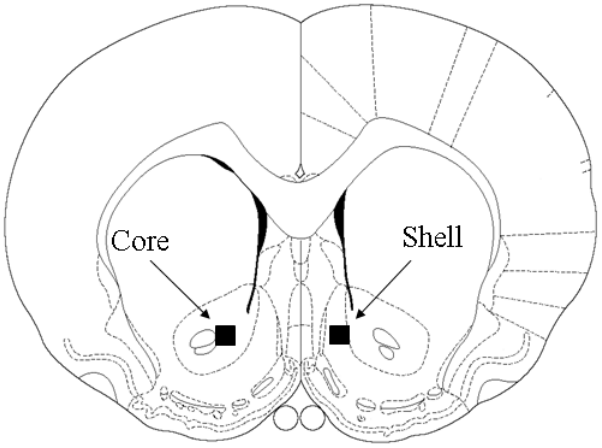

Figure 1.2. Schematic representation of a coronal section of the NAc in the mouse brain. The NAc (Bregma 1.10 mm) obtained from the mouse brain atlas (Franklin and Paxinos, 1997). The black boxes indicate the sample areas where quantifications were obtained for the core and shell NAc.

1.5.1 Shell and Core Compartments of the Nucleus Accumbens

The shell NAc receives dense dopaminergic innervations from the VTA and glutamatergic input from the hippocampus, amygdala, and PFC (Amalric and Koob, 1993; Gerfen et al., 1987), and sends GABAergic projections to the VTA, extended amygdala [comprised of the shell NAc, central nucleus of the amygdala (CNA), substantia innominata (SI), and bed nucleus of the stria terminalis (BNST)] (Alheid and Heimer, 1988; Koob, 1999), ventromedial ventral pallidum (VP) (Zahm and Heimer, 1990), lateral hypothalamus (LH), and mesencephalic tegmentum (Heimer et al., 1991). The shell is associated with limbic functions, via its efferent connections to the extended amygdala (Brog et al., 1993; Koob, 1999; Voorn et. al., 1986), the mediation of the

rewarding and reinforcing effects of drugs of abuse (McBride et al., 1999; Pontieri et al., 1995), and alcohol self-administration (Janak et al., 1999).

The core receives inputs from the SN, amygdala, and PFC (Zahm, 2000), and projects to the dorsolateral VP, subthalamic nucleus, SN and entopeduncular nucleus (Berendse et al., 1992; Heimer et al., 1991; Zahm and Heimer, 1990). The core has traditionally been associated with motor functions due to its connections with the SN and entopeduncular nucleus (Heimer et al., 1991; Nirenberg et al., 1996). Recent studies, however, associate the core NAc with drug-seeking, possibly due to glutamatergic influences from the PFC (Kalivas and Volkow, 2005), and it is hypothesized that the core mediates the expression of learned behaviors in response to motivationally relevant stimuli (Di Ciano and Everitt, 2001; Kelley, 2004). Additionally, the core NAc is associated with drug-induced sensitization (Li et al., 2004; Robinson and Kolb, 2004) and related synaptic rewiring (Berlanga et al., 2006).

1.5.2 Medium Spiny Projection Neurons of the Nucleus Accumbens

The major output neurons of the NAc are the GABAergic MSNs. Their cell bodies range in size from 15-20 microns with dendrites that can extend up to 500 microns. MSNs make up 95% of the total striatal population and are the synaptic targets of the majority of inputs to the striatum and give rise to the outputs of the striatum. These cells receive glutamatergic afferents from the PFC, thalamus, hippocampus, and amygdala, and dopaminergic afferents from the SN and VTA. These cells send projections to many different brain regions, including the CNA, SI, BNST (Alheid and Heimer, 1988; Koob, 1999), VP, LH, mesencephalic tegmentum, SN, and entopeduncular

nucleus (Heimer et al., 1991; Zahm and Heimer, 1990), and are reciprocally connected with striatal cholinergic interneurons and other interneurons. Within the circuitry of the basal ganglia two different subtypes of MSNs have been characterized into the direct- and indirect-pathways (Gerfen, 1992). Neurons in the direct pathway express DA D1 receptors and substance P and project primarily to the internal segment of the globus pallidus (GP) and SN, while neurons in the indirect pathway express DA D2 receptors and enkephalin and project to the external segment of the GP.

1.5.3 Aspiny Cholinergic Interneurons of the Nucleus Accumbens

Cholinergic interneurons of the NAc make up only 1-2% of the total striatal population yet they are the largest cells in this area. Their widespread dendritic arborizations and axonal fields position these cells as important integrators and modulators of striatal functioning (Kawaguchi et al., 1995; Wilson et al., 1990). Striatal cholinergic interneurons possess several receptors, including DA receptors, which have been linked to plasticity, learning, and drug abuse (Alcantara et al., 2003; Berlanga et al., 2005). These cells exhibit long-term potentiation (LTP) (Suzuki et al., 2001) and play a critical role in associative learning (Aosaki et al., 1994). Striatal cholinergic neurons receive synaptic input from the thalamus (Lapper and Bolam, 1992) in addition to glutamatergic inputs from the PFC, amygdala, and hippocampus (Kalivas, 2004). These cells then synapse onto MSNs and other striatal interneurons, thus providing a powerful influence on overall striatal signaling (Howe and Surmeier, 1995).

Pharmacological studies have demonstrated an effect of alcohol on acetylcholine (ACh) release, suggesting the importance of cholinergic interneurons in alcohol abuse

(Nestby et al., 1999; Stancampiano et al., 2004). Herring et al. (2004) revealed that these cells are activated by ethanol in a region-specific and dose-dependent manner. Studies included in this dissertation will focus on the effects of voluntary alcohol drinking on cholinergic interneurons in the NAc. A direct causal link between cholinergic interneurons and alcohol drinking, as demonstrated by selective destruction of these cells, is presented in Chapter 2 and neuroadaptive changes in cholinergic interneurons are examined throughout the remaining chapters.

1.6 Dorsal Striatum

The DS is a heterogeneous region that can be divided into medial and lateral subdivisions based on differential regulation of DA function (Cline et al., 1995), as well as connectivity. Dopaminergic cell bodies located in the VTA project primarily to the ventromedial region of the DS and shell NAc, while the dorsolateral region of the DS and core NAc are innervated by the SN (Gerfen et al., 1987). In addition to the VTA and SN, the DS also receives input from the dorsolateral PFC (Haber et al., 2000). The DS has traditionally been associated with motor function but recent studies have also elucidated its involvement in habit learning and long-term synaptic plasticity that underlies the progression towards habitual drug-seeking (Berke and Hyman, 2000; Everitt and Wolf, 2002). Cue-induced DA release in the DS can trigger relapse into drug-taking behavior (Ito et al., 2002) and previous work in our laboratory found cholinergic interneurons of the ventromedial DS to be dose-dependently activated as early as the first session of cocaine self-administration (Berlanga et al., 2003).

1.7 Prefrontal Cortex

The PFC has been implicated in a variety of brain processes including decision-making, attention, working memory, premotor planning (Dalley et al., 2004; Goldman-Rakic and Selemon, 1986), salience attribution, and inhibitory response control (Goldstein and Volkow, 2002). The PFC has also been associated with the motivational effects underlying drug-seeking, cue-induced drug craving, and vulnerability to relapse (Weiss et al., 2001). The prelimbic (PrL) region of the PFC receives dopaminergic projections from the VTA (Van Eden et al., 1987) and sends glutamatergic projections throughout the NAc which terminate most abundantly in the core NAc (Berendse et al., 1992; Brog et al., 1993; Vertes, 2004), while infralimbic (IL) PFC glutamatergic fibers are located mostly in the shell NAc (Brog et al., 1993). Dopaminergic input from the VTA is reported to exert an inhibitory influence on PFC neurons either directly, by influencing cortical pyramidal cells, or indirectly via GABAergic influences onto pyramidal cells (Penit-Soria et al., 1987; Pirot et al., 1992). In turn, the PFC is thought to control drug use by exhibiting an inhibitory influence on subcortical brain regions such as the NAc and CNA. It has been proposed that the transition from voluntary substance abuse to compulsive intake occurs through the disruption of this inhibitory influence (Goldstein and Volkow, 2002; Jentsch and Taylor; 1999; Kalivas and Volkow, 2005). This is further supported by fMRI studies reporting alcohol-induced hypofunction of the frontal cortex (Volkow et al., 1990; Volkow et al., 1992; Volkow et al., 1994).

1.8 Animal Models of Alcohol Drinking

Since the 1960s experimenters have used various methods to try and induce high alcohol drinking in rodents. Some examples include continuous-access two-bottle choice in unselected rats (Boyle et al., 1994), intravenous (Numan and Naparzewska, 1984), intragastric, schedule-induced polydipsia (Falk et al., 1972), and prandial drinking (Meisch, 1975). All of these methods have been criticized; some for producing low ethanol intake (two-bottle choice in unselected rats, intravenous, and intragastric), and others for maintaining rodents at a reduced body weight (schedule-induced polydipsia, and prandial drinking). Alcohol self-administration is the most homologous model of human alcohol drinking, and the characterization of animals that are selectively bred for high alcohol intake has produced a model with high clinical validity and suggests that ethanol may be reinforcing for some rats.

While it is recognized that no animal model fully emulates a human alcoholic it is widely accepted that the individual features of alcoholism can be studied in animal models in great detail. Selective breeding creates a powerful genetic tool by concentrating influential genetic factors into a single animal line. Such models mimic aspects of human dependence such as craving, relapse, and the loss of control over drinking, and provide a means with which to pursue the underlying neurological basis for this complex disease. It is important to understand the neuronal systems that underlie alcohol drinking in animals selectively bred for, or genetically predisposed for high levels of alcohol intake.

1.8.1 C57BL/6J Mice

A growing interest in the genetic mechanisms underlying alcohol drinking has lead to increased study of rodent models of alcohol drinking (George, 1988; Grahame, 2000; Risinger et al., 1998). Genetic variability exists in selectively outbred rodent models for alcohol consumption. Inbred mouse strains, such as the C57BL/6J mouse, minimize this variability by offering genetic homogeneity and provide important insights into the genetic and biochemical nature of alcohol abuse. The C57BL/6J mouse was not selectively bred for alcohol preference. Originally, the C57BL strain was used in cancer research (Stewart and Grupp, 1992) and was later found to be genetically predisposed for high alcohol drinking behavior (Ng et al., 1994), while also exhibiting a strong preference for morphine and cocaine (George and Goldberg, 1989; Horowitz et al., 1977). Out of 15 mouse strains studied, C57BL/6J mice have demonstrated the highest voluntary alcohol consumption and alcohol preference (Belknap et al., 1993).

1.8.2 Inbred Alcohol-Preferring (iP) Rats

Rodents that are not selected for ethanol preference typically consume low amounts of ethanol when given the two-bottle choice method. Alcohol-preferring (P) and alcohol-non-preferring (NP) rats were derived through selective breeding from a heterogeneous Wistar strain using the continuous-access, two-bottle choice procedure (described below, 1.8.4). P rats meet all the criteria for an animal model of alcoholism, including voluntary intake of alcohol, willingness to work for alcohol through operant responding, and the development of tolerance and dependence through free-choice drinking (Murphy et al., 2002). P rats have been reported to consume at least 5 g

alcohol/kg/d and attain blood alcohol concentrations of 50 to 200 mg% (Murphy et al., 2002). Inbred alcohol-preferring rats (iP) were developed from the P line and studies suggest that behaviors in iP rats, such as high-alcohol drinking, resemble those of the parent line (Edenberg et al., 2005). Inbred strains are useful for reducing genetic variability among individual animals, thus providing insight into the genetic and biochemical properties of alcohol drinking. Female iP rats were used in studies discussed in Chapter 5 of this dissertation. Females have been shown to consume more alcohol, in grams per kilogram of body weight, than male P rats (McKinzie et al., 1998a; McKinzie 1998b).

1.8.3 Initiation Alcohol Drinking

The studies presented in this dissertation use two different voluntary alcohol drinking models. The phrases 'initiation alcohol drinking' and 'binge alcohol drinking' are used to distinguish between the two models. Initiate can be defined as, "to cause or facilitate the beginning of" ("initiate," Merriam-Webster Online Dictionary, 2007). The initiation drinking paradigm employed in these studies was termed initiation drinking in an effort to model the time around when one begins to drink alcohol. This paradigm uses the standard continuous-access, two-bottle choice procedure. The two-bottle choice procedure is the most widely used and straightforward approach to model human alcohol consumption in animals. Using this method, animals in the experimental group are given continuous, 23-24 hours/day, access to two bottles; one containing 10% (v/v) ethanol and the other containing water. The control group has continuous access to two bottles but they both contain water, and food is available ad libitum for both groups. Since

experimental animals in the initiation drinking studies consume alcohol over the course of 23-24 hours a day it is not clear when or if the animals drink to pharmacologically significant levels (BECs > 0.8 mg.ml blood), because drinking is most likely episodic. The initiation drinking model used throughout the studies in this dissertation was therefore interested in examining the effects of moderate alcohol drinking on neuronal changes in DA D2 receptor and Cdk5 expression, specifically in cholinergic interneurons of the NAc.

1.8.4 Binge Alcohol Drinking

While traditional rodent models of alcohol self-administration allow for the investigation of neuroadaptive changes that occur over the course of days, weeks, or months, these models rarely produce pharmacologically significant blood ethanol concentrations (BECs › 0.8 mg/ml blood) over extended periods of time. A fundamental component of alcoholism is loss of control over alcohol consumption (National Institute on Alcohol Abuse and Alcoholism, 2001), which often involves binge drinking. Binge alcohol drinking is defined as a pattern of alcohol consumption that results in BECs of 0.08% (equivalent to 0.8 mg/ml blood) or above and usually corresponds to 4 or more drinks for women and 5 or more drinks for men in about 2 hours (National Institute of Alcohol Abuse and Alcoholism, 2004). In the United States alone an estimated 57 million people, aged 12 and over, participated in binge drinking in the past month (Substance Abuse and Mental Health Services Administration, 2007). Binge alcohol drinking produces high BECs in alcohol abusers which, when repeated, may ultimately lead to neuroadaptations that further drive behaviors such as compulsive drinking, craving and

relapse. One goal of alcohol research is to identify neuronal changes that occur with binge drinking. It therefore becomes important to develop an animal model of binge drinking.

A procedure, termed Drinking in the Dark (DID), was recently developed (Rhodes et al., 2005) in an effort to mimic binge alcohol drinking. Using this procedure a majority of C57BL/6J mice drink to a BEC above 1 mg/ml blood each day. This procedure will be discussed in more detail in Chapter 2 (section 2.3.3). Briefly, the home cage water bottle is replaced with a bottle containing 20% ethanol (v/v) for 2 hours (days 1 - 3) or 4 hours (days 4 - 28) starting 3 hours after lights off. At the end of the alcohol drinking session, intakes are recorded and the home cage water bottles are put back in place.

1.9 Cholinergic Cell Ablation

Previous studies have used mu p75-saporin, a ribosome-inactivating toxin, to study the functional role of the cholinergic system. This toxin has been shown to induce selective loss of cholinergic neurons in mice (Berger-Sweeney et al., 2001; Hunter et al., 2004). Studies investigating the role of the basal forebrain cholinergic system have found that this toxin not only selectively destroys cholinergic neurons but it also induces cognitive impairment in mice; two characteristics that are often used to model disorders such as Alzheimer's disease (Berger-Sweeney et al., 2001).

Selective removal of accumbal cholinergic interneurons provides a useful animal model for studying the role of these neurons in alcohol abuse. Recent studies have implicated cholinergic interneurons in the NAc as key substrates underlying drug self-

administration (Berlanga et al., 2003; Smith et al., 2004). Cholinergic cell ablation, by immunotoxin-mediated cell targeting, enhanced long-lasting behavioral changes of cocaine (Hikida et al., 2001) and morphine abuse (Hikida et al., 2003), impaired procedural learning and working memory (Kitabatake et al., 2003), and increased sensitivity to low doses of cocaine (Smith et al., 2004). Specifically, studies using cholinergic cell ablated rodents have measured cocaine induced conditioned place preference, motor sensitization (Hikida et al., 2001; Hikida et al., 2003), and cocaine self-administration (Smith et al., 2004), yet these studies have not been carried out in conjunction with voluntary alcohol drinking. Altogether these findings suggest that accumbal cholinergic neurons are directly involved in various behaviors associated with drugs of abuse, and implicate the potential role for these cells in alcohol drinking.

1.10 Motor Impairment

Motor impairment (ataxia) resulting from alcohol exposure is one of the most readily observable features of alcohol intoxication. While complex in nature, this behavior is often used to measure behavioral intoxication in rodents (Crabbe et al., 2003; Crabbe et al., 2005; Cronise et al., 2005; Rustay et al., 2003a; Rustay et al., 2003b). The DID model of binge alcohol drinking, which leads to BECs above 1 mg/ml, has been shown to cause behavioral intoxication, as measured by impaired performance on the rotarod and balance beam (Rhodes et al., 2005). Motor impairment resulting from alcohol drinking and cholinergic cell ablation, as measured by performance on the rotarod, was examined in Chapter 2 of this dissertation.

1.11 cFos

The immediate early gene (IEG) c-fos is often used as a marker for cellular activity (for review, see Curran and Morgan, 1995). Maximal expression occurs between 1 and 3 hours after stimulation, and detectable levels of Fos (the phosphoprotein product of c-fos) are low under basal conditions (Hughes et al., 1992). Studies examining activation of brain regions following alcohol exposure have used immunocytochemical labeling of c-fos (McBride, 2002).

In a previous study we investigated the effects of varying acutely administered intraperitoneal (i.p.) doses of alcohol on cellular activation using Fos immunoreactivity (IR) (Herring et al. 2004). This study focused on neuronal activation in brain regions that have been implicated in the reinforcing and anxiolytic effects of alcohol abuse; the extended amygdala and hypothalamus. Various neuroanatomical studies have examined Fos IR of the extended amygdala after systemic administration of alcohol (Chang et al., 1995; Knapp et al., 2001; McBride, 2002; Ryabinin et al., 1997), few have investigated the involvement of particular cell types within these regions (Criado and Morales, 2000; Morales et al., 1998), and none have examined neuronal activation in cholinergic interneurons. This study was therefore primarily interested in testing the hypothesis that cholinergic neurons in the NAc are putative targets for alcohol. Alcohol induced activation of cholinergic neurons was examined in the NAc (Figure 1.2), and other regions, using dual-label Fos ICC procedures. In addition, using single-label Fos ICC procedures, neuronal activation was measured in these brain regions irrespective of cell type. For the purposes of this discussion data from the dual-label [choline acetyltransferase (ChAT)/Fos] NAc are presented (Table 1.1), although significant

increases in single-label c-fos expression were also observed in other brain regions (Herring et al., 2004).

A 1 g/kg dose of ethanol lead to a significant increase in the percentage of Fos-positive cholinergic neurons in the shell NAc, while a 2 g/kg dose caused a significant increase in accumbal Fos-positive neurons, irrespective of cell type. These findings revealed a region-specific and dose-dependent change in Fos IR following acute ethanol exposure. This study identified the specific activation of cholinergic neurons of the shell NAc which lead to the development of studies designed to examine the effects of voluntary alcohol drinking in this brain region.

Table 1.1. Percent of Fos-Positive Cholinergic Neurons in the Shell NAc Following i.p. Administered Saline or Ethanol

Shell Nucleus Accumbens	
saline	28.6 ± 5.2
0.5 g/kg EtOH	32.8 ± 3.9
1.0 g/kg EtOH	46.5 ± 5.6**
2.0 g/kg EtOH	32.2 ± 4.6

Data are presented as mean number of Fos-positive cholinergic neurons per sample area ± SEM for the 7-8 rats per group.
**p <0.01 versus saline control; Dunnett's planned comparisons.

1.12 Dopamine Receptors

DA receptors are critical links between extracellular events and intracellular biochemical cascades that underlie long-term neuronal plasticity. DA exerts its effects through the activation of two pharmacologically distinct classes of receptors: DA D1-like receptors (D1 and D5) and D2-like receptors (D2, D3, and D4). These receptors are G-protein coupled and can be differentiated based on their coupling to adenylyl cyclase. In general, stimulation of the DA D1 receptor activates adenylyl cyclase while the DA D2 receptor inhibits adenylyl cyclase activity (Missale et al., 1998). ACh release is increased by DA D1-like agonists (Consolo et al., 1999), while DA D2-like activation has been shown both in vivo and in vitro to inhibit ACh release (Bertorelli and Consolo, 1990; Gorell and Czarnecki, 1986; Stoof et al., 1987).

1.13 Dopamine D2 Receptor Subfamily

The DA D2 receptor is the predominant D2-like subtype in the brain with the largest concentrations found in the striatum, substantia nigra (SN), and VTA (Diaz et al., 1995; Gurevich and Joyce, 1999; Landwehrmeyer et al., 1993). Studies utilizing animal models of alcoholism, as well as those with humans, have implicated the importance of DA receptors in alcoholism. DA D2 receptors in particular have been associated with alcohol drinking (Thanos et al., 2001; Thanos et al., 2004), withdrawal (Sousa et al., 1999), and the reinforcing effects of alcohol (Crabbe and Phillips, 1998; McBride et al., 1993; Nowak et al., 2000; Stefanini et al., 1992), as well as drug-seeking and relapse (De Vries and Shippenberg, 2002; De Vries et al., 2002; Self et al, 1996).

Numerous animal studies have shown that alcohol-preferring animals have lower DA D2 levels, particularly in the limbic areas, when compared to their non-preferring counterparts (for review see Tupala and Tiihonen, 2004). Low levels of DA D2 could predispose these animals to consume excessive amounts of alcohol in an effort to compensate for their subnormal level of DA function. Moreover, Thanos and colleagues (2001; 2004) reported that overexpression of the DA D2 gene in the core NAc attenuated alcohol drinking in P rats, suggesting that high DA D2 levels may be protective against alcohol abuse. Similarly, DA D2 agonists have been shown to decrease, while DA D2 antagonists increase alcohol intake in both high alcohol drinking (HAD) rats and P rats (Dyr et al., 1993*;* Levy et al., 1991). DA receptors in the NAc are targeted by dopaminergic terminals of the mesolimbic DA system, and the aforementioned studies suggest that these receptors are important components of alcohol drinking.

1.14 Cyclin dependent kinase 5

Fos-related antigens (FRAs), another class of Fos-like proteins, are induced by chronic drug administration (Hope et al., 1994; Nye et al., 1995; Nye and Nestler, 1996) and are more stable than the Fos-like proteins. ΔFosB, a splice variant of FosB, accumulates in the striatum in response to repeated exposure to drugs of abuse and persists for weeks or months even after the drug is withdrawn (Nestler et al., 2001).

Cdk5 is a downstream target of ΔFosB (Bibb et al., 2001) and it has been shown to regulate various signal transduction mechanisms associated with neuroplasticity (Bibb, 2003) including neurotransmitter release (Tomizawa et al., 2002), LTP induction (Li et

al., 2001), and associative learning (Fischer et al., 2002). Furthermore, Cdk5 is upregulated in the mouse striatum in response to chronic cocaine exposure and in transgenic mice overexpressing ΔFosB (Bibb et al., 2001), and is necessary for dendritic spine plasticity in the NAc of the rat (Norrholm et al., 2003). Cdk5 is a potential marker of neuronal plasticity, however the effects of alcohol drinking on Cdk5 expression has yet to be studied with the exception of previous work in our laboratory (Camp et al., 2006), and a study, using Western blot analysis, by Rajgopal and Vemuri (2001) that reported an increase in Cdk5 activity in the cerebral cortex and cerebellum of Wistar rats following chronic alcohol drinking.

Chapter 2: Cholinergic Cell Ablation in the Nucleus Accumbens Attenuates Binge Alcohol Drinking in C57BL/6J Mice

2.1 Abstract

Many studies have identified various brain regions that are targeted by alcohol and other drugs of abuse. One such brain region, the NAc, has long been identified as a primary area associated with the reinforcing effects of alcohol and other drugs of abuse (Di Chiara and Imperato, 1988; Koob, 2003; Koob and Bloom, 1988; Pontieri et al., 1995; Rassnick et al., 1992). While the NAc has been identified as an important brain region underlying alcohol drinking, the roles of specific cell types within this region have not been investigated. Cholinergic interneurons in the NAc are important neuronal integrators and modulators of striatal functioning (Kawaguchi et al., 1995; Wilson et al., 1990). Pharmacological studies demonstrate the effects of alcohol on ACh release in the NAc (Nestby et al., 1999) and PFC (Stancampiano et al., 2004), and cholinergic neurons are activated following acute alcohol exposure (Herring et al., 2004).

The purpose of this study was to examine whether cholinergic neurons of the NAc are directly involved in alcohol drinking by selectively destroying cholinergic cells with the immunotoxin mu p75-saporin. Mice received bilateral microinjections of either mu p75-saporin or saline. Animals then underwent 1 month of binge alcohol drinking using the Drinking in the Dark (DID) model. To examine motor impairment animals were placed on the fixed speed rotarod every other day immediately following the alcohol access period and latency to fall was recorded.

Selective destruction of cholinergic neurons of the NAc produced a 26% decrease in alcohol drinking compared to saline microinjected controls. Cholinergic cell eliminated mice also fell from the rotarod 24% sooner than controls. These findings provide evidence of a causal link between cholinergic interneurons and alcohol drinking and establish the basis for cholinergic targeted pharmacotherapeutic treatments designed to attenuate compulsive binge drinking.

2.2 Introduction

Various brain regions and molecular mechanism that underlie different stages of alcohol abuse have been identified, yet the role of specific cell types within these areas is not well understood. Identifying the involvement of specific cell types and neuroadaptations within those cells in voluntary alcohol drinking should improve our understanding of the cellular and molecular basis of behaviors such as compulsive alcohol drinking. One brain region that has long been associated with the reinforcing effects of alcohol is the NAc (Di Chiara and Imperato, 1988; Koob, 2003; Rassnick et al., 1992). The NAc contains a specific population of cells, cholinergic interneurons, which have largely been overlooked in alcohol research. Cholinergic interneurons have recently been implicated in alcohol and drug use (Berlanga et al., 2003; Camp et al., 2006; Herring et al., 2004; Hikida et al., 2001; Hikida et al., 2003; Mark et al., 1999; Nestby et al., 1999; Smith et al., 2004; Stancampiano et al., 2004), and are putative cells to examine as potential mediators of alcohol drinking.

Studies examining ablation of cholinergic neurons of the NAc demonstrate that destruction of these cells produce enhanced cocaine- and morphine-induced sensitivity to

drug-related place preference and cocaine-induced motor sensitization in mice (Hikida et al., 2001; Hikida et al., 2003). In addition, Smith et al. (2004) reported a leftward shift in cocaine self-administration in rats. Collectively, these studies suggest the importance of cholinergic interneurons in the modulation of drug-related behaviors, yet the effects of accumbal cholinergic cell ablation on alcohol drinking have not been examined.

The present study was therefore designed to establish a causal role of cholinergic interneurons in the NAc on alcohol drinking using a model of binge alcohol drinking. Cholinergic interneurons of this brain region were selectively destroyed, using the immunotoxin mu p75-saporin, to test the hypothesis that removal of the accumbal ACh signal would cause a decrease in alcohol drinking in C57BL/6J mice. A pilot ablation study was performed to verify correct placement of the injection sites and to determine if the immunotoxin mu p75-saporin successfully destroyed cholinergic interneurons in the NAc. Mu p75-saporin is a compound that consists of a molecule of saporin, a ribosome-inactivating toxin, combined with a rat monoclonal antibody that binds to the extracellular domain of murine p75 (Berger-Sweeney et al., 2001). This immunotoxin destroys cholinergic neurons by inhibiting protein synthesis (Wiley et al., 1991).

While it was hypothesized that cholinergic cell ablation would cause a decrease in alcohol drinking, the possibility existed for an increase in drinking. C57BL/6J mice were used in this study because they innately consume high amounts of alcohol and have been shown to drink enough alcohol to produce pharmacologically significant BECs (above 0.8 mg/ml blood) in the DID paradigm (Rhodes et al., 2005). If accumbal cholinergic cell ablation caused an increase in drinking, a ceiling effect was likely to occur where increases would not be detected due to the high amount of alcohol innately consumed by

these animals. In order to detect either an increase or a decrease in drinking, effects of the ablation were also examined in BALB/cByJ mice, a strain that consumes intermediate amounts of alcohol.

Collectively, the studies presented in this dissertation hypothesize that alcohol-induced neuroadaptive changes on cholinergic neurons of the NAc regulate striatal neurons and signaling mechanisms which translate to behaviors such as drug-seeking or compulsive alcohol drinking. The aim of the present study was to establish a causal role for accumbal cholinergic interneurons in alcohol drinking. These data have been presented in preliminary form (Camp and Alcantara, 2007).

2.3 Materials and Methods

2.3.1 Animals

Fourteen male C57BL/6J mice (18.7-22.9 g) and fourteen male BALB/cByJ mice (20.6-25.2 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Cholinergic cell ablation did not produce a change in alcohol drinking in the BALB/cByJ mice. These animals consume intermediate amounts of alcohol. We potentially observed a floor effect in this strain where differences may not have been detected due to the low to intermediate quantities of alcohol consumed by this strain. Therefore, only data from the C57BL/6J mice are presented. Mice were individually housed in standard cages in a temperature- and humidity-controlled room under a reverse 12 hour light/dark cycle (lights on 21:00 h). Mice were between 7 and 8 weeks old at the start of the experiment and food was available ad libitum. All experimental procedures complied with guidelines

specified by the National Institutes of Health and were performed under an institutionally reviewed and approved research protocol.

2.3.2 Surgical Procedures and Cholinergic Cell Ablation

2.3.2.1 Stereotaxic Surgery

All animals were anesthetized with a ketamine (117 mg/kg) and xylazine (7.92 mg/kg) solution (from Thiele et al., 2003) administered i.p. When the animals were fully anesthetized, as verified by tail pinch and corneal response, they were placed in a stereotaxic apparatus. The scalp was swabbed with betadine, and Lidocaine HCL was injected subcutaneously at the surgical site. An incision into the skin overlying bregma was made and the scalp was pulled back and secured with surgical clamps. Small holes were made in the skull aimed at the shell NAc using the following stereotaxic coordinates: anteroposterior, +1.1; mediolateral, ±1.0; dorsoventral, -4.5 (Franklin and Paxinos, 1997).

2.3.2.2 Cholinergic Cell Ablation

The immunotoxin 192 IgG-saporin, directed against the rat p75 nerve growth factor receptor, has been used to study the functions of the cholinergic system in rats (Beaulé and Amir, 2002; Smith et al., 2004; Wiley, 1992; Wrenn and Wiley, 1998). Saporin, bound to an antibody against the mouse p75 receptor, was used in the present experiment to selectively destroy cholinergic neurons in mice (Berger-Sweeney et al., 2001). Animals received bilateral microinjections into the NAc of either anti-murine-p75-saporin or saline. The neurotoxin was dissolved in saline immediately before injection

and saline alone was injected into controls. A volume of 0.5 μL of saporin (3.6 μg) or saline was injected into each hemisphere. Microinjections were administered using a precision micro infusion pump at a rate of 0.2 μl/min. [0.5 μL into each hemisphere for a total of 1 μL (3.6 μg of toxin)]. The injection needle was left in place for 5 minutes to allow for diffusion of the liquid. The scalp was then sutured using about 5 closely placed and trimmed sutures of 3-0 nylon monofilament. Antibiotic ointment containing Pramoxine HCL (an analgesic) was then applied to the skin at and near the incision site. Mice were monitored and allowed to recover for 1 week.

2.3.2.3 Verification of Effective Ablation

After animals were perfused, brain tissue was immunolabeled for ChAT and all remaining cholinergic cells were quantified in order to demonstrate the effectiveness of the ablation (Figure 2.1). A thorough anterior to posterior examination of the NAc (Bregma 1.54 mm - 0.86 mm) was performed for each animal. This enabled us to compare the number of cholinergic interneurons between the saline-treated controls and the experimental animals throughout the full extent of the NAc. A visual examination of the striatum, including the DS, was performed and the lesion appears to be confined to the region of the NAc medial and ventral to the anterior commissure (Figure 2.2). Verification of cannula placement was visible with ChAT IR.

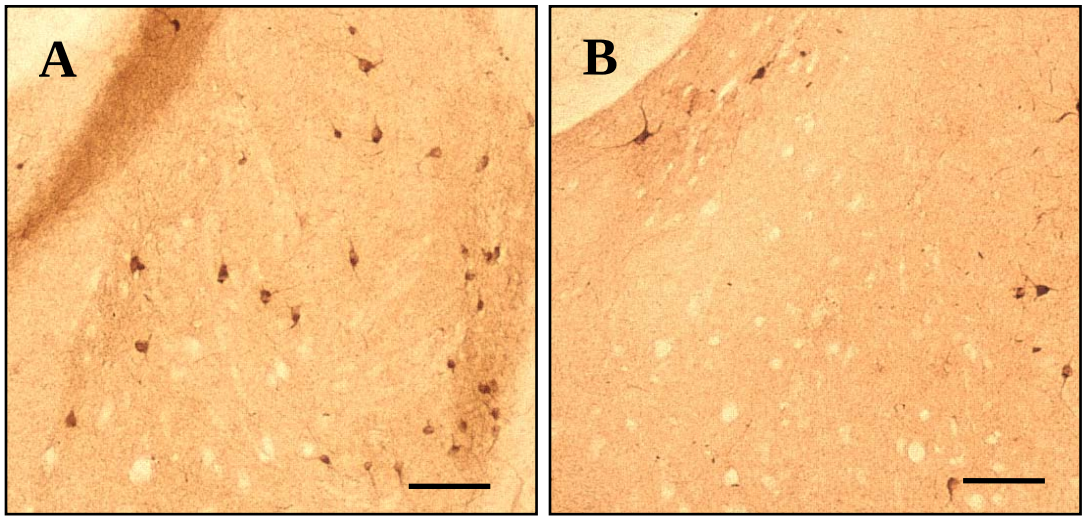

Figure 2.1. Representative photomicrographs from the NAc (Bregma 1.10 mm) following microinjections of saline or mu p75-saporin. (A) saline-treated control and (B) saporin-treated C57BL/6J mouse. Scale bars = 100 μm.

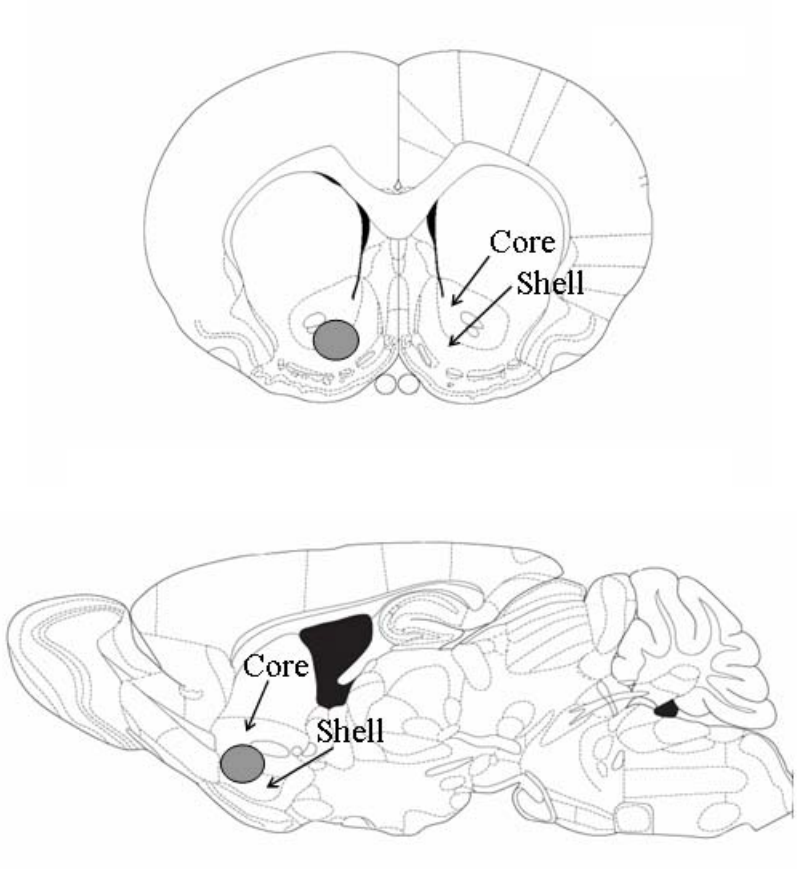

Figure 2.2. Schematic representation of the extent of saporin lesions in the NAc of C57BL/6J mice. The spread of the lesion is indicated by the grey circles. Top, coronal brain section (Bregma 1.10 mm); bottom, sagittal brain section (Lateral 0.96 mm). Figures adapted from Franklin and Paxinos (1997).

2.3.3 Model of Binge Alcohol Drinking Design and Procedures

One week after surgeries animals were administered alcohol using the Drinking in the Dark (DID) schedule of binge alcohol drinking. For the first three days the DID procedure was as follows: Three hours into the dark cycle (12:00 h) the home cage water bottle was replaced with an identical bottle containing 20% (v/v) ethanol solution. The ethanol bottle remained in place for 2 hours. At the end of the 2 hour session intakes were recorded, animals were weighed, and home cage water bottles were put back into place. On the 4th day procedures were the same as before except that the 20% ethanol solution was left in place for 4 hours. Mice remained on the 4 hour ethanol schedule for the remainder of the month. BECs were collected and analyzed immediately upon removal of ethanol on the last drinking day.

2.3.4 Blood Ethanol Concentrations

Retro-orbital blood samples were collected from each animal immediately following the alcohol access period on the last drinking day using 25 µL heparinized capillary tubes. Next, 10 µL of blood from each animal were pipeted into separate gas chromatography vials containing 90 µL of saline. BECs were determined using gas chromatography as previously described by Tang et al. (2003). The blood samples were analyzed by a Varian CP 3800 gas chromatograph with a Varian 8200 headspace autosampler and flame ionization detector. An INNOWAX capillary column (30 m X 0.53 mm X 0.1 µm film thickness) was used along with an absorbing/desorbing SPME fiber assembly. The fiber coating was 75 µm Carboxen-PDMS. The retention time of ethanol was approximately 1.8 min. The concentration of ethanol was quantified by

constructing a standard curve consisting of 0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 mM ethanol.

2.3.5 Rotarod Design and Procedures

An Economex Rota Rod (Columbus Instruments, Columbus, OH) was used for the fixed speed rotarod test and the design of this study was adapted from Cronise and colleagues (2005). The rotating rod was 4 cm in diameter with a 40 cm fall height. The day before surgeries to microinject either saporin or saline into the NAc all mice were trained to criterion at 10 RPM. Specifically, mice were given 6, 30-second (s) trials at 10 RPM with 30 s intervals between trials. Next, mice had to remain on the rod at 10 RPM for 3, 3-minute (min) trials. Latencies to fall were recorded. A criterion of a maximum of 16 training trials for the 3 x 3-min was used. If mice did not reach criterion within 16 trials they were to be excluded from the study. After training at 10 RPM, mice were given 1 trial at a fixed speed of 15 RPM. Mice were then tested following each water or alcohol access period on days 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 at both 10 RPM and 15 RPM. They were given 3 trials (1 trial at 10 RPM and 2 at 15 RPM) with a ceiling of 3 min each. Latencies to fall were recorded at each RPM, and latencies were averaged in the 15 RPM condition to derive daily performance.

2.3.6 Tissue Preparation

The day after the last drinking day animals were anesthetized with an i.p. injection of sodium pentobarbital and perfused transcardially with 15 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.4), followed by 185 mL of 4%

paraformaldehyde/0.1% glutaraldehyde in PBS. The brains were immediately removed and postfixed for 2 hours in 4% paraformaldehyde in PBS. Vibratome sections (50 μm thick) were taken and placed in a cryoprotectant solution and stored for 24 hours at 4°C and then stored at -20°C. Tissue was later processed for light microscopy using single ChAT immunoperoxidase labeling procedures.

2.3.7 Single ChAT Immunoperoxidase Labeling

Light microscopy single-labeling immunoperoxidase procedures were performed on free-floating tissue sections that were rinsed in 0.1 M PBS (2 × 10, 3 × 5 minutes) and preincubated for 1 hour in a PBS blocking solution containing 5% normal goal serum (NGS) and 0.01% H_2O_2. Sections were then incubated overnight at 4°C in an affinity-purified mouse anti-ChAT monoclonal antibody (1:1,000; Chemicon, Temecula, CA) diluted in the 5% NGS/PBS blocking solution. The tissue was subsequently rinsed in PBS (4 × 5 minutes and similarly rinsed after each step). Sections were then incubated in secondary biotinylated donkey anti-mouse immunoglobulin (IgG) antiserum (1:500; Jackson ImmunoResearch, West Grove, PA), diluted in 2% NGS/PBS for 2 hours, and then incubated in avidin–biotin complex (ABC; Vectastain Elite kit, Vector Laboratories, Burlingame, CA) for 1 hour. ChAT IR was visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in PBS, which resulted in a brown reaction product.

2.3.8 Quantitative and Statistical Analysis

For each animal, quantifications of single ChAT immunoperoxidase-labeled brain tissue were performed in the shell and core regions throughout the NAc in regions corresponding to Bregma 1.54 mm – 0.86 mm (Franklin and Paxinos, 1997). The left and right hemispheres were quantified from an average of 3 brain sections per animal. Digital images representing areas of 0.45 × 0.45 mm were acquired with a 20 × objective (800 × working magnification) from both hemispheres of the NAc. Two digital images were acquired for quantification from left and right hemispheres of the core NAc (Bregma 1.10 mm), and three digital images were acquired from both hemispheres of the shell NAc (1.10 mm). All images were stored and analyzed by observers blind to group assignment. In quantifying these single ChAT IR regions, the observers counted all cells that expressed ChAT staining within the left and right hemispheres of both the core and shell NAc. An average number for the sample areas per brain region, averaged across both hemispheres, was calculated for each animal. The group averages were subsequently calculated for each brain area of interest.

Daily session intakes of alcohol and water were calculated for each animal. Alcohol intakes were calculated in grams of alcohol per kilogram of body weight (g/kg). Latencies to fall from the rotarod were recorded at each RPM. All values are reported as means ± standard error of the mean (SEM). Statistical analysis of the alcohol drinking data was performed for days 8-28. This allowed 1 week for the immunotoxin, saporin, to be completely effective. A two-way repeated measures analysis of variance (ANOVA) was used to analyze alcohol and water drinking across days and between groups, behavioral measures of motor impairment, and body weight. Group (saporin or saline)

was used as the between-subjects factor and day, or trial, was used as the within-subjects factor. Student's *t*-test was used identify a significant change in ChAT expression following bilateral microinjections of either saporin or saline.

2.4 Results

2.4.1 Single ChAT Immunoreactivity

Single ChAT IR was examined in the shell and core regions of the NAc to verify effectiveness of the mu p75-saporin ablation. Mean number of ChAT IR neurons in saline treated controls was 5.16 ± 0.12, while in the saporin-treated the mean was 1.29 ± 0.05 (Figure 2.3). Mu p75-saporin produced an average loss of 75% [$t(12) = 29.27$, $p<0.0001$, 2-tailed].

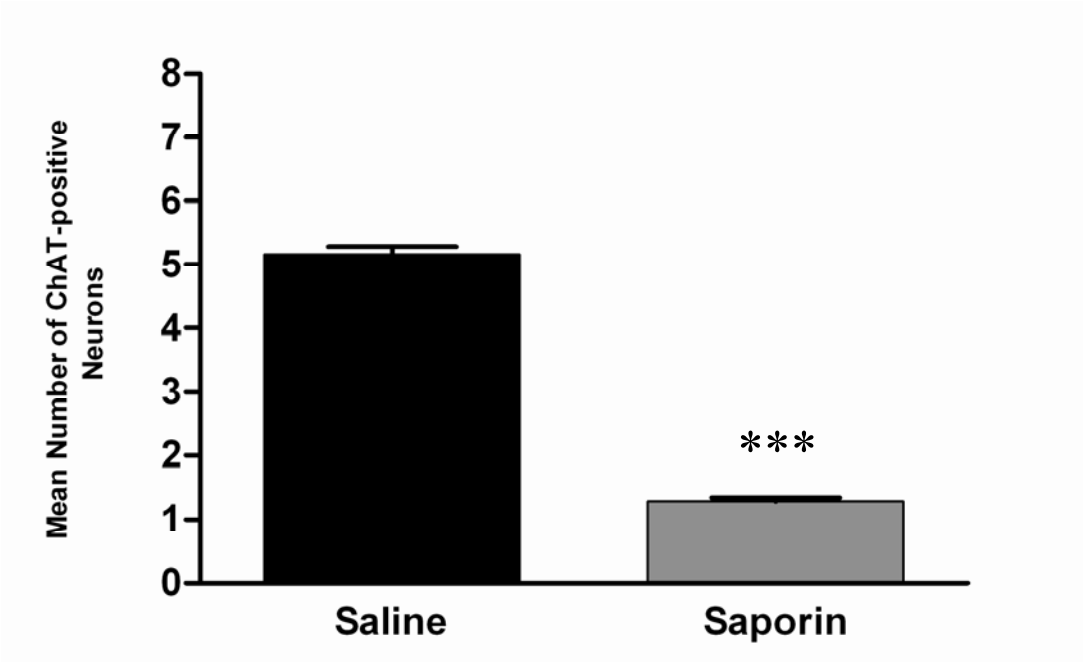

Figure 2.3. Average number of ChAT immunoreactive neurons following bilateral microinjections of saline or mu p75-saporin. Mu p75-saporin caused a 75% loss of cholinergic neurons in the NAc. Data are presented as mean number of cholinergic neurons per optical field ± SEM in C57BL/6J mice (***p<0.0001).

2.4.2 Model of Binge Alcohol Drinking

Average daily intake of ethanol ± SEM for cholinergic cell eliminated C57BL/6J mice was 5.71 ± 0.5 g/kg, while saline treated mice consumed 7.76 ± 0.58 g/kg. Cholinergic cell eliminated mice showed a 26% decrease in alcohol drinking compared to saline microinjected controls ($p<0.05$) (Figure 2.3). There was no significant difference in water drinking (Figure 2.4), suggesting that changes seen in alcohol drinking were not due to overall fluid consumption.

A blood sample was taken from each animal immediately following the alcohol access period on the last drinking day to determine BECs. In the saline treated control group BECs were above 1.0 mg/ml in three out of seven mice and one BEC was 0.93 mg/ml. In the saporin treated group the BEC of one animal was 2.23 mg/ml and another was 0.86 mg/ml. BECs of the other five animals were much lower.

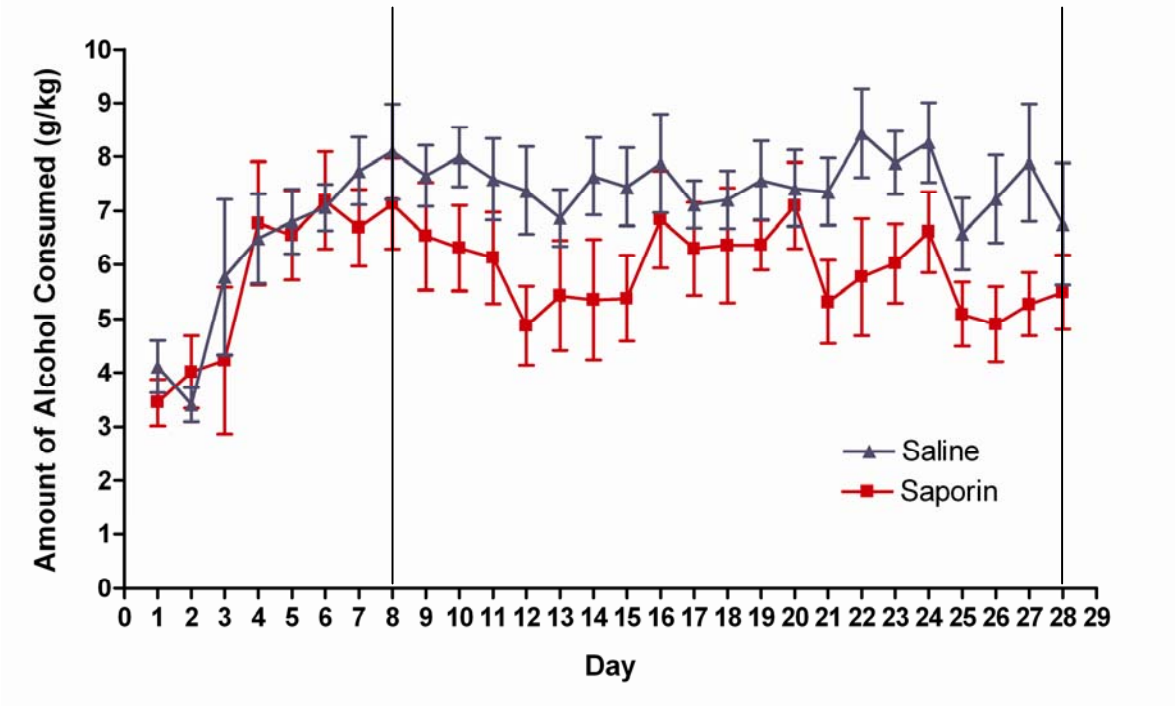

Figure 2.4. Alcohol consumption in saline- and saporin-treated C57BL/6J mice. All animals underwent 1 month of binge alcohol drinking. Main effect of group for days 8-28, $p<0.05$. Mean values ± SEM are shown.

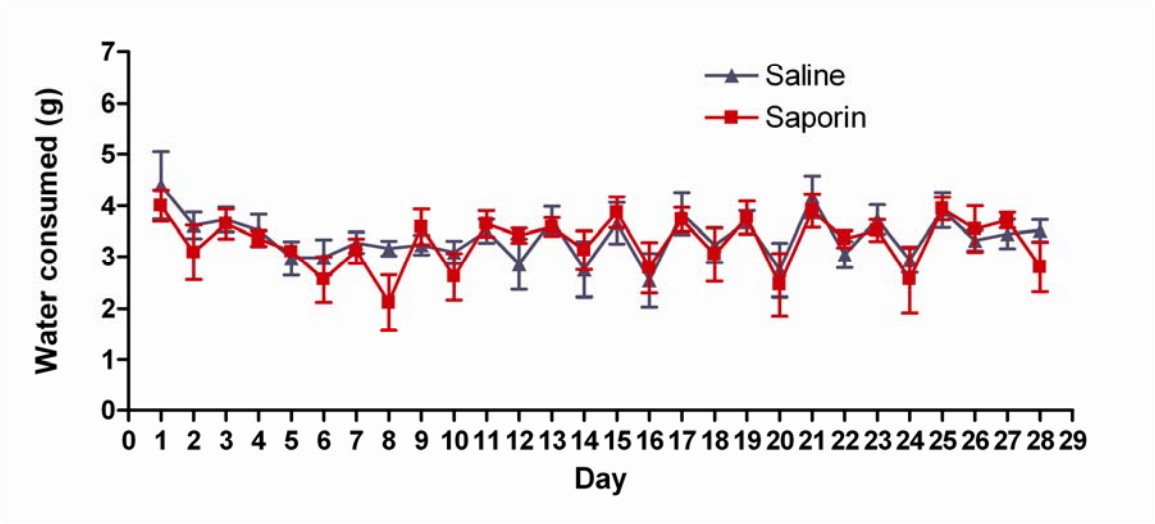

Figure 2.5. Water consumption in saline- and saporin-treated C57BL/6J mice. Mean values ± SEM are shown.

2.4.3 Rotarod

Cholinergic cell eliminated mice fell from the 15 RPM rotarod 24% sooner than controls ($p<0.05$). Latency to fall increased in both groups over the course of 14 trials ($p<0.0001$), and there was no interaction between group and trial (Figure 2.5). There were no significant differences between groups at 10 RPM.

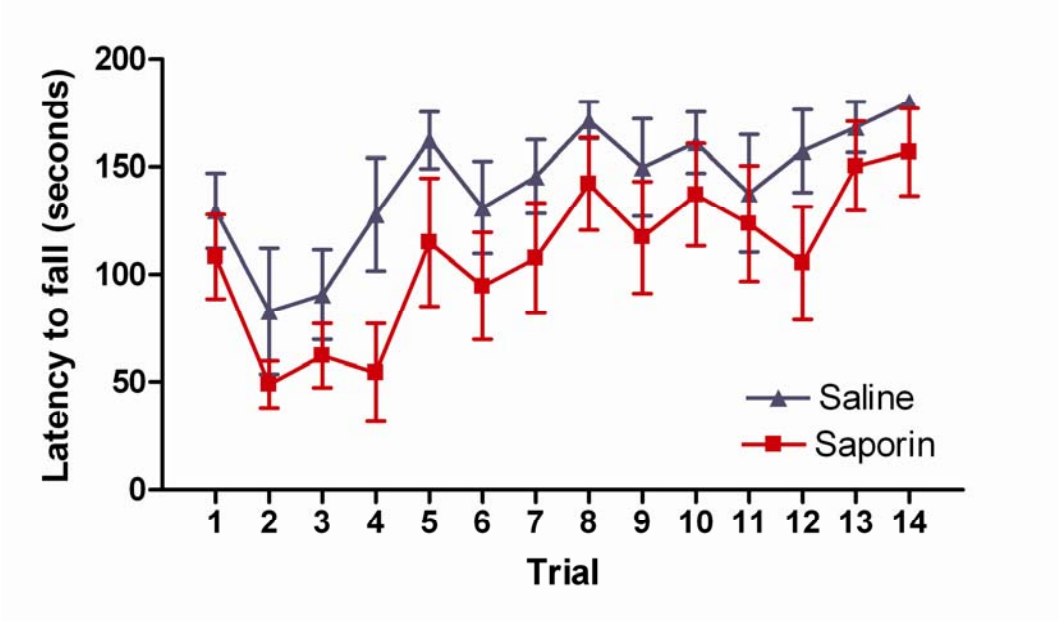

Figure 2.6. Latency to fall from the fixed speed rotarod at 15 RPM. Main effects of group ($p<0.05$) and main effect of time ($p<0.0001$). Mean values ± SEM are shown.

2.5 Discussion

The present study examined the effects of accumbal cholinergic cell ablation on binge alcohol drinking in C57BL/6J mice. Many procedures have been developed to increase ethanol intake in rodents but they often involve periods of water and/or food restriction, or sucrose fading, and they rarely produce pharmacologically significant BECs. The DID model of binge alcohol drinking employed in this study is a simple procedure that does not require food or water restriction and leads to pharmacologically significant drinking in C57BL/6J mice (Rhodes et al., 2005).

One major goal of alcoholism research is to identify the underlying cellular and molecular basis of this complex disease in order to develop improved pharmacotherapeutic treatments. The NAc, a brain region involved in the reinforcing effects of alcohol (Di Chiara and Imperato, 1988; Koob, 2003; Rassnick et al., 1992), contains a cell population, cholinergic interneurons, which have not been thoroughly studied in alcohol research. Cholinergic interneurons are important integrators and modulators of striatal functioning (Kawaguchi et al., 1995; Wilson et al., 1990), and have recently been implicated in studies on alcohol (Camp et al., 2006; Herring et al., 2004; Nestby et al., 1999; Stancampiano et a., 2004) and other drugs of abuse (Berlanga et al., 2003; Hikida et al., 2001; Hikida et al., 2003; Mark et al., 1999; Smith et al., 2004). These cells were therefore of interest to examine as potential underlying mediators of alcohol drinking.

Previous cholinergic cell ablation studies revealed an increase in cocaine-induced locomotor activity, and robust conditioned place preference with a lower dose of cocaine (Hikida et al., 2001) or morphine (Hikida et al., 2003), when compared to controls.

Additionally, cholinergic cell elimination has been shown to produce a leftward shift in cocaine self-administration (Smith et al., 2004). These findings show that ACh from cholinergic cells of the NAc is involved in the neural responses that underlie cocaine- and morphine-induced behaviors. It is hypothesized that accumbal cholinergic interneurons are potentially one of the key elements that contribute to homeostatic regulation of the NAc in response to drugs of abuse. Removing ACh from the NAc potentially strengthens the DA response to drugs of abuse, thus increasing sensitivity to drug-induced behavioral changes. Ablation studies have not been carried out in conjunction with alcohol. Alcohol, however, shares the characteristic with these other drugs of abuse of being rewarding in animals and humans. When the NAc undergoes plasticity, as may occur through alcohol drinking, the function of the cholinergic system may become compromised and drive such behaviors as compulsive alcohol drinking. In the present study, cholinergic cell ablation caused a decrease in ACh which was hypothesized to increase sensitivity to alcohol, thus resulting in a decrease in alcohol drinking.

The rotarod is one of the most commonly used behavioral tests of motor incoordination (Bogo et al., 1981; Rustay et al., 2003b). Rhodes et al. (2005) demonstrated that DID produces behavioral intoxication in C57BL/6J mice, as measured by motor impairment on a rotarod test similar to the one employed in the present study. Motor impairment was measured both within and between groups at 10 RPM and 15 RPM. Since it has been established that DID produces behavioral intoxication, the present study sought to examine if cholinergic cell ablation causes further motor impairment. Cholinergic cell eliminated mice fell from the rotarod significantly sooner at 15 RPM, but not at 10 RPM. The rotarod task at 10 RPM may not have been challenging

enough to detect a significant difference between the groups. Alternatively, a difference at 10 RPM may not have been detected since the majority of training was conducted at this speed. Animals had more practice on the rotarod at 10 RPM which may have increased their latencies to fall. Differences detected between saporin- and saline-treated mice at 15 RPM were most likely due to a motor deficit rather than intoxication. It took about 7 g/kg alcohol in 4 hours to reach BECs of 1.0 mg/ml. On average, animals in the saporin group consumed less than 7 g/kg suggesting that the shorter latencies to fall were due to cholinergic cell ablation-induced motor deficits and not intoxication. There was also a significant main effect of time indicating that, over the course of 1 month, mice in both groups improved with intoxicated practice.

Results from the present study indicate that cholinergic interneurons of the NAc are directly involved in alcohol drinking and demonstrate a role for these cells in motor impairment. Subsequent studies can now examine the intricate details of alcohol-induced neuroadaptations that occur within the cholinergic neuronal circuits of the NAc that may potentially underlie alcohol drinking. Those findings will then lead to improved site-specific pharmacotherapies that can target these circuits at specific stages of alcohol abuse and dependence.

Chapter 3: Dopamine D2 Receptor Neuroadaptation in Cholinergic Interneurons of the Nucleus Accumbens of C57BL/6J Mice Occurs Following Binge Alcohol Drinking But Not After Initiation Alcohol Drinking

3.1 Abstract

Identifying alcohol-induced receptor neuroadaptations that occur within specific cell populations will help to elucidate the underlying neurobiology of this complex disease. The present study tested the hypothesis that DA D2 receptor changes occur in cholinergic neurons of the NAc following initiation and binge alcohol drinking.

In the initiation model of alcohol drinking the experimental group self-administered ethanol using a two-bottle choice procedure with unlimited access to 10% (v/v) ethanol and water for 23 h/d for 1 month. Control animals received identical treatment, except that both bottles contained water. In the binge model of alcohol drinking experimental animals were given limited access to 20% (v/v) ethanol in the dark cycle, and mice remained on this schedule for 1 month. All animals were perfused and brain sections were processed for ICC procedures.

The binge model of alcohol drinking produced a 33% decrease in DA D2 positive cholinergic interneurons in the core NAc. No significant changes were observed following the initiation model of alcohol drinking indicating that high BECs, produced by the binge drinking model, were necessary for DA D2 receptor changes in this brain region. These findings identify region- and cell-specific DA D2 receptor neuroplasticity that can be targeted by improved genetic, pharmacotherapeutic and behavioral treatment programs for alcohol abuse and alcoholism.

3.2 Introduction

Alcoholism is a chronic disorder that is characterized by a specific set of behaviors, including persistent alcohol seeking and the inability to stop drinking, which can lead to tolerance, withdrawal, and vulnerability to relapse (American Psychiatric Association, 1994). Because this devastating disease affects millions of people worldwide, and poses serious medical, economic, and social consequences, it is important to identify the critical brain regions and specific cell types involved in alcohol drinking and the neuroadaptations within those circuits that underlie alcohol abuse and dependence.

The mesocorticolimbic DA system, comprised of dopaminergic projections from the VTA to limbic and cortical regions including the NAc and PFC, is widely accepted as a critical component of alcohol self-administration (Pierce and Kumaresan, 2006). Studies show that alcohol produces increased cell body firing in the VTA both in vitro (Brodie et al., 1990) and in vivo (Gessa et al., 1985), which results in elevated extracellular DA levels in terminal areas of the mesolimbic DA system (Di Chiara and Imperato, 1988; Gronier et al., 2000; Imperato and Di Chiara, 1986; Yim and Gonzales, 2000). Voluntary alcohol drinking also increases DA activity in the NAc (Doyon et al., 2003; Doyon et al., 2005; Weiss et al., 1993). DA, therefore, is thought to be a key neurotransmitter underlying the initiation of alcohol use (Czachowski et al., 2001; Czachowski et al., 2002; Gonzales et al., 2004) and the reinforcing effects of alcohol (Koob and Bloom, 1988; Rassnick et al., 1992).

Alcohol effects on DA signaling can also lead to DA receptor neuroadaptation. DA receptors are important links between extracellular events and intracellular signaling

mechanisms that underlie long-term neuronal plasticity. Neuroadaptive changes in DA D2 receptors have been reported to occur following alcohol drinking and withdrawal and have been implicated in drug-seeking behavior and relapse (De Vries and Shippenberg, 2002; De Vries et al., 2002; Self et al., 1996). Thanos and colleagues (2001; 2004) found that overexpression of the DA D2 receptor gene in the core NAc resulted in a decrease in alcohol drinking in P rats. This finding suggests that high levels of DA D2 receptors are protective against alcohol abuse. Similarly, DA D2 receptor agonists have been shown to decrease alcohol intake in C57BL/6J mice (Ng and George, 1994). While these findings implicate DA D2 receptors in specific brain areas to be critically involved in alcohol drinking, alcohol-induced changes in DA D2 receptor expression in specific cell types within these brain areas have not been previously investigated.

Although DA D2 receptors are located on a variety of cell types, cholinergic interneurons were of particular interest to examine for possible alcohol mediated changes in DA D2 receptor changes in the present study for several reasons. Namely, these cells have been associated with the initial effects of alcohol (Herring et al., 2004) as well as voluntary alcohol drinking (Camp et al., 2006; Camp and Alcantara, 2007). They are important integrators and modulators of striatal functioning (Kawaguchi et al., 1995; Wilson et al., 1990) and possess several key receptors, including DA receptors, which have been linked to plasticity, learning, and drug abuse (Alcantara et al., 2003; Berlanga et al., 2005). In addition, alcohol self-administration has been shown to increase ACh in the NAc, suggesting the importance of cholinergic interneurons in alcohol abuse (Nestby et al., 1999). Furthermore, Chapter 2 of this dissertation revealed a direct causal link between cholinergic interneurons and alcohol drinking as demonstrated by selective

destruction of these cells (Camp and Alcantara, 2007). Understanding the effects of alcohol drinking on neuroadaptations of DA D2 receptors in cholinergic neurons should provide insight into the effects of alcohol mediated dopaminergic modulation of ACh neurotransmission and its effects on specific alcohol-mediated behaviors.

The present study examined accumbal DA D2 receptor expression in cholinergic interneurons following initiation and binge alcohol drinking in C57BL/6J mice. This study tested the hypothesis that 1 month of initiation alcohol drinking causes a decrease in D2-positive cholinergic neurons of the core NAc, while binge alcohol drinking produces a more robust decrease in D2-positive cholinergic cells in this brain region. Comparing initiation and binge alcohol drinking may potentially give insight into neuroadaptive changes that occur both in moderate alcohol drinking (initiation) and in binge alcohol drinking, when BECs are driven to pharmacologically significant levels. Identifying neuroadaptations that occur in the transition from causal alcohol drinking to compulsive drinking is important for future research aimed to prevent the development of behaviors such as compulsive drinking. Identifying alcohol-driven neuroplasticity of receptor subtypes located on specific cell populations in brain areas implicated in the rewarding effects of alcohol and compulsive alcohol drinking may aid in improving the understanding of the underlying neurobiology of alcoholism.

3.3 Materials and Methods

3.3.1 Animals

3.3.1.1 Model of Initiation Alcohol Drinking

Twenty male C57BL/6J mice (18.7-23.3 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). Each mouse was individually housed in a standard cage in a temperature- and humidity-controlled room under a 12 hour light/dark cycle (lights on 07:00 h). Mice were 7 weeks old at the start of the experiment and food and water were available ad libitum. All experimental procedures complied with guidelines specified by the National Institutes of Health and were performed under an institutionally reviewed and approved research protocol.

3.3.1.2 Model of Binge Alcohol Drinking

Twenty-six male C57BL/6J mice (17.3-22.5 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). Each mouse was individually housed in a standard cage in a temperature- and humidity-controlled room under a reverse 12 hour light/dark cycle (lights on 19:00 h). Mice were 7 weeks old at the start of the experiment and food was available ad libitum. All experimental procedures complied with guidelines specified by the National Institutes of Health and were performed under an institutionally reviewed and approved research protocol.

3.3.2 Model of Initiation Alcohol Drinking Design and Procedures

A continuous-access, two-bottle choice procedure was used for this study. Animals were randomly assigned to either the ethanol group or control group. In the

alcohol group, food pellets, water, and 10% (v/v) alcohol were available continuously for 23 hours/day, while the control group had continuous access to food pellets and water. One hour was used to service the cages. During this hour, the fluid volumes consumed were recorded to the nearest 0.1 g, the bottles were refilled, the left-right positions of the 2 bottles were alternated to counterbalance among subjects to control for side preference, and the mice were weighed. An alcohol bottle and a water bottle were placed on an empty cage on each of the animal racks. These bottles were weighed each day to estimate the fluid that was lost due to leakage or evaporation. Average volume lost from these bottles was subtracted from the individual volumes each day. Alcohol intake was converted into average g alcohol/kg body weight/day. The data were recorded and stored for later analysis. Both groups remained under these conditions for 28 days (i.e., 1 month).

3.3.3 Model of Binge Alcohol Drinking Design and Procedures

The Drinking in the Dark (DID) model of binge alcohol drinking used in this study was the same as previously described in Chapter 2 (page 34, section 2.3.3). Briefly, animals were randomly assigned to either the alcohol group or control group. For the alcohol group the home cage water bottle was replaced with a bottle containing 20% (v/v) ethanol for 2 hours (days 1 – 3) or 4 hours (days 4 – 28) starting 3 hours after lights off (12:00 h). The control group received identical treatment except that the home cage water bottle was replaced with another water bottle. Ethanol and water bottles were weighed upon removal from the cages and animals were weighed daily after alcohol access. Animals remained on this schedule for 1 month.

3.3.4 Tissue Preparation

At the end of the 1 month drinking period, animals were perfused and tissue was sectioned as previously described in Chapter 2 (page 36, section 2.3.6).

3.3.5 Light Microscopy Immunocytochemistry

3.3.5.1 Dual ChAT and Dopamine D2 Receptor Immunoperoxidase Labeling

Light microscopy dual-labeling immunoperoxidase procedures were performed on free-floating tissue sections that were rinsed in 0.1 M PBS (2 × 10, 3 × 5 minutes) and preincubated for 1 hour in a PBS blocking solution containing 5% NGS and 0.01% H_2O_2. Sections were then incubated simultaneously in both primary antibodies: affinity-purified mouse anti-ChAT monoclonal antibody (1:1,000; Chemicon) and affinity-purified rabbit anti-DA D2 receptor polyclonal antibody (1:350, Chemicon). Sections were then rinsed in PBS (4 × 5 minutes and similarly rinsed after each step). The tissue was subsequently incubated in secondary biotinylated donkey anti-mouse IgG antiserum (1:500; Jackson ImmunoResearch), diluted in 2% NGS/PBS for 2 hours, and then incubated in ABC (Vector Laboratories) for 1 hour. ChAT IR was visualized with DAB and 0.01% H_2O_2 in PBS, which resulted in a brown reaction product. Sections were then incubated in secondary biotinylated goat anti-rabbit IgG antiserum (1:200; Vector Laboratories) diluted in 2% NGS/PBS for 1 hour and then incubated in ABC (Vector Laboratories) for 1 hour. DA D2 receptor IR was visualized with an SG substrate kit (Vector Laboratories), which resulted in a blue/black reaction product.

To confirm specificity of the labeling procedures, all combinations of single-labeled ChAT and D2 were visualized with DAB or Vector SG. Specific labeling for the

respective antigens was observed under all of these conditions. Control sections for single-labeling and dual-labeling procedures were processed identically, with the exception that primary antibodies were omitted from the incubation solution. Immunolabeling was not observed in any of the control conditions. All photomicrograph images were processed for contrast and brightness with Adobe Photoshop 8.0.

3.3.5.2 Single Dopamine D2 Receptor Immunoperoxidase Labeling

Light microscopy single-labeling immunoperoxidase procedures were performed on free-floating tissue sections that were rinsed in 0.1 M PBS (2 × 10, 3 × 5 minutes) and preincubated for 1 hour in a PBS blocking solution containing 5% NGS and 0.01% H_2O_2. Sections were then incubated overnight at 4°C in an affinity-purified rabbit anti-DA D2 receptor polyclonal antibody (1:350, Chemicon) diluted in the 5% NGS/PBS blocking solution. The tissue was subsequently rinsed in PBS (4 × 5 minutes and similarly rinsed after each step). Sections were then incubated in secondary biotinylated goat anti-rabbit IgG antiserum (1:200; Vector Laboratories), diluted in 2% NGS/PBS for 2 hours, and then incubated in ABC (Vector Laboratories) for 1 hour. DA D2 receptor IR was visualized with DAB and 0.01% H_2O_2 in PBS, which resulted in a brown reaction product.

3.3.6 Quantitative and Statistical Analysis

3.3.6.1 Dual ChAT and Dopamine D2 Receptor Quantification

Digital images representing areas of 0.45 × 0.45 mm were acquired with a 20 × objective (800 × working magnification) from brain tissue that was dual

immunoperoxidase labeled for ChAT and DA D2 receptor. For each animal, the shell and core regions of the NAc were examined from coronal brain sections corresponding to the appropriate levels from the Franklin and Paxinos (1997) mouse brain atlas. Two digital images were acquired from both left and right hemispheres of the core NAc (Bregma 1.10 mm) and three digital images were acquired from both hemispheres of the shell NAc (Bregma 1.10 mm). Images were acquired on a Nikon (Melville, NY) Eclipse E800 light microscope equipped with a digital camera interfaced with a personal computer. All images were stored and analyzed by observers blind to group assignment. In quantifying these dual-labeled regions, the observers counted cholinergic cells and then determined which of these cells coexpressed D2 receptor labeling. The percentage of cholinergic cells that expressed D2 receptor IR was calculated for each sample area. An average number for the sample areas per brain region, averaged across both hemispheres, was calculated for each animal. The group averages were subsequently calculated for each brain area of interest.

3.3.6.2 Single Dopamine D2 Receptor Quantification

For single immunoperoxidase labeled brain tissue sections, digital images representing an area of 0.225 × 0.225 mm were acquired using a 20 × objective (800 × working magnification) for the shell and core regions of the NAc (Franklin and Paxinos, 1997). Two digital images were acquired for quantification from both left and right hemispheres of the core NAc (Bregma 1.10 mm), and three digital images were acquired from both hemispheres of the shell NAc (Bregma 1.10 mm). All images were stored and analyzed by observers blind to group assignment. In quantifying these single

immunoperoxidase labeled regions, the observers counted all cells that expressed DA D2 receptor IR within the left and right hemispheres of each brain region of interest. An average number for the sample areas per brain region, averaged across both hemispheres, was calculated for each animal. The group averages were subsequently calculated for each brain area of interest.

3.3.6.3 Statistical Analysis

Daily session intakes were calculated in grams of alcohol per kilogram of body weight (g/kg). All values are reported as means ± SEM. Statistical analysis of data was carried out using Student's *t*-test for individual comparisons between alcohol-treated rats and control groups. A value of $p<0.05$ was considered significant for these analyses.

3.4 Results

3.4.1 Home Cage Fluid Intake

In the initiation drinking paradigm animals in the experimental group self-administered an average of 8.5 ± 1.4 g/kg alcohol per day, while in the control group animals self-administered an average of 1.9 ± 0.3 mL of water a day.

In the binge drinking paradigm animals in the experimental group self-administered an average of 7.8 ± 0.8 g/kg alcohol per day, while in the control group animals self-administered an average of 2.0 ± 0.7 mL of water a day.

3.4.2 Model of Initiation Alcohol Drinking

3.4.2.1 Dual ChAT and Dopamine D2 Receptor Immunoreactivity

Dual ChAT and D2 receptor IR was examined in the shell and core regions of the NAc. One month of continuous-access, two-bottle choice initiation alcohol drinking did not elicit significant changes in either region. The percentage of D2-positive cholinergic neurons is presented in Table 3.1.

3.4.2.2 Single Dopamine D2 Receptor Immunoreactivity

Single D2 receptor IR was examined in the shell and core regions of the NAc. One month of voluntary initiation alcohol drinking did not elicit significant changes in the general population of accumbal cells in either of these brain regions. The average number of DA D2 receptor-positive neurons per sample area in the core NAc was 33.9 ± 1.8 in the control group and 35.7 ± 1.7 in the alcohol group, while in the shell NAc the average number of DA D2 receptor-positive neurons per sample area was 28.8 ± 1.1 in the control group and 28.2 ± 2.0 in the alcohol group. These data are reported as means ± SEM.

Table 3.1 DA D2 Receptor Neuroadaptation in the NAc of C57BL/6J Mice Following 1 Month of Voluntary Initiation Alcohol Drinking

Percent of DA D2 receptor-positive cholinergic neurons		
	Water	Alcohol
Nucleus Accumbens		
Core	65.8 ± 14.1	65.8 ± 8.1
Shell	45.9 ± 5.1	59.9 ± 8.0

Data are presented as mean number of DA D2-positive neurons per sample area ± SEM.

3.4.3 Model of Binge Alcohol Drinking

3.4.3.1 Dual ChAT and Dopamine D2 Receptor Immunoreactivity

Dual ChAT and D2 receptor IR was examined in the shell and core regions of the NAc (Figure 3.1). Quantitative analysis of the percent of cholinergic neurons that coexpressed D2 receptor, as reported in Table 3.2 and Figure 3.2, was calculated from immunoperoxidase labeled tissue. At the end of the 1 month binge alcohol drinking period, a 33% decrease in the percentage of D2 receptor-positive cholinergic neurons was observed in the core NAc [$t(22) = 2.61$, $p<0.05$, 2-tailed] (Figure 3.2). The average number of DA D2 receptor-positive cholinergic neurons per sample area in the core NAc was 59.3 ± 6.6 in the control group and 39.7 ± 4.1 in the alcohol group. These data are reported as means ± SEM. Alcohol did not elicit significant changes in the shell NAc.

3.4.3.2 Single Dopamine D2 Receptor Immunoreactivity

Single D2 receptor IR was examined in the shell and core regions of the NAc.

Alcohol drinking did not cause significant changes in the general population of accumbal neurons in either of these brain regions. The average number of DA D2 receptor-positive neurons per sample area in the core NAc was 14.1 ± 1.0 in the control group and 14.8 ± 1.5 in the alcohol group, while in the shell NAc the average number of DA D2 receptor-positive neurons per sample area was 15.6 ± 1.3 in the control group and 17.3 ± 1.4 in the alcohol group. These data are reported as means ± SEM.

Table 3.2 DA D2 Receptor Neuroadaptation in the NAc of C57BL/6J Mice Following 1 Month of Voluntary Binge Alcohol Drinking

Percent of DA D2 receptor-positive cholinergic neurons		
	Water	Alcohol
Nucleus Accumbens		
Shell	57.1 ± 5.8	41.5 ± 5.9

Data are presented as mean number of DA D2-positive neurons per sample area ± SEM.

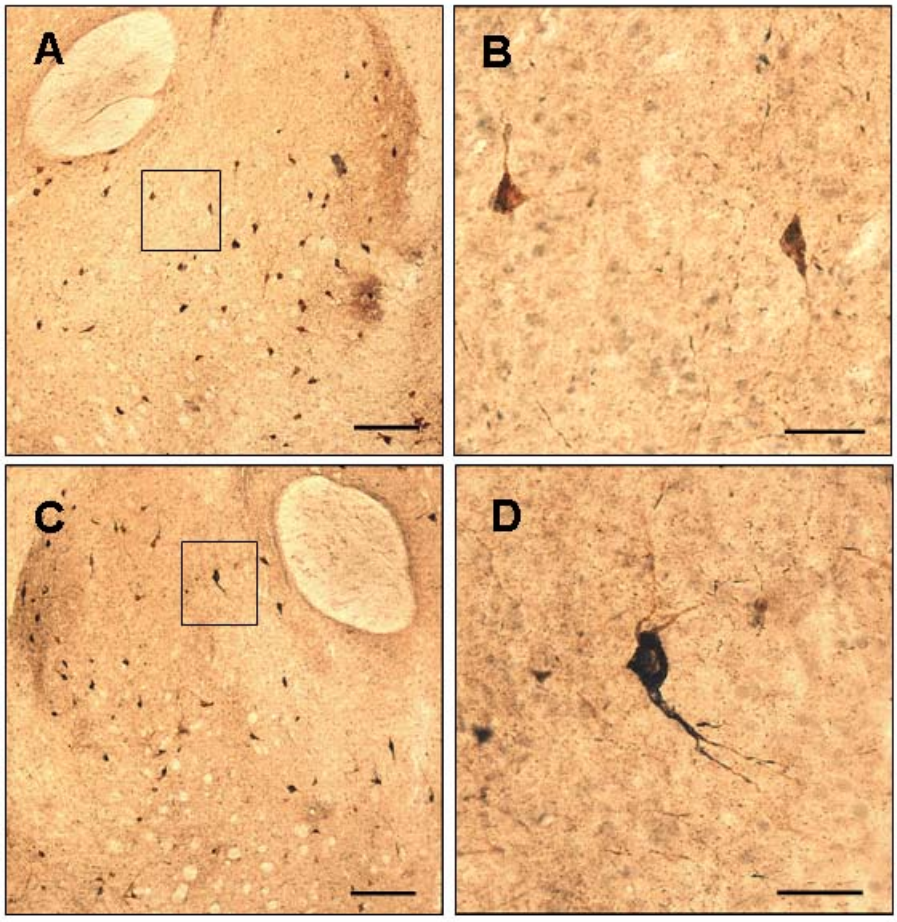

Figure 3.1. Representative photomicrographs of dual-immunoperoxidase labeled tissue illustrating the localization of DA D2 receptors in cholinergic interneurons of the NAc in C57BL/6J mice. (A) Low-magnification of the NAc from an animal that underwent 1 month of binge alcohol drinking. (B) High-magnification of the core NAc from the area outlined in the box shown in A. (C) Low-magnification of the NAc from a control animal. (D) High-magnification of the core NAc from the area outlined in the box shown in C. The brown DAB label identified ChAT IR cholinergic neurons, whereas the blue/black SG label indicated DA D2 receptor IR. Scale bars = 100 μm A, C; 30 μm B, D.

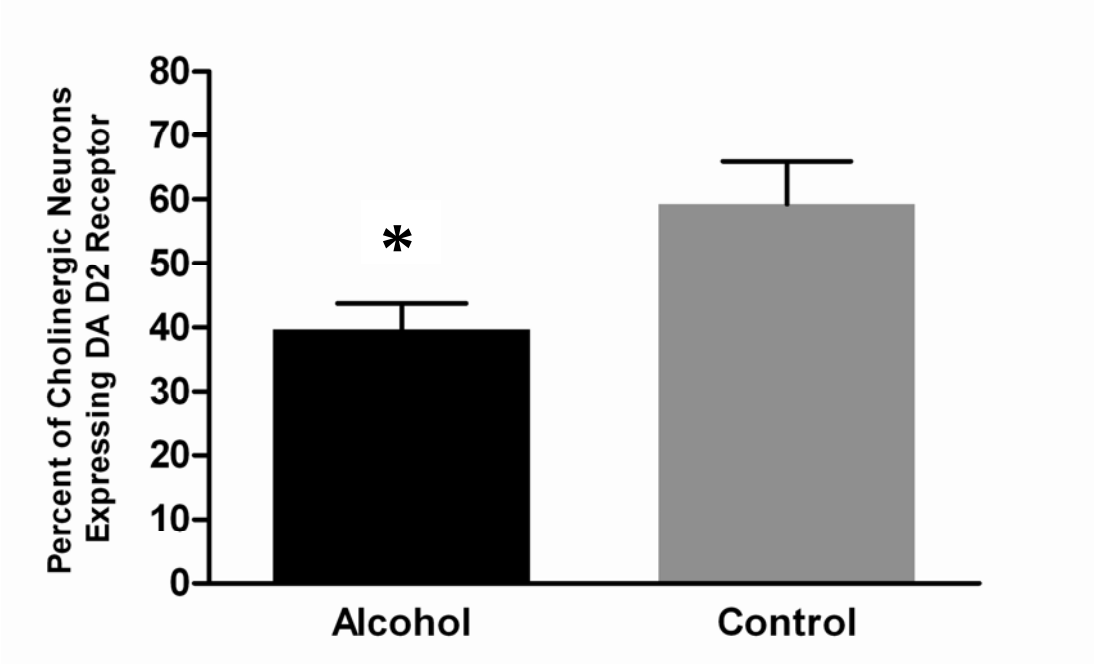

Figure 3.2. Percent of DA D2 receptor-positive cholinergic interneurons in the core NAc of C57BL/6J mice following 1 month of voluntary binge alcohol drinking. Binge drinking elicited a 33% decrease in the percentage of DA D2 receptor-positive cholinergic neurons in the core NAc. Data are presented as mean number of DA D2-positive cholinergic neurons per optical field ± SEM (*p<0.05).

3.5 Discussion

The present study focused on identifying alcohol mediated DA D2 receptor changes on cholinergic neurons of the NAc. Binge alcohol drinking resulted in a decrease in the percentage of D2 IR cholinergic neurons in the core NAc. Significant changes in D2 receptor expression were not observed in non-cholinergic neurons following the binge drinking model. Additionally, D2 receptor changes in cholinergic or non-cholinergic neurons were not found following the initiation drinking model. Altogether, this study demonstrates that changes in D2 receptor expression in C57BL/6J mice are specifically influenced by binge alcohol drinking, when BECs are repeatedly driven to intoxicating levels. Furthermore, these findings suggest that neuroadaptation of D2 receptors previously reported to occur in association with alcohol drinking may be occurring primarily in cholinergic neurons.

Studies utilizing animal models of alcoholism, as well as those in human alcoholics, have identified the importance of D2 receptors. Thanos and colleagues (2001; 2004) varied the levels of DA D2 receptors in the NAc of P rats using an adenoviral vector and found that increases in accumbal DA D2 receptors reduced alcohol preference and intake. Furthermore, human neuroimaging studies have shown that chronic alcohol drinking results in significantly lower amounts of available DA D2 receptors (Hietala et al., 1994; Volkow et al., 1996) and nonalcoholic members of alcoholic families have higher than normal levels of DA D2 receptors (Volkow et al., 2006). These findings were consistent with earlier work by Blum and colleagues (1990) who found a positive correlation between the A1 allele of the D2 receptor gene and an increased susceptibility to alcoholism. These studies demonstrate the importance of reduced levels of D2

receptors in alcohol abuse, but none focus on examining receptor changes in specific cell types. These findings suggest that alcohol mediated dopaminergic facilitation of ACh release, occurring through DA D2 receptor down-regulation, may influence neuronal circuits that facilitate compulsive alcohol drinking.

Cholinergic interneurons of the striatum have previously been implicated in acute alcohol exposure (Herring et al., 2004), voluntary alcohol drinking (Camp et al., 2006; Camp and Alcantara, 2007) and drug abuse (Berlanga et al., 2003; Mark et al., 1999). In the striatum, these large, aspiny, local circuit neurons are involved in associative learning (Aosaki et al., 1994), synaptic plasticity, LTP induction (Calabresi et al., 2000; Suzuki et al., 2001), and modulation of prefronto-striatal information processing (Alcantara et al., 2001). Their widespread dendritic arborizations (Wilson et al., 1990) integrate inputs from regions such as the PFC and thalamus (Dimova et al., 1993; Lapper and Bolam, 1992), and their extensive axonal fields synapse onto several striatal cells including MSNs, thereby providing a powerful influence on overall striatal signaling and motor output behavior (Howe and Surmeier, 1995).

The goal of the present study was to identify receptor neuroadaptation on cholinergic interneurons following voluntary alcohol drinking. As has been discussed throughout the chapters of this dissertation, the NAc is a key brain area associated with the reinforcing effects of alcohol and other drugs of abuse (Di Chiara and Imperato, 1988; Koob, 2003; Koob and Bloom, 1988; Pontieri et al., 1995; Rassnick et al., 1992) and drug-seeking behavior (Kalivas and Volkow, 2005). Its connectivity with areas such as the PFC and DS position this region to serve as an integrator of cortical and limbic information and to generate goal-directed behaviors (Kalivas, 2004; Mogenson et al.,

1980; Voorn et al., 1986). The core NAc is associated with drug-seeking (Kalivas and Volkow, 2005), as well as the expression of learned behaviors in response to motivationally relevant stimuli (Di Ciano and Everitt, 2001; Kelley, 2004). Additionally, the core NAc is associated with sensitization to cocaine and other drugs of abuse (Li et al., 2004; Robinson and Kolb, 2004) and related synaptic rewiring (Berlanga et al., 2006). The findings that DA D2 IR was down-regulated in cholinergic cells of the core NAc are consistent with previous reports of decreased DA D2 receptor availability in striatal areas of alcohol abusers.

These findings demonstrate that cholinergic neurons targeted by alcohol undergo changes in DA D2 receptor neuroadaptation in the NAc following voluntary binge alcohol drinking. The fact that DA D2 receptor changes did not occur in other neurons of this region identify the importance of cholinergic interneurons as key components of alcohol drinking. This also suggests that striatal DA D2 receptor changes previously reported, such as in neuroimaging studies, could potentially be influenced by changes occurring in cholinergic neurons. Significant changes were observed in the core NAc and, while not statistically significant, there was a tendency for a down-regulation of D2-positive cholinergic cells in the shell NAc following binge alcohol drinking. The core NAc has recently been implicated in drug-seeking (Kalivas and Volkow, 2005) and is a component of a striatal circuitry where ventral regions influence more dorsal regions (Haber et al., 2000). The core NAc could potentially be recruited in binge drinking as drinking behavior becomes more compulsive and habitual. It is also noteworthy that changes did not occur following 1 month of initiation drinking. This suggests that in C57BL/6J mice DA D2 receptor changes are contingent upon repeated, intoxicating

BECs. These findings, in addition to previous findings reported in this dissertation, identify cholinergic interneurons as critical components of alcohol drinking. These results may contribute to a better understanding of the cellular and molecular mechanisms that lead to alcoholism, thus leading to improved pharmaceutical and behavioral treatment programs for alcoholism.

Chapter 4: Cdk5 Neuroadaptation in Cholinergic Interneurons of the Nucleus Accumbens of C57BL/6J Mice Occurs Following Initiation and Binge Alcohol Drinking

4.1 Abstract

Neurobiological studies have identified brain areas and related molecular mechanisms involved in alcohol abuse and dependence. Specific cell types in these brain areas and their role in alcohol-related behaviors, however, is not well understood. This study examined plasticity-related intracellular changes that occur in cholinergic neurons of C57BL/6J mice following a model of initiation drinking and a model of binge alcohol drinking. Cdk5 IR was examined in cholinergic neurons of the NAc, using dual ICC procedures, and single Cdk5 IR was examined in this brain region to determine the potential role of other cells within the NAc network on alcohol drinking.

Brain tissue examined in this study was performed on adjacent sections to those examined in the DA D2 receptor study (Chapter 3). In the initiation model of alcohol drinking the experimental group self-administered ethanol using a two-bottle choice procedure with unlimited access to 10% (v/v) ethanol and water for 23 h/day for 1 month. Experimental animals in the binge model were given limited access to 20% (v/v) ethanol in the dark cycle. Control animals in both groups received only water bottles and all animals remained on these schedules for 1 month. All animals were perfused and brains were processed for ICC procedures.

The initiation model of alcohol drinking produced a 38% increase in Cdk5 IR cholinergic interneurons in the core NAc, while the model of binge alcohol drinking

caused a 65% increase in the percent of Cdk5 IR cholinergic interneurons in the core NAc. Additionally, the binge model of alcohol drinking revealed a 29% increase in single Cdk5 IR in the core NAc.

This study identified Cdk5 neuroadaptation in cholinergic interneurons and other neurons of the NAc following 1 month of initiation alcohol drinking and 1 month of binge alcohol drinking. These findings contribute to our understanding of the cellular and molecular basis of alcohol drinking.

4.2 Introduction

Cdk5 is a neuronal serine/threonine protein kinase that has been reported to control a number of signal transduction mechanisms that regulate both functional and structural neuronal plasticity (Bibb, 2003), including neurotransmitter release (Tomizawa et al., 2002), LTP induction (Li et al., 2001), associative learning (Fisher et al., 2002), and synapse formation (Johansson et al., 2005). Neuronal plasticity is a critical component of the neurobiological responses to alcohol and other drugs of abuse. Neurotransmitter release can induce the activation of receptors which are then coupled to intracellular signaling pathways in the postsynaptic cell. These second messenger signal transduction pathways then cause a plethora of cellular alterations, such as gene transcription, that can lead to the development of such behaviors as compulsive alcohol drinking. Cdk5 has been identified as a downstream target of ΔFosB (Bibb et al., 2001), a transcription factor that accumulates and persists in the striatum in response to repeated exposure to drugs of abuse and persists for weeks or months even after the drug is withdrawn (Nestler et al., 2001). Cdk5 has also recently been identified as an important

mediator of drug addiction (Benavides and Bibb, 2004), and was therefore used in this study to examine neuroadaptive changes that result from voluntary alcohol drinking.

Cholinergic interneurons of the NAc are putative cells to study as potential key mediators of initiation and binge alcohol drinking. These cells undergo plasticity and learning (Aosaki et al., 1994; Suzuki et al., 2001), integrate a variety of cognitive, limbic, and motor functions (Calabresi et al., 2000), and provide a powerful influence on striatal output neurons. In addition, pharmacological studies have demonstrated an effect of alcohol on ACh release in the NAc (Nestby et al., 1999) and PFC (Stancampiano et al., 2004), suggesting the importance of cholinergic interneurons in alcohol abuse. A previous study in our laboratory demonstrated a dose-dependent activation of accumbal cholinergic interneurons following an acute i.p. administered does of alcohol (Herring et al., 2004).

The purpose of this study was to investigate the involvement and neuroplasticity of cholinergic interneurons and other neurons of the NAc as potential substrates underlying voluntary initiation and binge alcohol drinking in C57BL/6J mice. The study discussed in Chapter 2 identified the involvement of cholinergic neurons in alcohol drinking, and Chapter 3 examined alcohol-induced DA D2 receptor neuroadaptations. The present study tested the hypothesis that 1 month of initiation alcohol drinking causes an increase in Cdk5 IR in cholinergic neurons of the core NAc, and that binge alcohol drinking causes a more robust increase in Cdk5 IR cholinergic neurons of this region. Greater neuroplastic changes were predicted with the binge model where BECs repeatedly reach pharmacologically significant levels.

4.3 Materials and Methods

4.3.1 Animals

4.3.1.1 Model of Initiation Alcohol Drinking

Twenty male C57BL/6J mice (18.7-23.3 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were individually housed in standard cages in a temperature- and humidity-controlled room under a 12 hour light/dark cycle (lights on 07:00 h). Mice were 7 weeks old at the start of the experiment and food and water were available ad libitum. All experimental procedures complied with guidelines specified by the National Institutes of Health and were performed under an institutionally reviewed and approved research protocol.

4.3.1.2 Model of Binge Alcohol Drinking

Twenty-six male C57BL/6J mice (17.3-22.5 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were individually housed in standard cages in a temperature- and humidity-controlled room under a reverse 12 hour light/dark cycle (lights on 19:00 h). Mice were 7 weeks old at the start of the experiment and food was available ad libitum. All experimental procedures complied with guidelines specified by the National Institutes of Health and were performed under an institutionally reviewed and approved research protocol.

4.3.2 Model of Initiation Alcohol Drinking Design and Procedures

The experimental design for the model of initiation drinking was identical to that described in Chapter 3 (page 50, section 3.3.2)

4.3.3 Model of Binge Alcohol Drinking Design and Procedures

The experimental design for model of binge alcohol drinking was identical to that described in Chapter 2 (page 34, section 2.3.3).

4.3.4 Tissue Preparation

At the end of the 1 month drinking period, animals were perfused and tissue was sectioned as previously described in Chapter 2 (page 36, section 2.3.6).

4.3.5 Light Microscopy Immunocytochemistry

4.3.5.1 Dual ChAT and Cdk5 Immunoperoxidase Labeling

Light microscopy dual-labeling immunoperoxidase procedures for ChAT and Cdk5 were performed on adjacent sections to those examined for dual ChAT and D2 IR. Procedures were identical to those described in Chapter 3 (page 52, 3.3.5.1), except that the primary antibody cocktail contained mouse anti-ChAT monoclonal antibody (1:000, Chemicon) and affinity-purified rabbit anti-Cdk5 polyclonal antibody [1:250, Santa Cruz Biotechnology Inc., Santa Cruz, CA; Cdk5 (C-8); epitope mapping at C-terminal amino acid residues 284–291 of human Cdk5] diluted in the 5% NGS/PBS blocking solution overnight at 4°C. Cdk5 antibody specificity has been demonstrated by blotting analysis to recognize specifically Cdk5 of mouse, rat, and human origin. Cdk5 IR was visualized with an SG substrate kit (Vector Laboratories).

4.3.5.2 Single Cdk5 Immunoperoxidase Labeling

Light microscopy single-labeling immunoperoxidase procedures for Cdk5 were performed on adjacent sections to those examined for single DA D2 receptor IR. Procedures were identical to those described in Chapter 3 (page 53, section 3.3.5.2), except that the primary antibody was affinity-purified rabbit anti-Cdk5 polyclonal antibody (1:250, Santa Cruz Biotechnology, Inc.)

4.3.6 Quantitative and Statistical Analysis

4.3.6.1 Dual ChAT and Cdk5 Quantification

Dual quantification procedures were identical to those described in Chapter 3 (page 53, section 3.3.6.1), except that when quantifying the dual-labeled regions observers determined which cholinergic cells coexpressed Cdk5 IR.

4.3.6.2 Single Cdk5 Quantification

Single quantification procedures were identical to those described in Chapter 3 (page 54, section 3.3.6.2), except that observers counted all cells that expressed Cdk5 IR.

4.3.6.3 Statistical Analysis

Daily session intakes were calculated in grams of alcohol per kilogram of body weight (g/kg). All values are reported as means ± SEM. Statistical analysis of data was carried out using Student's *t*-test for individual comparisons between alcohol-treated rats and control groups. A value of $p<0.05$ was considered significant for these analyses.

4.4 Results

4.4.1 Home Cage Fluid Intake

In the initiation drinking paradigm animals in the experimental group self-administered an average of 8.5 ± 1.4 g/kg alcohol per day, while in the control group animals self-administered an average of 1.9 ± 0.3 mL of water a day.

In the binge drinking paradigm animals in the experimental group self-administered an average of 7.8 ± 0.8 g/kg alcohol per day, while in the control group animals self-administered an average of 2.0 ± 0.7 mL of water a day.

4.4.2 Model of Initiation Alcohol Drinking

4.4.2.1 Dual ChAT and Cdk5 Immunoreactivity

Dual ChAT and Cdk5 IR was examined in the shell and core regions of the NAc. Quantitative analysis of the percent of cholinergic neurons that co-expressed Cdk5, as reported in Table 4.1 and Figure 4.1, was calculated from immunoperoxidase labeled tissue. At the end of the 1 month initiation drinking period, a 38% increase in the percentage of Cdk5-positive cholinergic neurons was observed in the core NAc [t (16)= 2.73, $p<0.01$, 2-tailed] (Figure 4.1). The average number of Cdk5-positive cholinergic neurons per sample area in the core NAc was 42.7 ± 4.0 in the control group and 59.2 ± 4.3 in the alcohol group. These data are reported as means ± SEM. Alcohol did not elicit significant changes in the shell NAc.

4.4.2.2 Single Cdk5 Immunoreactivity

Single Cdk5 IR was examined in the shell and core regions of the NAc. Quantitative analysis of Cdk5-positive neurons was calculated from immunoperoxidase labeled tissue. One month of initiation alcohol drinking did not elicit significant changes in the general accumbal cell population in either the shell or core NAc. The average number of Cdk5-positive neurons per sample area in the core NAc was 62.4 ± 1.9 in the control group and 61.8 ± 2.6 in the alcohol group, while in the shell NAc the average number of Cdk5-positive neurons per sample area was 56.9 ± 1.1 in the control group and 57.4 ± 0.9 in the alcohol group. These data are reported as means ± SEM.

Table 4.1 Cdk5 Neuroadaptation in the NAc of C57BL/6J Mice Following 1 Month of Voluntary Initiation Alcohol Drinking

Percent of Cdk5-positive cholinergic neurons

	Water	Alcohol
Nucleus Accumbens		
Shell	52.5 ± 3.0	44.8 ± 3.3

Data are presented as mean number of Cdk5-positive neurons per sample area ± SEM.

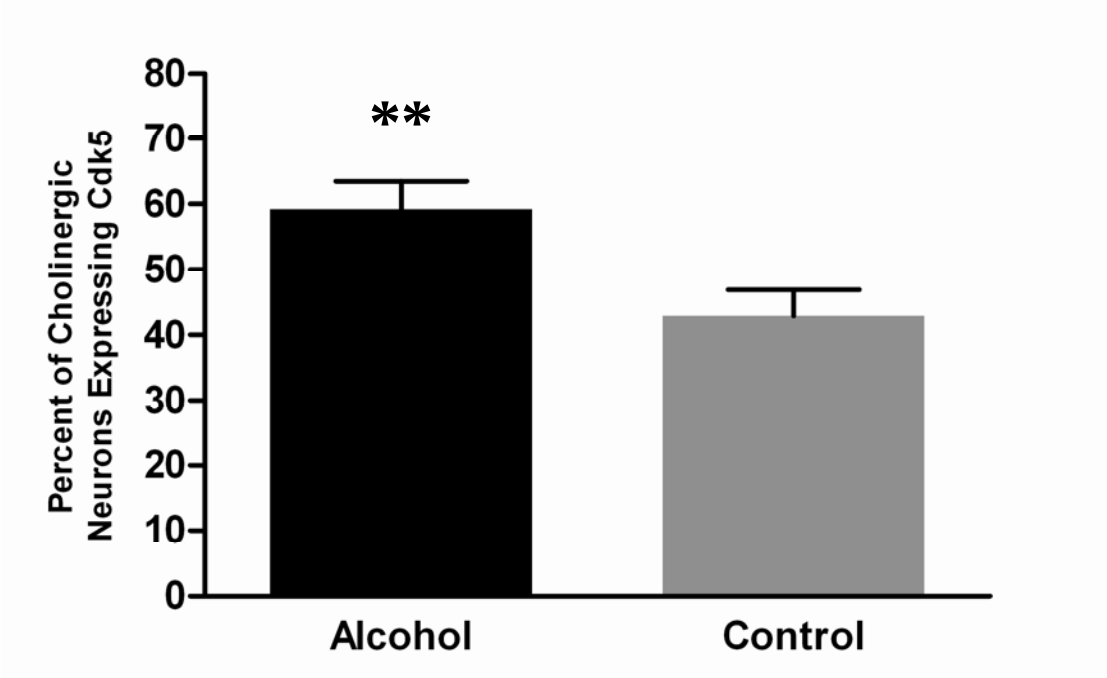

Figure 4.1. Percent of Cdk5-positive cholinergic interneurons in the core NAc of C57BL/6J mice following 1 month of voluntary initiation alcohol drinking. Initiation drinking elicited a 38% increase in the percentage of Cdk5-positive cholinergic neurons in the core NAc. Data are presented as mean number of Cdk5-positive cholinergic neurons per optical field ± SEM (**$p<0.01$).

4.4.3 Model of Binge Alcohol Drinking

4.4.3.1 Dual ChAT and Cdk5 Immunoreactivity

Dual ChAT and Cdk5 IR was examined in the shell and core regions of the NAc (Figure 4.2). Quantitative analysis of the percent of cholinergic neurons that co-expressed Cdk5, as reported in Table 4.2 and Figure 4.3, was calculated from immunoperoxidase labeled tissue. At the end of the 1 month DID drinking period, a 65% increase in the percentage of Cdk5-positive cholinergic neurons was observed in the core NAc [$t(23) = 2.22$, $p<0.05$, 2-tailed] (Figure 4.3). The average number of Cdk5-positive cholinergic neurons per sample area in the core NAc was 31.4 ± 7.1 in the control group and 51.8 ± 5.9 in the alcohol group. These data are reported as means ± SEM. Alcohol did not elicit significant changes in the shell NAc.

4.4.3.2 Single Cdk5 Immunoreactivity

Single Cdk5 IR was examined in the shell and core regions of the NAc. Quantitative analysis of Cdk5-positive neurons was calculated from immunoperoxidase labeled tissue. One month of DID binge alcohol drinking resulted in a 29% increase above baseline values in the core NAc [$t(20) = 2.71$, $p<0.01$, 2-tailed] (Figure 4.4). The average number of Cdk5-positive neurons per sample area in the core NAc was 18.9 ± 1.3 in the control group and 24.4 ± 1.5 in the alcohol group. Alcohol drinking did not produce significant changes in the number of Cdk5-labeled neurons in the shell NAc. The average number of Cdk5-positive neurons per sample area in the shell NAc was 15.4 ± 2.2 in the control group and 19.8 ± 1.7 in the alcohol group. These data are reported as means ± SEM.

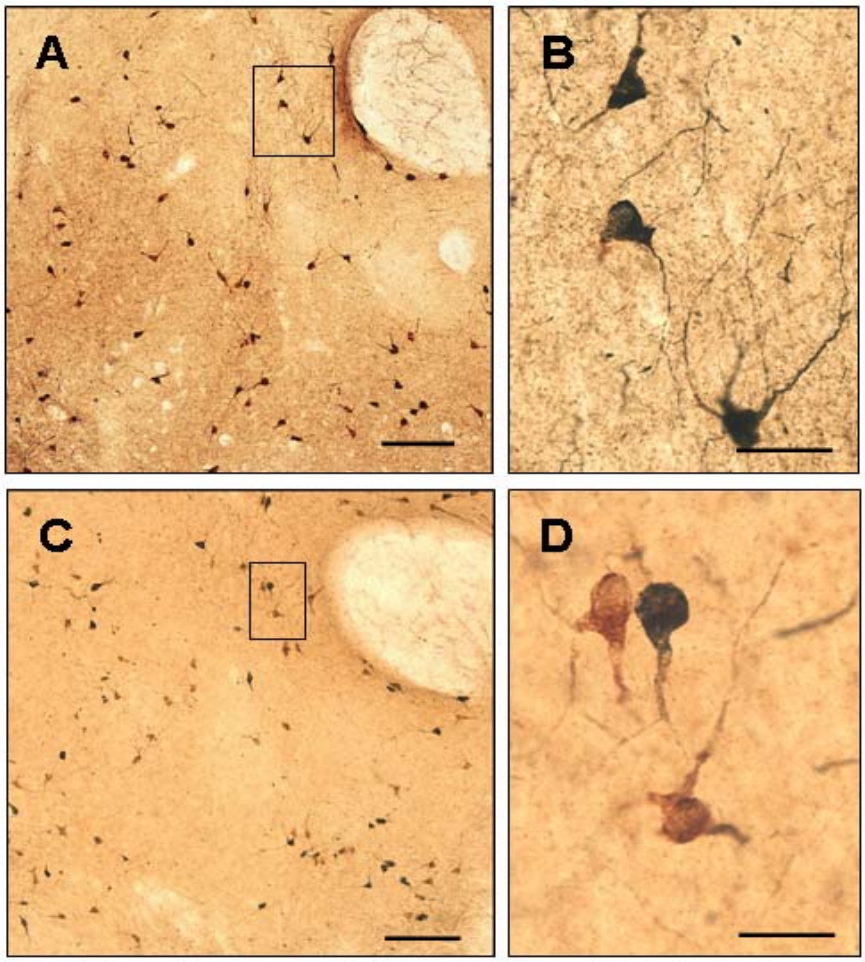

Figure 4.2. Representative photomicrographs of dual-immunoperoxidase labeled tissue illustrating the localization of Cdk5 in cholinergic interneurons of the NAc in C57BL/6J mice. (A) Low-magnification of the NAc from an animal that underwent 1 month of binge alcohol drinking. (B) High-magnification of the core NAc from the area outlined in the box shown in A. (C) Low-magnification of the NAc from a control animal. (D) High-magnification of the core NAc from the area outlined in the box shown in C. The brown DAB label identified ChAT IR cholinergic neurons; whereas the blue/black SG label indicated Cdk5 IR. Scale bars = 100 μm A, C; 30 μm B, D.

Table 4.2 Cdk5 Neuroadaptation in the NAc of C57BL/6J Mice Following 1 Month of Voluntary Binge Alcohol Drinking

Percent of Cdk5-positive cholinergic neurons		
	Water	Alcohol
Nucleus Accumbens		
Shell	40.6 ± 3.8	46.5 ± 5.5

Data are presented as mean number of Cdk5-positive neurons per sample area ± SEM.

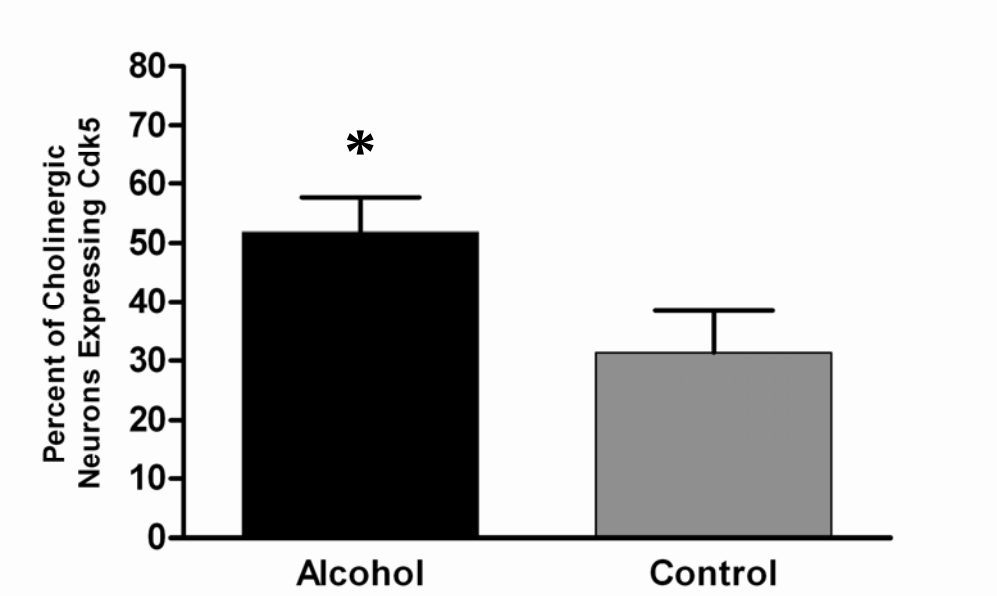

Figure 4.3. Percent of Cdk5-positive cholinergic interneurons in the core NAc of C57BL/6J mice following 1 month of voluntary binge alcohol drinking. Binge drinking elicited a 65% increase in the percentage of Cdk5-positive cholinergic neurons in the core NAc. Data are presented as mean number of Cdk5-positive cholinergic neurons per optical field ± SEM (*p<0.05).

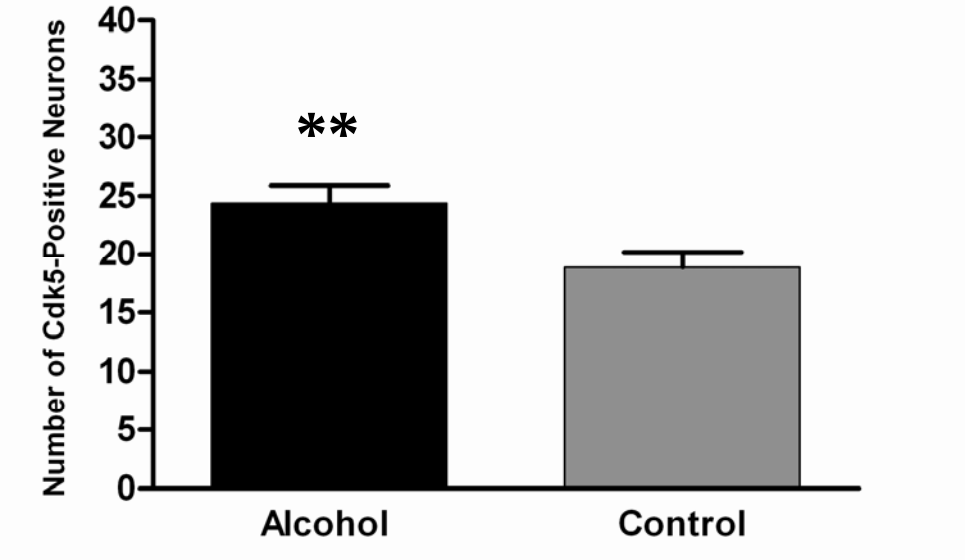

Figure 4.4. Number of Cdk5-positive neurons in the core NAc of C57BL/6J mice following 1 month of voluntary binge alcohol drinking. Binge drinking elicited a 29% increase in the percentage of Cdk5-positive cholinergic neurons in the core NAc. Data are presented as mean number of Cdk5-positive neurons per optical field ± SEM (**p<0.01).

4.5 Discussion

The present study examined the effects of initiation drinking and binge alcohol drinking on the expression of Cdk5 in cholinergic neurons and other neurons of the NAc. The identification of specific cell types in brain regions involved in alcohol drinking is essential for understanding the precise neurobiological basis of initial drinking that may ultimately progress to compulsive drinking. It was hypothesized that alcohol drinking in the continuous access, two-bottle choice initiation drinking procedure would cause an increase in Cdk5-positive cholinergic interneurons of the core NAc, and that excessive alcohol drinking in the binge model would cause a more robust increase in Cdk5-positive cholinergic interneurons of this region. The data show that, in fact, alcohol drinking in the initiation model causes a 38% increase in Cdk5-positive cholinergic neurons of the core NAc, while alcohol drinking in the binge model causes an almost 2-fold increase, 65%, in Cdk5-positive cholinergic cells of this region when compared with baseline values.

Cdk5 controls a number of mechanisms that regulate neuronal plasticity such as synapse formation, which may underlie long-term changes in the brain that mediate compulsive alcohol drinking, craving, or relapse. A previous study reported that inhibiting Cdk5 decreased spine density in the NAc (Norrholm et al. 2003). The findings of the present study suggest a potential increase in synapse formation in the core NAc with initiation alcohol drinking, and to a greater extent with binge drinking.

As continuously highlighted in the studies of this dissertation, cholinergic interneurons of the NAc are putative cells to study as potential mediators of alcohol drinking since they have recently been implicated in alcohol and drug use (Berlanga et

al., 2003; Camp et al., 2006; Herring et al., 2004; Hikida et al., 2001; Hikida et al., 2003; Mark et al., 1999; Nestby et al., 1999; Smith et al., 2004; Stancampiano et al., 2004). The NAc has been implicated in the rewarding and reinforcing properties of alcohol and other drugs of abuse (Di Chiara and Imperato, 1988; Koob, 2003; Koob and Bloom, 1988; Pontieri et al., 1995; Rassnick et al., 1992), however, this is not to say that the NAc is the only brain region affected by alcohol drinking. Many studies, in addition to work from our laboratory have found selective brain regions to be associated with exposure to alcohol (Bachtell et al., 1999; Camp et al., 2006; Herring et al., 2004; Lyons et al., 1998; Porrino et al., 1998; Williams-Hemby and Porrino, 1994). Bachtell and colleagues (1999) found that out of 23 brain regions c-fos expression was significantly induced in only 3: the core NAc, medial posteroventral portion of the CNA, and the Edinger-Westphal nucleus. Additionally, Porrino and colleagues (1998) found increases in cerebral metabolism in only 9 of 39 regions examined. These studies and studies from our laboratory indicate that alcohol selectively targets specific brain areas, suggesting that critical molecular transduction mechanisms that underlie alcohol drinking are region and cell specific.

Traditional animal models of alcohol drinking rarely produce pharmacologically significant BECs over extended periods of time. These methods are important for identifying changes that occur with the initial effects of alcohol drinking, but in order to understand neuroplasticity that occurs with compulsive drinking one must use binge models. This study shows that binge alcohol drinking causes an almost 2-fold increase in Cdk5-positive cells in the core NAc, which suggests importance of this brain region as casual alcohol drinking progresses to compulsive drinking. This finding is fundamental to

alcohol research since a primary component of alcoholism is loss of control over alcohol consumption (American Psychiatric Association, 1994). The present study identifies cholinergic neurons as critical sites for alcohol-mediated neuroadaptations which may facilitate the underlying characteristics of alcoholism such as compulsive alcohol drinking. These sites can then be targeted for the development of improved pharmacotherapeutic and behavioral interventions in the treatment of this complex disease.

Chapter 5: Dopamine D2 Receptor and Cdk5 Neuroadaptation in Cholinergic Interneurons of the Nucleus Accumbens, Dorsal Striatum, and Prefrontal Cortex of Inbred Alcohol-Preferring (iP) Rats Occurs Following Initiation Alcohol Drinking

5.1 Abstract

Previous studies in this dissertation have focused on the involvement and neuroplasticity of cholinergic interneurons in C57BL/6J mice. In order to further our understanding of this complex disease it is important to identify alcohol-induced similarities and differences across species. The goal of the present study, therefore, was to examine changes in DA D2 receptor and Cdk5 expression in cholinergic interneurons and other neurons of iP rats. Neuroadaptive changes were examined in various brain regions that have been implicated in the rewarding and reinforcing effects of alcohol and alcohol drinking following the initiation model of voluntary alcohol drinking.

Alcohol was self-administered using the two-bottle choice procedure with unlimited access to 10% (v/v) ethanol and water for 23 h/day for 1 month. Control animals received identical treatment, except both bottles contained water. Rats were perfused and brain sections were processed for ICC procedures.

Alcohol drinking resulted in a 25% and 46% decrease in DA D2 positive cholinergic interneurons in the core NAc and PrL PFC, respectively. A 46% increase in DA D2 positive cholinergic interneurons was also observed in the ventromedial DS. In addition, voluntary alcohol drinking produced a 51% increase in Cdk5-positive cholinergic interneurons in the shell NAc. Alcohol drinking also produced a 51% decrease in Cdk5-positive cholinergic neurons in the IL PFC and a 46% decrease in

Cdk5-positive cholinergic neurons in the PrL PFC. This study identifies cholinergic neuronal circuits that undergo DA D2 receptor and Cdk5 neuroadaptations following voluntary alcohol drinking in iP rats.

5.2 Introduction

Previous studies in this dissertation have focused on examining the involvement and neuroadaptations of cholinergic interneurons in the NAc following voluntary alcohol drinking in C57BL/6J mice. An important goal for research examining human conditions, including alcoholism, is to extrapolate results from animal studies to humans in an effort to better understand the underlying neurobiology of the disease and to develop improved treatments. Since there is no perfect animal model for alcoholism similarities and differences across species have to be considered when modeling different aspects of this disease.

There are many neuroanatomical and neurochemical similarities between mice and rats which make both important in alcohol studies. It has previously been identified that the boundaries of the shell and core subcompartments of the NAc of the mouse and rat are similar (Zocchi et al., 2003). In addition, ethanol and other drugs of abuse cause an increase in DA release in both the mouse (Zocchi et al., 2003) and the rat (Yim and Gonzales, 2000). While C57BL/6J mice are genetically predisposed to consume high amounts of ethanol, iP rats have been selectively bred for this behavior.

This study examined neuroadaptive changes in DA D2 receptor and Cdk5 expression in iP rats following 1 month of initiation alcohol drinking. iP rats are selectively bred to consume alcohol and meet the criteria for an animal model of

alcoholism. They voluntary drink alcohol, display a willingness to work for alcohol through operant responding, and develop tolerance and dependence through free-choice drinking (Murphy et al., 2002). P rats consume at least 5 g alcohol/kg/day and attain BECs of 50 - 200 mg% (Murphy et al., 2002). The iP strain was developed from the P line and it is suggested that the high-alcohol drinking behavior of the iP rat resembles that of the parent line (Edenberg et al. 2005).

The present study examined neuroadaptations in a number of different brain regions in the iP rat. This chapter will highlight changes in the NAc, DS, and PFC. The DS and PFC are intricately linked to the NAc through the nigrostriatal and mesocorticolimbic DA pathways, respectively. The DS is associated with habit learning (Ito et al., 2002; Jog et al., 1999) and is hypothesized to be involved in long-term synaptic plasticity as casual drug use progresses towards habitual drug-seeking (Berke and Hyman, 2000; Everitt et al., 2001; Everitt and Wolf, 2002). The PFC has been implicated in various higher-order cognitive tasks including, decision-making, attention, working memory, premotor planning (Dalley et al., 2004; Goldman-Rakic and Selemon, 1986), salience attribution, and inhibitory response control (Goldstein and Volkow, 2002). Additionally, the PFC is associated with the motivational effects underlying drug-seeking, cue-induced drug craving, and vulnerability to relapse (Weiss et al., 2001). Neuroadaptive changes in D2 receptor and Cdk5 expression were examined in the present study. Cdk5 was used as a marker of neuronal plasticity, and alternate brain sections were used to examine changes in D2 IR.

5.3 Materials and Methods

5.3.1 Animals

Thirty female iP rats (250-300 g) derived from strain 10C were obtained from the Indiana University Alcohol Research Center. Each rat was individually housed in a standard cage with food and water available ad libitum.

5.3.2 Model of Initiation Alcohol Drinking Design and Procedures

The initiation drinking paradigm was identical to that described in Chapter 3 (page 50, section 3.3.2).

5.3.3 Tissue Preparation

At the end of the 1 month drinking period, animals were anesthetized with an i.p. injection of sodium pentobarbital and perfused transcardially with 50 mL of 0.1 M PBS; pH 7.4), followed by 200 mL of 4% paraformaldehyde/0.1% glutaraldehyde in PBS. Alcohol and water bottles were removed immediately before each animal was anesthetized. Ten perfusions were performed each day, alternating between animals from alcohol and control group, for a total of 3 perfusion days. Brains were immediately removed and postfixed for 2 hours in 4% paraformaldehyde in PBS. Vibratome sections (100 μm thick) were taken and placed in 30% sucrose in PBS, frozen in liquid nitrogen, and stored in a -70°C freezer. Tissue was then thawed and processed for dual immunoperoxidase, single immunoperoxidase, or dual immunofluorescence labeling procedures. All efforts were made to minimize animal suffering and the number of animals used in the present study.

5.3.4 Dopamine D2 Receptor Immunoperoxidase Labeling

5.3.4.1 Dual ChAT and Dopamine D2 Receptor Immunoperoxidase Labeling

Light microscopy dual-labeling immunoperoxidase procedures for ChAT and D2 receptor were identical to those described in Chapter 3 (page 52, section 3.3.5.1).

5.3.4.2 Single Dopamine D2 Receptor Immunoperoxidase Labeling

Light microscopy single-labeling immunoperoxidase procedures for D2 receptor were identical to those described in Chapter 3 (page 53, section 3.3.5.2).

5.3.5 Cdk5 Immunoperoxidase Labeling

5.3.5.1 Dual ChAT and Cdk5 Immunoperoxidase Labeling

Light microscopy dual-labeling immunoperoxidase procedures for ChAT and Cdk5 were identical to those described in Chapter 4 (page 67, section 4.3.5.1).

5.3.5.2 Single Cdk5 Immunoperoxidase Labeling

Light microscopy single-labeling immunoperoxidase procedures for Cdk5 were identical to those described in Chapter 4 (page 68, section 4.3.5.2).

5.3.6 Dual ChAT and Cdk5 Immunofluorescence Labeling

Light microscopy dual-labeling immunofluorescence procedures were performed on free-floating, coronal tissue sections to verify colocalization of Cdk5 and ChAT (Figure 5.1). Sections were rinsed in 0.1 PBS (2 × 10, 3 × 5 minutes) and preincubated for 1 hour in a PBS blocking solution containing 5% normal donkey serum (NDS) and

2% bovine serum albumin (BSA). Sections were then incubated simultaneously in both primary antibodies: affinity-purified mouse anti-ChAT monoclonal antibody (1:50; Chemicon) and affinity-purified rabbit anti-Cdk5 polyclonal antibody (1:250; Santa Cruz Biotechnology, Inc.), diluted in the 5% NDS/2% BSA/PBS blocking solution for 48 hour at 4°C. Sections were rinsed in PBS (4 × 5 minutes). The tissue was then incubated in a cocktail of both secondary antibodies: fluorescein (FITC) affinipure donkey anti-mouse IgG and Texas Red affinipure donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch), diluted in 2% NDS/PBS solution for 1 hour. Sections were washed with PBS (3 × 5 minutes) and placed back in the primary cocktail for 1 hour, followed by incubation in the secondary cocktail for 1 hour. The incubation in primary and secondary cocktails was repeated a third time with washes (PBS 3 × 5 minutes) between each hour of incubation. The sections were then mounted and dried overnight and coverslipped with Krystalon (EM Science Harleco, Gibbstown, NJ) mounting media.

Fluorescence images were acquired on a Leica SP2 AOBS laser-scanning confocal microscope with a 40 × 1.25NA objective. Mouse anti-ChAT monoclonal antibody labeled with FITC (1:500; Jackson ImmunoResearch) was visualized using 488-nm excitation and a 535/70 emission filter. Rabbit anti-Cdk5 polyclonal antibody labeled with Texas Red (1:500; Jackson ImmunoResearch) was visualized using 594-nm excitation and a 650/100 emission filter. Fluorescent channels were acquired sequentially.

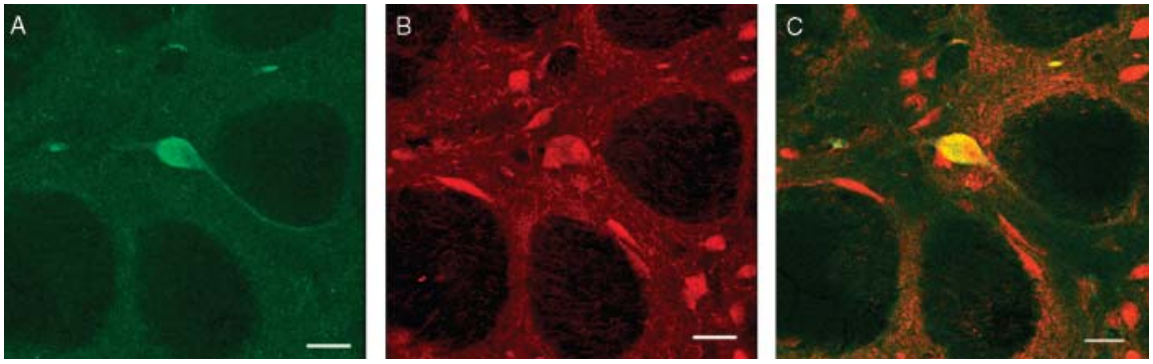

Figure 5.1. Confocal images of tissue processed for dual-immunofluorescence procedures. Maximum projections reveal ChAT IR neurons (A, FITC/green) and Cdk5-labeled cells (B, Texas Red). Each 512x512-pixel image represents a 177x177-µm area. A *z*-series of 25 images was acquired over a 3.9-µm distance. Coexpression of ChAT and Cdk5 in these neurons is shown with an overlay image at a single *z* position and is evident by the yellow color (C). Their intensities were normalized using Adobe Photoshop 7.0. Scale bars = 20 µm.

5.3.7 Quantitative and Statistical Analysis

5.3.7.1 Dual ChAT and Dopamine D2 Receptor Quantification

Dual quantification procedures were identical to those described in Chapter 3 (page 53, section 3.3.6.1). In the present study, areas of the NAc, DS, and PFC were examined. For each animal, brain areas of interest were examined from coronal brain sections corresponding to the appropriate Bregma levels from the Paxinos and Watson rat brain atlas (1998) (Figure 5.2). Two digital images were for quantification from left and right hemispheres of the IL and PrL PFC (Bregma 3.20 mm), and core NAc (Bregma 1.20 mm). Three digital images were acquired from both hemispheres of the shell NAc (Bregma 1.20 mm), and dorsolateral and ventromedial regions of the DS (Bregma 1.20 mm).

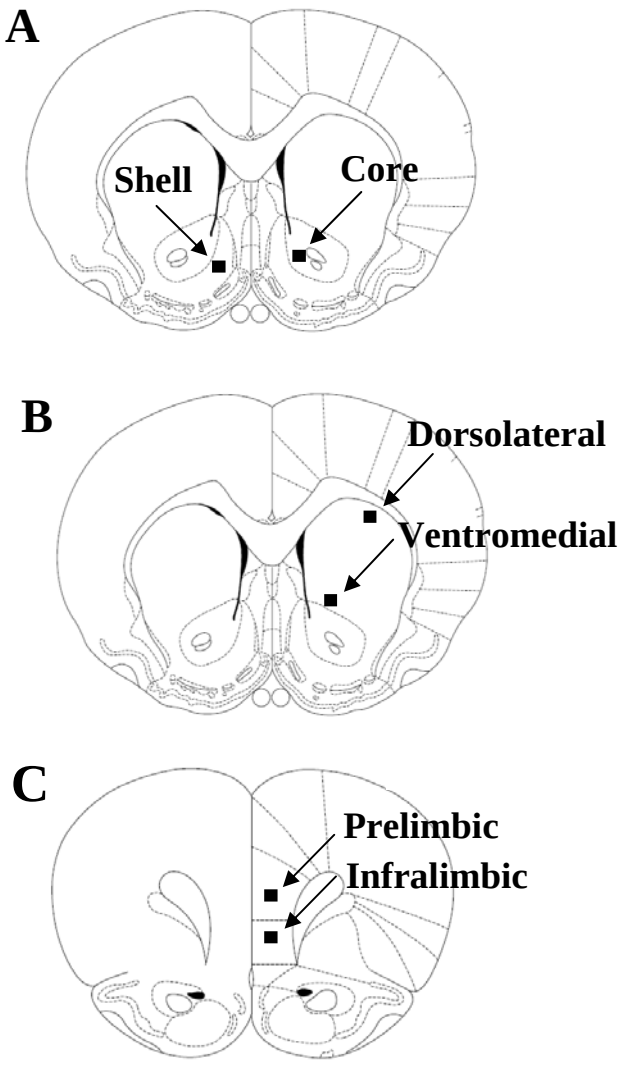

Figure 5.2. Schematic representations of coronal sections of the rat brain. Sections obtained from the Paxinos and Watson atlas (1998). The black boxes indicate the sample areas were quantifications were obtained from each area of interest. Samples were taken from (A) the shell and core NAc (Bregma 1.20 mm); (B) the dorsolateral and ventromedial regions of the DS (Bregma 1.20 mm); (C) the prelimbic and infralimbic regions of the PFC (Bregma 3.20 mm).

5.3.7.2 Single Dopamine D2 Receptor Quantification

Single quantification procedures were identical to those described in Chapter 3 (page 54, section 3.3.6.2), except that coronal brain sections corresponded to the Paxinos and Watson rat brain atlas (1998) as described above (section 5.3.7.1).

5.3.7.3 Dual ChAT and Cdk5 Quantification

Dual quantification procedures were identical to those described in Chapter 4 (page 68, section 4.3.6.1), except that coronal brain sections corresponded to the Paxinos and Watson rat brain atlas (1998) as described above (section 5.3.7.1).

5.3.7.4 Single Cdk5 Quantification

Single quantification procedures were identical to those described in Chapter 4 (page 68, section 4.3.6.2), except that coronal brain sections corresponded to the Paxinos and Watson rat brain atlas (1998) as described above (section 5.3.7.1).

5.3.7.5 Statistical Analysis

Daily session intakes were calculated in grams of alcohol per kilogram of body weight (g/kg). All values are reported as means ± SEM. Statistical analysis of data was carried out using Student's *t*-test for individual comparisons between alcohol-treated rats and control groups. A value of $p<0.05$ was considered significant for the D2 receptor analyses. Because of the large number of brain areas examined in the Cdk5 analyses, a significance of $p<0.01$ was used in an effort to reduce any false positives.

5.4 Results

5.4.1 Home Cage Fluid Intake

Animals in the ethanol group self-administered an average of 6.15 ± 0.81 g/kg ethanol per day. In the control group, animals self-administered an average of 9.5 ± 0.35 mL water per day. One animal was excluded from the study due to a leaking bottle. The data from that animal, therefore, was not used in the statistical analysis of this study. Also, due to occasional tissue shredding, data were collected and analyzed only from those areas of sampling that were intact and therefore appropriate for data quantification.

5.4.2 Dopamine D2 Receptor Immunoreactivity

5.4.2.1 Dual ChAT and Dopamine D2 Receptor Immunoreactivity

Dual ChAT and DA D2 immunoperoxidase labeling was examined in the shell and core NAc (Figure 5.3), the dorsolateral and ventromedial regions of the DS, and the IL and PrL regions of the PFC. Quantitative analysis of the percent of cholinergic neurons that co-expressed DA D2 in alcohol treated and control animals is reported in Table 5.1 and Figure 5.4. Baseline levels of DA D2 immunoperoxidase-labeled cholinergic cells were determined by calculating the percentage of cholinergic cells that expressed DA D2 in control animals. After 1 month of voluntary alcohol drinking, there was a 25% decrease in the percentage of DA D2 receptor-positive cholinergic neurons, as compared to baseline values, in the core NAc [$t(13)=2.19$, $p<0.05$, two-tailed] (Figure 5.4A), and a 46% decrease in the percentage of DA D2 receptor-positive cholinergic cells in the PrL PFC [$t(17)=2.41$, $p<0.05$, two-tailed] (Figure 5.4B). A 46% increase in the percentage of DA D2 receptor-positive cholinergic neurons, as compared to baseline

values, was observed in the ventromedial DS [t(15)=2.60, $p<0.05$, two-tailed] (Figure 5.4C). In the core NAc the average number of DA D2 receptor-positive cholinergic neurons per sample area was 32.6 ± 2.5 in the control group and 24.3 ± 3.0 in the alcohol group, while in the PrL PFC the average number of DA D2 receptor-positive cholinergic neurons per sample area was 11.0 ± 1.3 in the control group and 5.9 ± 1.7 in the alcohol group. In the ventromedial DS the average number of DA D2 receptor-positive cholinergic neurons per sample area was 19.2 ± 2.3 in the control group and 28.1 ± 2.4 in the alcohol group. Alcohol did not elicit significant changes in the percentage of DA D2 receptor-positive cholinergic neurons in the shell NAc, IL PFC, or the dorsolateral region of the DS.

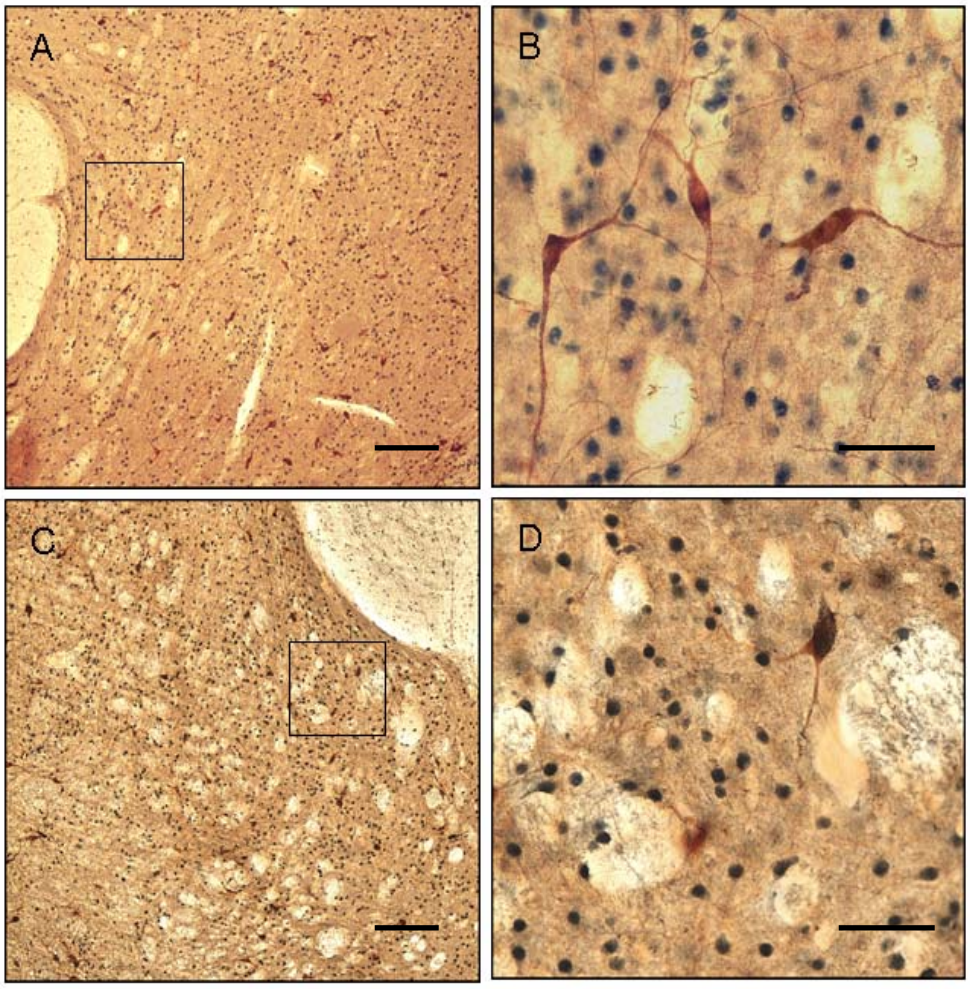

Figure 5.3. Representative photomicrographs of dual-immunoperoxidase labeled tissue illustrating the localization of DA D2 receptors in cholinergic interneurons of the NAc in iP rats. (A) Low-magnification of the NAc from an animal that underwent 1 month of initiation alcohol drinking. (B) High-magnification of the core NAc from the area outlined in the box shown in A. (C) Low-magnification of the NAc from a control animal. (D) High-magnification of the core NAc from the area outlined in the box shown in C. The brown DAB label identified ChAT IR cholinergic neurons, whereas the blue/black SG label indicated D2 receptor IR. Scale bars = 100 μm A, C; 40 μm B, D.

Table 5.1. Percent of DA D2 Receptor-Positive Cholinergic Neurons in iP Rat Brain Following 1 Month of Initiation Alcohol Drinking

	Water	Alcohol
Nucleus Accumbens		
Shell	31.0 ± 2.6	29.0 ± 2.0
Dorsal Striatum		
Dorsolateral	25.4 ± 3.4	28.8 ± 4.7
Prefrontal Cortex		
Infralimbic	5.9 ± 2.2	13.4 ± 4.8

Data are presented as mean number of DA D2 receptor-positive cholinergic neurons per sample area ± SEM.

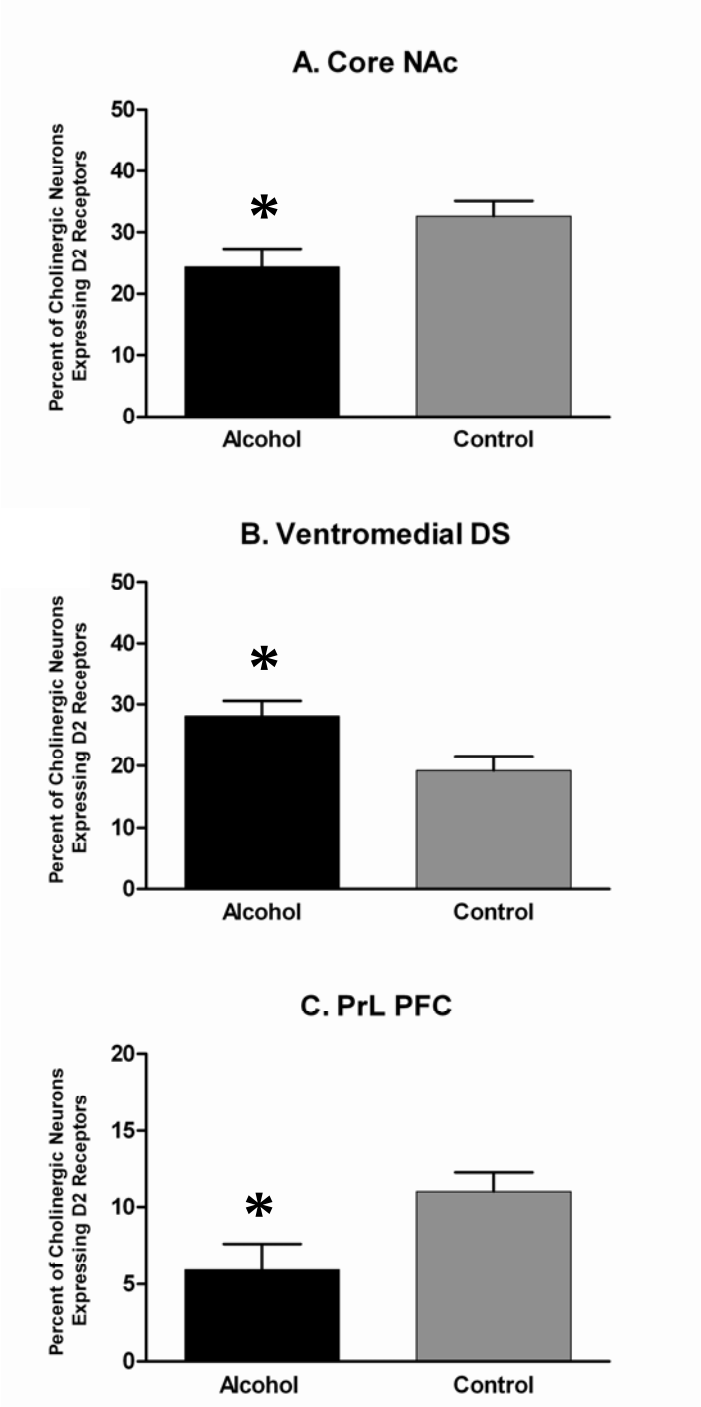

Figure 5.4. Percent of DA D2 receptor-positive cholinergic neurons in iP rats following 1 month of initiation alcohol drinking. Initiation drinking elicited (A) a 25% decrease in the core NAc, (B) a 46% increase in the ventromedial DS, and (C) a 46% decrease in the PrL PFC in D2-positive cholinergic neurons as compared to baseline values. Data are presented as mean number of DA D2 receptor-positive cholinergic neurons per optical field ± SEM (*p<0.05).

5.4.2.2 Single Dopamine D2 Receptor Immunoreactivity

Single DA D2 receptor immunoperoxidase labeling was examined in the shell and core NAc, the dorsolateral and ventromedial regions of the DS, and the IL and PrL regions of the PFC (Table 5.2). One month of alcohol drinking did not elicit significant changes in any of the brain regions examined.

Table 5.2. Number of DA D2 Receptor-Positive Neurons in iP Rat Brain Following 1 Month of Initiation Alcohol Drinking

	Water	Alcohol
Nucleus Accumbens		
Core	40.0 ± 2.9	38.6 ± 5.5
Shell	35.1 ± 2.8	35.4 ± 3.4
Dorsal Striatum		
Dorsolateral	20.1 ± 1.5	17.0 ± 3.0
Ventromedial	16.0 ± 1.3	16.8 ± 2.1
Prefrontal Cortex		
Prelimbic	29.4 ± 3.0	29.1 ± 4.1
Infralimbic	31.4 ± 4.6	28.7 ± 4.2

Data are presented as mean number of DA D2 receptor-positive neurons per sample area ± SEM.

5.4.3 Cdk5 Immunoreactivity

5.4.3.1 Dual ChAT and Cdk5 Immunoreactivity

Dual ChAT and Cdk5 IR was examined in the shell and core regions of the NAc (Figure 5.5), dorsolateral and ventromedial regions of the DS, and IL and PrL PFC.

Additional brain regions examined but not discussed in the present study are presented in Table 5.3. Quantitative analysis of the percent of cholinergic neurons that co-expressed Cdk5 was calculated from immunoperoxidase labeled tissue. At the end of the 1 month drinking period, a 51% increase in the percentage of Cdk5-positive cholinergic neurons was observed in the shell NAc [$t(19) = 2.70$, $p<0.01$, 2-tailed] (Figure 5.6A). In the PFC a 51% decrease in the percentage of Cdk5-positive cholinergic neurons compared with baseline values was observed in the IL region [$t(14) = 3.42$, $p<0.01$, 2-tailed] (Figure 5.6B), and a 46% decrease was observed in the PrL region [$t(18) = 3.02$, $p<0.01$, 2-tailed] (Figure 5.6C). In the shell NAc the average number of Cdk5-positive cholinergic neurons was 29.4 ± 3.7 in the control group and 44.4 ± 3.0 in the alcohol group. In the IL PFC the average number of Cdk5-positive cholinergic neurons was 49.7 ± 6.8 in the control group and 24.2 ± 4.0 in the alcohol group, while in the PrL PFC the average number of Cdk5-positive cholinergic neurons was 45.4 ± 5.7 in the control group and 24.2 ± 4.1 in the alcohol group. These data are reported as means ± SEM. Alcohol did not elicit significant changes in the percentage of Cdk5-positive cholinergic neurons in the core NAc, dorsolateral DS or ventromedial DS.

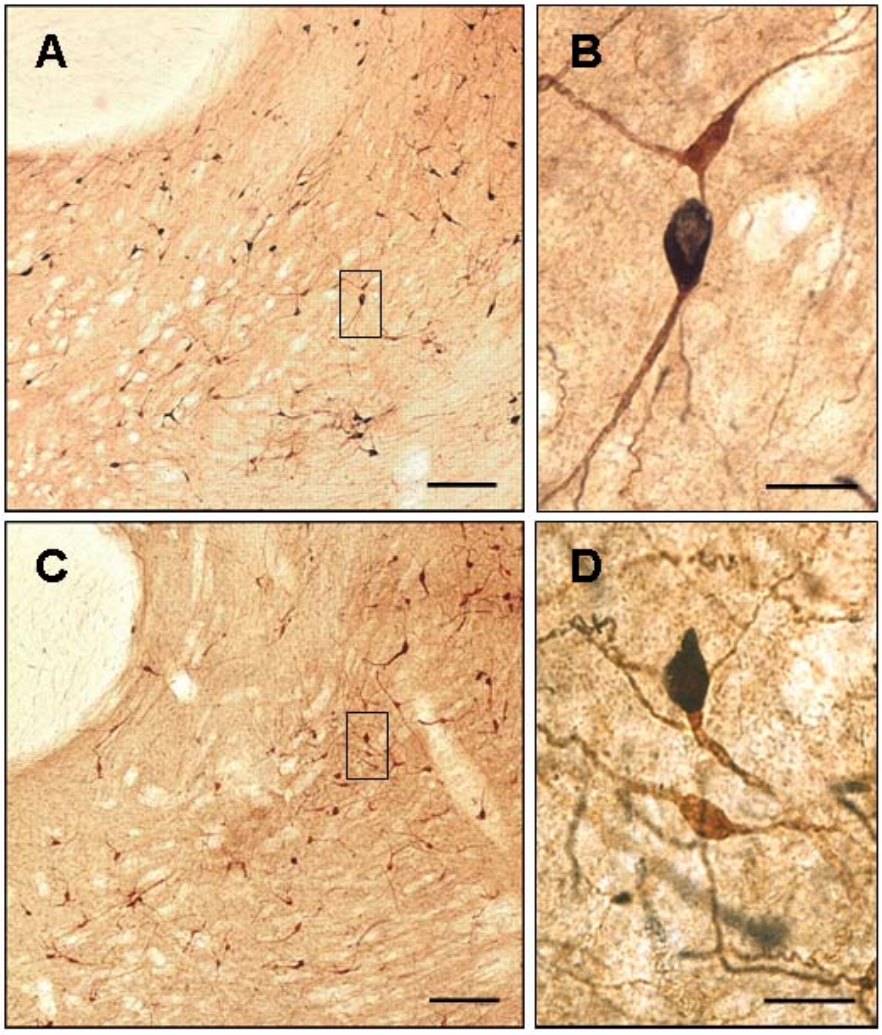

Figure 5.5. Representative photomicrographs of dual-immunoperoxidase labeled tissue illustrating the localization of Cdk5 in cholinergic interneurons of the NAc in iP rats. (A) Low-magnification of the NAc from an animal that underwent initiation alcohol drinking. (B) High-magnification of the shell NAc from the area outlined in the box shown in A. (C) Low-magnification of the NAc from a control animal. (D) High-magnification of the shell NAc from the area outlined in the box shown in C. The brown DAB label identified ChAT IR cholinergic neurons; whereas the blue/black SG label indicated Cdk5 IR. Scale bars = 100 μm in A, C; 30 μm in B, D.

Table 5.3 Percent of Cdk5-Positive Cholinergic Neurons in iP Rat Brain Following 1 Month of Initiation Alcohol Drinking

	Water	Alcohol
Prefrontal Cortex		
Cg1	43.5 ± 7.3	61.4 ± 6.0
Striatum		
Core NAc	30.1 ± 3.2	23.0 ± 3.8
Dorsolateral DS	39.0 ± 4.0	35.9 ± 5.5
Ventromedial DS	33.0 ± 3.7	39.1 ± 5.3
Olf	37.2 ± 4.9	49.8 ± 7.3
VP	50.1 ± 6.0	55.0 ± 5.3
Basal Forbrain		
MS	58.5 ± 3.2	55.5 ± 4.6
Extended Amygdala		
SI	41.2 ± 2.9	41.2 ± 1.4
Hypothalamus		
LH	36.8 ± 9.2	39.8 ± 14.1

Data are presented as mean number of Cdk5-positive cholinergic neurons per sample area ± SEM.

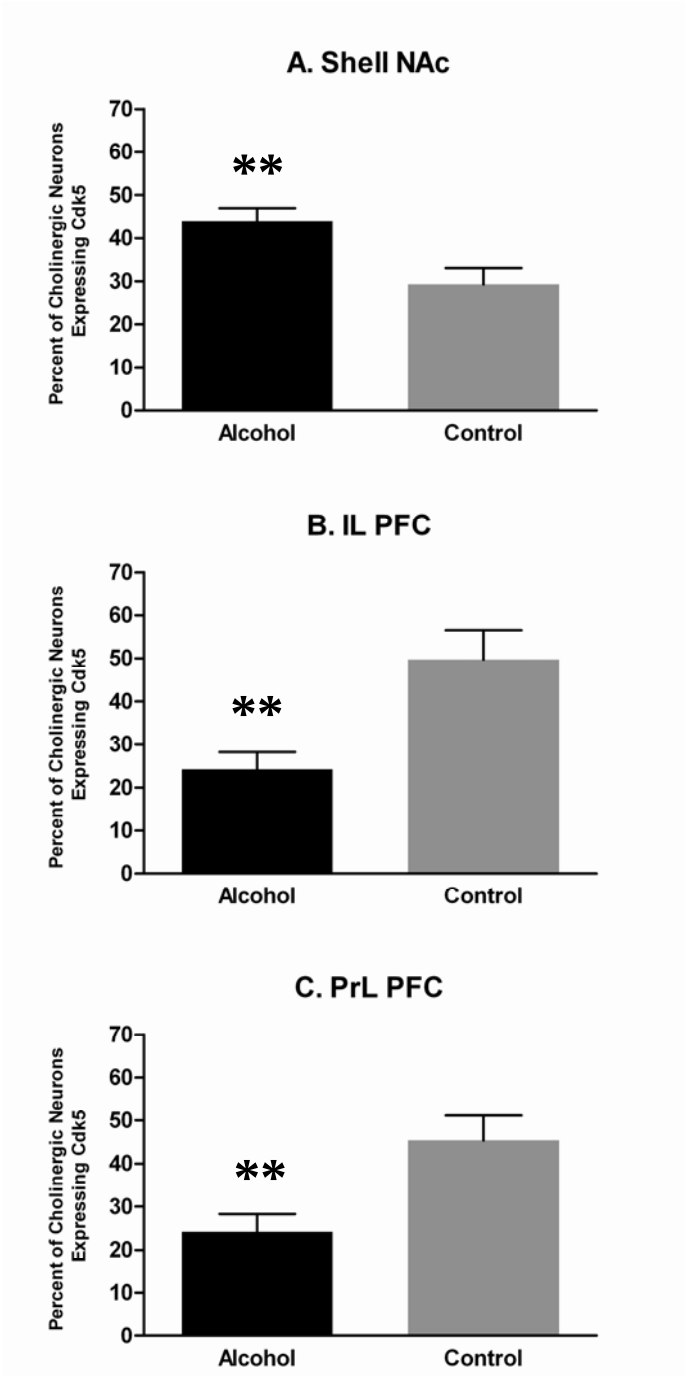

Figure 5.6. Percent of Cdk5-positive cholinergic neurons in iP rats following 1 month of initiation alcohol drinking. Initiation drinking elicited (A) a 51% increase in the shell NAc, (B) a 51% decrease in the IL PFC, and (C) a 46% decrease in the PrL PFC in Cdk5-positive cholinergic neurons as compared to baseline values. Data are presented as mean number of DA D2 receptor-positive cholinergic neurons per optical field ± SEM (**p<0.01).

5.4.3.2 Single Cdk5 Immunoreactivity

Single Cdk5 IR was examined in the shell and core regions of the NAc, dorsolateral and ventromedial regions of the DS, and IL and PrL PFC. Quantitative analysis of Cdk5-positive neurons, as reported in Table 5.4, was calculated from immunoperoxidase labeled tissue. Alcohol drinking did not produce significant changes in the number of Cdk5-labeled neurons in either region of the NAc, DS, or PFC.

Table 5.4 Number of Cdk5-Positive Neurons in iP Rat Brain Following 1 Month of Initiation Alcohol Drinking

	Water	Alcohol
Prefrontal Cortex		
IL	44.1 ± 6.8	47.4 ± 1.9
PrL	41.4 ± 3.3	46.4 ± 2.4
Cg1	19.6 ± 1.8	20.1 ± 1.7
Striatum		
Shell Nac	57.6 ± 2.1	63.1 ± 1.3
Core Nac	69.1 ± 4.4	67.3 ± 3.4
Dorsolateral DS	49.9 ± 3.5	54.7 ± 4.4
Ventromedial DS	55.4 ± 4.5	63.1 ± 3.6
Olf	13.0 ± 2.0	18.4 ± 3.2
VP	46.3 ± 4.4	53.9 ± 4.7
Basal Forebrain		
MS	7.3 ± 0.6	7.1 ± 0.8
Extended Amygdala		
BNST	51.8 ± 5.1	58.1 ± 7.6
CNA	48.0 ± 1.7	68.0 ± 8.1**
Hypothalamus		
LH	0.9 ± 0.1	0.8 ± 0.2
PVN	48.7 ± 4.1	46.1 ± 5.5
Hippocampus		
CA1	32.5 ± 1.8	32.6 ± 1.8
CA3	28.5 ± 2.1	27.0 ± 1.8
Dentate gyrus	90.6 ± 5.8	85.4 ± 4.8

Data are presented as mean number of Cdk5 positive neurons per sample area ± SEM. ** indicates $p < 0.01$.

5.5 Discussion

The present study examined changes in DA D2 receptor and Cdk5 expression in iP rats following the 1 month, continuous-access, initiation alcohol drinking model. Previous studies in this dissertation have highlighted changes in D2 receptor and Cdk5 expression in C57BL/6J mice. It is important to perform similar experiments across species in an effort to better extrapolate findings in animal models to human conditions. Comparing similarities in alcohol-induced neuroadaptations across species helps to identify key brain regions that underlie alcohol drinking. Differences across species aid in identifying the appropriate animal model for the question at hand. Different species can express differences in behavior, neuronal connectivity, and neurochemical content in various brain regions. The present study examined neuroadaptive changes in D2 receptor and Cdk5 expression in the NAc, DS, and PFC of iP rats. Additional brain areas that have been reported to be involved in various alcohol related behaviors were also examined (see Table 5.3 and Table 5.4.), however the discussion is focused on the aforementioned brain regions.

5.5.1 Dopamine D2 Receptor Neuroadaptation in Cholinergic Interneurons of iP Rats Following Initiation Alcohol Drinking

The present study aimed to identify D2 receptor changes in cholinergic interneurons, and other neurons, of the NAc, DS, and PFC of iP rats following 1 month of voluntary initiation alcohol drinking. Findings revealed a 25% decrease in D2-positive cholinergic interneurons in the core NAc, a 46% decrease in D2-positive cholinergic neurons in the PrL PFC, and a 46% increase in D2-positive cholinergic neurons of the

ventromedial DS, as compared to baseline values, following 1 month of voluntary initiation alcohol drinking.

D2 receptors have been implicated in the reinforcing effects of alcohol (Crabbe and Phillips, 1998; McBride et al., 1993; Nowak et al., 2000; Stefanini et al., 1992), alcohol drinking (Thanos et al., 2001; Thanos et al., 2004), drug-seeking behavior and relapse (De Vries and Shippenberg, 2002; De Vries et al., 2002; Self et al., 1996) as well as withdrawal (Sousa et al., 1999). Low densities of D2 receptors have been observed in drug abusers (Volkow et al., 1990; Volkow et al., 2001; Wang et al., 1997), however, it is currently unknown whether decreases in D2 receptors are a consequence to drug use or if low D2 levels predispose an individual to drug abuse and dependence. Volkow and colleagues (1999) addressed this question by measuring DA D2 receptor availability in healthy human subjects before administering intravenous methylphenidate (i.e. Ritalin). The subjects who reported the drug as pleasant had significantly lower striatal DA D2 receptor availability than those who reported it as unpleasant (Volkow et al., 1999). In addition, low striatal DA D2 receptor availability was demonstrated to be predictive of increased cocaine self-administration in monkeys (Morgan et al., 2002). The aforementioned studies suggest that the effects of a drug are produced as a combination of the pharmacologic action of the drug and the neurochemistry of a subject's brain and suggest that high D2 levels may be protective against substance abuse.

Studies comparing various strains of alcohol-preferring rats to their nonpreferring counterparts have generally found that DA D2 receptor density, especially in limbic areas, is lower in the alcohol-preferring animals (Korpi et al., 1987; McBride et al., 1993; Stefanini et al., 1992). It has been hypothesized that this difference may predispose these

animals to high alcohol intake (McBride et al., 1993). These results, in conjunction with the fact that P rats have been shown to have moderate to low levels of DA D2 receptors (McBride et al., 1993), suggest that high levels of DA D2 receptors could be protective against alcohol abuse. Thanos and colleagues (2001; 2004) found that overexpression of the DA D2 receptor gene in the core NAc of P rats attenuated alcohol drinking, suggesting that high levels of DA D2 receptors may be protective against alcohol abuse. Furthermore, human neuroimaging studies have shown that chronic alcohol drinking results in significantly lower amounts of available DA D2 receptors (Hietala et al., 1994; Volkow et al., 1996) and nonalcoholic members of alcoholic families have higher than normal levels of DA D2 receptors (Volkow et al., 2006). None of these studies however narrowed the focus to examine these receptors in specific cell types, which makes the present examination of D2 receptors in cholinergic interneurons key to revealing the underlying neurobiology of alcoholism. The fact that D2 receptor changes were observed in the core NAc in both the C57BL/6J mice and iP rats further exemplifies this area as a key region involved in alcohol drinking.

The core NAc has traditionally been characterized as an area involved primarily with motor functions. Recent studies, however, associate the core NAc with drug-seeking (Kalivas and Volkow, 2005), and it is hypothesized that the core mediates the expression of learned behaviors in response to motivationally relevant stimuli (Di Ciano and Everitt, 2001; Kelley, 2004). The core NAc is also associated with drug-induced sensitization (Li et al., 2004; Robinson and Kolb, 2004) and related synaptic rewiring (Berlanga et al., 2006). The findings that DA D2 IR was reduced in cholinergic cells of the core NAc are

consistent with previous reports of decreased DA D2 receptor availability in striatal areas of alcohol abusers.

The DS is a region composed of a medial and a lateral subdivision. The ventromedial DS receives dopaminergic afferents from the VTA, while the dorsolateral DS is innervated primarily by the SN (Gerfen et al., 1987). The DS has traditionally been associated with motor function, however recent studies demonstrate its role in habit learning and the synaptic plasticity that underlies the progression from casual drug use to habitual drug-seeking (Berke and Hyman, 2000; Everitt and Wolf, 2002). Cue-induced DA release in the DS can trigger relapse into drug-taking behavior (Ito et al., 2002) and previous work in our laboratory found cholinergic interneurons of the ventromedial DS to be dose-dependently activated by the acute self-administration of cocaine (Berlanga et al., 2003). The present study provides evidence of receptor neuroadaptation in the ventromedial DS following voluntary alcohol drinking, as measured by an increase in DA D2 IR cholinergic neurons. These findings, along with the previous work of others, suggest that the DS undergoes alcohol-induced cellular and receptor changes that may underlie a repertoire of alcohol-mediated behaviors.

The PFC has been implicated in various cognitive processes such as decision-making and goal-directed learning (Dalley et al., 2004; Ostlund and Balleine, 2005) as well as alcohol drinking (Hodge et al., 1996). Connectivity between the PFC and subcortical regions, and the neurotransmitter release that occurs within these areas, are thought to control behaviors such as the transition from voluntary substance abuse to compulsive intake (Goldstein and Volkow, 2002; Jentsch and Taylor; 1999; Kalivas and Volkow, 2005). Down-regulation of DA D2 receptors in the PrL PFC observed in the

present study is consistent with previous reports indicating that alcohol produces hypofrontality (Lyons et al., 1998) and impairment in PFC executive function and memory tasks (O'Mahony and Doherty, 1996; Sullivan et al., 2000). The present work provides further insight into the mechanisms that may contribute to this PFC hypofunction and impairment of PFC mediated functions.

5.5.2 Cdk5 Neuroadaptation in Cholinergic Interneurons of iP Rats Following Initiation Alcohol Drinking

Adjacent sections to those examined for dual ChAT and DA D2 receptor IR were examined for dual ChAT and Cdk5 IR. Cdk5 has been shown to regulate various mechanisms associated with neuroplasticity, such as synapse formation (Bibb, 2003; Johansson et al., 2005; Norrholm et al., 2003), and was therefore used in this study as a potential marker of neuronal plasticity. The findings from this study revealed a 51% increase in Cdk5-positive cholinergic neurons of the shell NAc, and a 51% and 46% decrease in Cdk5-positive cholinergic neurons of the IL PFC and PrL PFC, respectively, as compared to baseline values, following 1 month of voluntary initiation alcohol drinking. Additional brain areas were examined (see Table 5.3, Table 5.4, and Camp et al., 2006), but for the purpose of this discussion significant data from the NAc and PFC are presented.

As has been extensively discussed in this dissertation, the NAc is a critical region for mediating the reinforcing properties of alcohol and other drugs of abuse (Koob, 2003; Koob and Bloom, 1988; Pontieri et al., 1995; Rassnick et al., 1992). Many studies have been conducted that reveal differential responsiveness of the shell and core NAc to drugs

of abuse. For example, acute administration of a variety of drugs of abuse causes an increase in extracellular DA levels in the shell but not the core (Pontieri et al., 1995), and rats will lever press for cocaine into the shell but not the core (Carlezon et al., 1995). Porrino and colleagues (1998) reported an increase in activity of various brain regions, including the PFC and shell NAc, but not the core NAc following voluntary alcohol consumption in rats. Alternatively, Smith and colleagues (2001) reported a decrease in functional activity, as measured by the 2-DG method, in 49 of 57 brain regions examined, including the medial PFC, shell NAc, and core NAc. The finding that alcohol elicited significant changes in Cdk5 expression in only 3 of the 12 brain regions examined with dual-labeling procedures, and only 1 of the 17 regions examined with single-labeling procedures suggests that alcohol exerts its effects in a region-specific manner. Furthermore, these findings of significant changes in Cdk5-positive cholinergic interneurons suggest that alcohol also acts in a cell-specific manner.

The PFC is associated with numerous behaviors including drug-seeking, and alcohol drinking (Dalley et al., 2004; Hodge et al., 1996; McFarland et al., 2003). It is hypothesized that the transition from casual drug use to compulsive intake occurs through disruption of the inhibitory influence of the PFC on subcortical brain areas such as the NAc (Goldstein and Volkow, 2002; Jentsch and Taylor; 1999; Kalivas and Volkow, 2005). Dopaminergic afferents from the VTA have been shown to exert an inhibitory influence on PFC neurons. For example, electrical stimulation of the VTA and application of DA into the PFC induces inhibition of the spontaneous firing rate of cortical cells (Bunney and Aghajanian, 1976; Ferron et al., 1984; Godbout et al., 1991; Mantz et al., 1988; Penit-Soria et al., 1987; Peterson et al., 1987; Sesack and Bunney,

1989). This inhibition may be related not only to the direct effect of DA on cortical pyramidal cells but also to an indirect action of DA via GABA interneuronal influences on pyramidal cells (Penit-Soria et al., 1987; Pirot et al., 1992). Consistent with these findings, hypofrontality has been reported to occur, as measured by the 2-DG method, following long-term self-administration of cocaine (Macey et al., 2004) and in a time-dependent manner following an intragastric dose of alcohol (Lyons et al., 1998). The present study provides evidence that voluntary alcohol drinking results in a down-regulation of Cdk5 IR cholinergic neurons of the PFC. These findings provide evidence of alcohol-mediated hypofrontality at a cellular, mechanistic level and are in accordance with reports of VTA-mediated cortical cell inhibition, reduced ACh levels (Stancampiano et al., 2004), and alcohol-induced hypofrontality (Berglund and Ingvar, 1976; Berglund and Risberg, 1977; Lyons et al., 1998; Volkow et al., 1990; Volkow et al., 1992; Volkow et al., 1994).

Chronic alcohol drinking did not induce changes in general cell activation, as measured by single-label Cdk5 IR, in the IL PFC, PrL PFC, shell NAc, core NAc, ventromedial DS, or dorsolateral DS, suggesting that previous reports of striatal hyperactivity or prefrontal hypoactivity changes are occurring specifically in cholinergic neurons. It is possible, however, that changes in Cdk5 in these areas may occur at earlier or later stages of alcohol drinking that were not examined in the present study.

The studies performed in iP rats identified the involvement of cholinergic interneurons of the NAc, DS and PFC in initiation alcohol drinking. These findings are consistent with previous reports on a ventral to dorsal recruitment of striatal areas (Haber et al., 2000) with continued alcohol drinking, and further identify the PFC as a critical

influence over subcortical activity that underlies the progression from controlled drug use to addiction.

Chapter 6: General Discussion

Alcoholism is a complex relapsing disease that is characterized by the preoccupation with obtaining alcohol, excessive alcohol drinking, the development of tolerance and dependence, and an impairment in social and occupational functioning (American Psychiatric Association, 1994). Identifying the neurobiological mechanisms involved in alcohol drinking, specifically the transition from initiation drinking to binge drinking, is critical for the development of treatments for alcoholics and the vulnerability for relapse in those recovering. Neuroplasticity underlies the development of alcoholism and refers to neuroadaptations and the capability of brain reorganization in response to various stimuli, including alcohol drinking. The studies in this dissertation focused on identifying the involvement of a specific cell type, cholinergic interneurons, and the neuroadaptive changes that occur within these cells following alcohol drinking. These studies tested the overarching hypothesis that cholinergic interneurons in the NAc are critical substrates that underlie alcohol drinking, and that the plasticity that occurs within these cells then facilitates behaviors that are hallmarks of alcoholism such as compulsive alcohol drinking.

The study presented in Chapter 2 is the first study to examine the effects of accumbal cholinergic cell ablation on binge alcohol drinking in C57BL/6J mice. Results revealed a direct role for cholinergic interneurons in alcohol drinking by demonstrating that selective destruction of these cells causes a decrease in alcohol drinking. Once it was established that accumbal cholinergic interneurons are involved in alcohol drinking the next step was to investigate specific neuroadaptations that occur within these cells that

may underlie the transition from casual alcohol use to compulsive drinking. The next two studies were therefore designed to examine specific receptor and molecular neuroadaptations that occur within cholinergic neurons following the initiation and binge alcohol drinking models.

The second study (Chapter 3) examined the effects of initiation and binge alcohol drinking on DA D2 receptor expression in accumbal cholinergic neurons in C57BL/6J mice. One month of binge alcohol drinking lead to a decrease in D2-positive cholinergic cells in the core NAc, implicating this region as critically involved in compulsive alcohol drinking. Striatal D2 receptors have been shown to be down-regulated in addicted individuals (Volkow et al., 2004), however previous reports used imaging technology and report global changes in particular brain regions. The present study was able to pinpoint D2 receptor changes on a specific cell type, suggesting that significant findings reported in earlier studies could potentially be occurring on cholinergic interneurons. Since D2 receptors are inhibitory, a down-regulation potentially causes an overall increase in ACh release when compared to water drinking controls. It is hypothesized that this increase in ACh release then regulates striatal neurons and intracellular signaling mechanisms which translate to behaviors such as drug-seeking or compulsive alcohol drinking.

Cdk5 controls a number of signal transduction mechanisms that regulate neuronal plasticity (Bibb, 2003). Cdk5 expression was examined in cholinergic interneurons of the core NAc of C57BL/6J mice following the initiation and binge alcohol drinking models (Chapter 4). One month of initiation alcohol drinking lead to a significant increase in Cdk5-positive cholinergic neurons in the core NAc of C57BL/6J mice, while binge drinking lead to a more robust increase in Cdk5 expression in these cells. The progression

from initiation alcohol drinking to compulsive, binge drinking is a key behavior underlying the development of alcoholism. These data suggest that cholinergic interneurons, particularly in the core NAc, are critically involved in the transition from initial alcohol drinking to binge drinking. Neuroplasticity within these cells, as measured by Cdk5 IR, could mediate a potential mechanistic switch that facilitates this transition. This study also revealed an increase in single-label Cdk5 expression in the core NAc. This finding is presumably indicative of an increase in Cdk5-positive MSNs and suggests a recruitment of these cells in binge drinking. Future work would need to address earlier time points to identify when exactly these cells are being recruited.

Animal models are invaluable tools for examining the underlying neurobiology of human diseases. They afford experimental control and neurochemical analysis that is not possible in humans. When modeling human conditions such as alcoholism it becomes important to compare similarities and differences between species to more precisely identify the underlying neuronal mechanisms that contribute to this disease. Animal models have been developed to address characteristics of alcoholics such as craving, loss of controlled drinking, and relapse. Comparing similar neuroadaptive changes between animal models helps to identify key brain regions that could serve as potential targets for improved treatments. While previous studies have identified neuroadaptive changes in C57BL/6J mice, the final study (Chapter 5) assessed changes in both D2 receptor and Cdk5 expression in cholinergic cells of the NAc, DS, and PFC in iP rats following 1 month of initiation alcohol drinking.

One month of voluntary initiation alcohol drinking in iP rats caused a significant decrease in D2-positive cholinergic neurons of the core NAc and PrL PFC, and an

increase in D2-positive cholinergic cells in the ventromedial DS. A down-regulation of D2-positive cholinergics in the core NAc and PrL PFC is consistent with previous findings of decreased D2 receptor availability in striatal areas of alcoholics (Hietala et al., 1994; Volkow et al., 1996), and with the idea that the transition from casual drug use to compulsive intake occurs through a disruption of the PFC influence on subcortical brain regions (Goldstein and Volkow, 2002; Jentsch and Taylor; 1999; Kalivas and Volkow, 2005). The increase in D2-positive cholinergic cells in the ventromedial DS is in accordance with the underlying role of this area in habit learning and habitual drug-seeking (Berke and Hyman, 2000; Everitt and Wolf, 2002). The down-regulation of D2-positive cholinergic neurons in the core NAc of C57BL/6J mice and iP rats, reported in these dissertation studies, could facilitate future behaviors characteristic of alcoholics such as compulsive alcohol drinking. Thanos and colleagues showed that overexpression the D2 gene in the core NAc attenuated alcohol drinking, further suggesting that low receptor levels in this area could lead to compulsive drinking. A disruption in the normal circuitry between the NAc and PFC could lead to increased drinking as well as relapse. The deficit in inhibitory control from the PFC could influence other compulsive behaviors such as abuse of other drugs, gambling, and response to cues associated with drugs of abuse.

An increase in Cdk5-positive neurons of the shell NAc was observed following 1 month of initiation drinking in the iP rat while a concomitant down-regulation of Cdk5-positive neurons was observed in the PrL and IL regions of the PFC. These results are in accordance with fMRI measured overactivity of subcortical brain areas and a corresponding hypofrontality that occurs with alcohol and drug abuse. Results from iP

rats specifically identify cholinergic neuroadaptations in brain regions previously identified in the complex circuitry that underlies alcohol drinking and the progression to habitual drinking. Cdk5 has been shown to be necessary for various mechanisms associated with neuroplasticity including increased spine formation in the NAc (Norrholm et al., 2003). Increases in Cdk5 expression in the NAc, observed in the present studies, may be predictive of increased synapse formation that underlies long-term persistent changes in the brain that facilitates behaviors such as compulsive alcohol drinking. Initiation and binge alcohol drinking lead to a 38% and 65% increase, respectively, in Cdk5-positive cholinergic neurons in the core NAc of C57BL/6J mice. High BECs produced by the binge drinking model caused an almost 2-fold increase in the percentage of cholinergic cells that expressed Cdk5. This suggests that if drinking continues, to the point where intoxication is reach frequently, long-term synaptic changes are likely to occur in regions that mediate the transition from casual to compulsive alcohol use. Similar to the discussion above, the down-regulation of Cdk5-positive cholinergic cells observed in the PFC of iP rats could lead to a reduction of synaptic connections which would normally serve to inhibit addiction related circuits and behaviors, leading to the future potential for relapse.

The NAc is well established as a key region that underlies the reinforcing effects of alcohol and other drugs of abuse. The shell of the NAc is involved in the acute administration of alcohol (Di Chiara and Imperato, 1988; Herring et al., 2004; Imperato and Di Chiara, 1986) while the core is associated with drug seeking and the maintenance of instrumental behavior (Everitt and Robbins, 2005; Kalivas and Volkow, 2005). The DS has been implicated in habitual behavior as casual drug use progresses toward

compulsive use and addiction. The transition from initial drug use to habitual drug seeking is thought to involve a ventral to dorsal transfer of information processing. One such mechanism that might be controlling this ventral to dorsal movement is a cascading loop circuitry in which output from the shell NAc influences DA projections to the core, and similarly, output from the core influences projections to the DS (Haber et al., 2000).

Altogether, the studies of this dissertation indicate that cholinergic interneurons of the NAc are critical mediators of alcohol drinking and that drinking, either in an initiation or binge model, causes neuroadaptive receptor and molecular changes that underlie behaviors such as compulsive drinking. These studies integrate molecular, cellular and circuitry information and contribute to the understanding of the neurobiological mechanisms that underlie alcohol drinking. The findings are in accordance with recent work emphasizing the role of the striatum as a whole (including the shell NAc, core NAc, and DS) in the processes leading first to drug abuse then to addiction through an interconnected spiraling loop with ventral striatal regions influencing more dorsal striatal regions (Haber et al., 2000). It remains to be determined whether these cholinergic specific neuroadaptations can lead to structural changes, such as synaptic remodeling, that may underlie long-term alcohol mediated behaviors such as compulsive alcohol drinking, which may drive the switch that occurs from recreational drug use to long-term drug addiction. Future work will determine whether synaptic rewiring occurs in this brain tissue following these models of alcohol drinking and will provide critical insight into the neurobiological basis of alcohol drinking and relapse. Altogether, these data provide the basis for cholinergic targeted treatments designed to attenuate compulsive binge drinking and provide the groundwork for future studies examining region- and cell-specific

receptor and intracellular changes that underlie excessive alcohol drinking, craving, and relapse. These findings may lead to improved cell- and molecular-specific targeted genetic, pharmaceutical and behavioral treatment programs for alcoholism.

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