MUSCARINIC ACETYLCHOLINE M2 RECEPTORS INCREASE IN CHOLINERGIC INTERNEURONS OF THE NUCLEUS ACCUMBENS FOLLOWING BINGE ALCOHOL DRINKING IN C57BL/6J MICE

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A Thesis

Presented to

The Faculty of the Department

of Psychology

University of Houston

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In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

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By

Naomi Inoue

May, 2012

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ABSTRACT

Alcoholism is a complex disease that affects millions of people in the world.

While many animal models of alcoholism exist, it is extremely difficult, if not impossible, to capture every aspect of alcoholism in an animal model. Utilizing an animal model that is reported to produce pharmacologically significant levels of blood ethanol concentration, the present study examined the cholinergic interneurons in the nucleus accumbens, a brain region that is affected by drugs of abuse including alcohol. Cholinergic interneurons in the nucleus accumbens were of particular interest in this study since previous findings have reported that these neurons are involved in binge alcohol drinking. Specifically, cholinergic neurons are shown to possess muscarinic M2 receptors that autoregulate acetylcholine release. The study presented in this thesis examined the effect of binge alcohol drinking on muscarinic M2 autoreceptors on accumbal cholinergic interneurons. The present findings report an increase in the number of M2 receptor-positive cholinergic neurons in the nucleus accumbens following binge alcohol drinking in C57BL/6J mice. Binge alcohol drinking, however, had no significant effect on the volume of the nucleus accumbens or the volume of accumbal cholinergic interneurons. These findings may provide the groundwork for future studies that may aim to develop novel muscarinic receptor subtype targeted treatments for alcohol abuse and alcoholism.

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INTRODUCTION

Alcoholism is a chronic relapsing disease that alters physiological and psychological states of individuals. The American Psychiatric Association (APA) Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) defines alcohol abuse as alcohol consumption that has negative consequences (American Psychiatric Association, 1994). Alcohol dependence (synonymous with alcoholism) is defined as having three or more of the following symptoms: tolerance, withdrawal, compulsive alcohol consumption, preoccupation with obtaining alcohol, drinking as the focal point of life, and continued use despite problems (American Psychiatric Association, 1994). Alcohol abuse and dependence impose a devastating impact on society. Approximately 18 million adults in the United States (U.S.) meet the medical criteria for diagnosis of alcohol abuse or alcoholism, and nearly 53% of the adult population in the U.S. have a family history of alcoholism (NIH/NIAAA, 2006). More than 700,000 people are treated daily for alcoholism in the United States alone (Chen et al., 2009). The economic cost of alcohol abuse and dependence was estimated to be $184.6 billion in 1998 with an average annual increase of 3.8% (Harwood, 2000). Monetary costs do not include the pain and suffering of alcoholics themselves and people they affect. Alcoholism is associated with other psychiatric disorders, and approximately 18% of alcoholics commit suicide (Wilson & Kolander, 2003).

Binge alcohol drinking is the most prevalent pattern of heavy alcohol consumption in the U.S. (NIH/NIAAA, 2009). Out of the roughly 127 million Americans who drink alcohol, about 23% classify themselves as binge drinkers; this rate is higher in 18-25 year olds, for which 41% binge drink (Substance Abuse and Mental Health Services Administration; SAMHSA, 2009). Given the significant impact that alcohol has on society, it is imperative to

understand the underlying neuronal mechanisms of alcohol abuse and alcoholism in order to develop better pharmacotherapeutic, cognitive, and behavioral treatments.

Effects of Alcohol on Mammalian CNS

Alcohol is an organic compound in which a hydroxyl functional group is bound to a carbon atom. Upon administration, alcohol, more specifically, ethanol (or drinking alcohol), induces physiological changes. Ethanol is a straight chain alcohol, and its molecular formula is C_2H_5OH. Ethanol acts as a depressant to the central nervous system (CNS) that induces sedative, hypnotic, and anesthetic effects (Davies, 2003). Historically, ethanol was believed to disrupt non-specifically the neuronal lipid bilayer. However, it is now generally agreed that ethanol targets specific ligand- and voltage- gated ion channels on membranes (Littleton & Little, 1994; Tabakoff & Hoffman, 1996; Chandler et al., 1998; Harris, 1999). These ethanol-targeted ligand-gated ion channels are linked to neural receptors including gamma-aminobutyric acid type A ($GABA_A$), N-methyl-D aspartate (NMDA), glycine, neuronal nicotinic, and 5-hydroxytryptamine type 3 (5-HT3) (Valenzuela et al., 1998; Cardoso et al., 1999; Lovinger, 1999). Ethanol also modulates voltage-gated calcium channels (Narahashi et al., 2001). Ethanol exerts specific effects on these channels with specific outcomes. For example, ethanol exposure is responsible for potentiating $GABA_A$ and glycine receptors (Mihic, 1999), while blocking NMDA receptors (Hoffman et al., 1989) and voltage-gated calcium ion channels (Wang et al., 1994; Widmer et al., 1998). Through its action on ion channels, therefore, ethanol can affect a number of cellular and molecular effects and neuroadaptations and behavioral outcomes.

Model of Binge Alcohol Drinking: C57BL/6J Mice and Drinking in the Dark Paradigm

Binge alcohol drinking is a pattern of drinking that brings blood ethanol

concentration (BEC) to 0.08 gram% or above (National Institute of Alcohol Abuse and Alcoholism, 2004). This typically results by consuming 5 or more drinks for males and 4 or more drinks for females in about 2 hours. One of the hallmarks of alcoholism is compulsive ethanol intake (Everitt & Robbins, 2005). Indeed, compulsive drug-seeking behaviors and drug intake are critical criteria for the definition of drug dependence (American Psychiatric Association, 1994). Crews and Braun (2003) state that it is common for alcohol abusers and alcoholics to consume a large quantity of alcohol per drinking session. In fact, binge alcohol drinking to intoxication is strongly associated with alcohol abuse (Kim et al., 2008) and dependence (Robin et al., 1998). According to Hill et al. (2000), adolescent binge drinkers are likely to suffer from alcohol abuse and/or dependence in their early adulthood.

Alcoholism is a complex disease with multiple factors. While it is extremely unrealistic, if not impossible, to produce an animal model that captures every aspect of human alcoholics, individual features of alcoholism can be reflected in partial animal models that include tolerance, sensitization, and withdrawal (Crabbe et al. 1987; Crabbe, 1989; Crabbe et al. 2003). The proposed study utilized a drinking in the dark (DID) procedure in C57BL/6J (B6) mice as an animal model of binge alcohol drinking since this model is capable of producing BEC levels of 0.08% or above. Examining the effects of binge alcohol drinking on neuroadaptations in cell specific mechanisms, in an animal model of binge alcohol drinking with clinical relevance, can contribute to developing improved treatments for alcohol abuse and dependence.

B6 mice are known to voluntarily consume large quantity of ethanol. Pierce and Kumaresan (2006) note that self-administration of drugs is the animal model most homologous to human drug taking. Of 12 inbred strains of mice, B6 has the highest ethanol

intake when a DID paradigm is utilized (Rhodes et al., 2007). The DID paradigm was developed to produce pharmacologically significant BEC >1.0mg/ml for B6 mice (Rhodes et al., 2005). The DID model restricts ethanol access to the dark phase when activity (eating, drinking and exercise) is heightened (Rhodes, et al., 2005; Rhodes et al., 2007). The DID paradigm is relatively simple as it does not require extensive food or fluid restriction or a lengthy procedure of sucrose fading to obtain the necessary BEC levels (Rhodes et al., 2005).

Ethanol and the Dopaminergic Mesolimbic Pathway

Dopamine (DA) projections of the mesolimbic pathway originate in the ventral tegmental area (VTA) of the midbrain. DA neurons in the VTA innervate the nucleus accumbens (NAc), amygdala, hippocampus, ventral pallidum, and medial prefrontal cortex (PFC) (Pierce & Kumaresan, 2006). The DA mesolimbic system is implicated in the rewarding and reinforcing properties of drugs of abuse, including ethanol (Pierce & Kumaresan, 2006). Upon administration, ethanol triggers dopamine (DA) release in the brain (Di Chiara et al., 2004; Oscar-Berman & Marinkovic, 2007) and activates the DA mesolimbic system (Di Chiara and Imperato, 1988; Di Chiara et al., 2004; Pierce and Kumaresan, 2006). Striatal cholinergic interneurons possess several receptors, including DA receptors; these neurons have been linked to plasticity, learning, and drug abuse (Alcantara et al., 2003; Berlanga et al., 2005). Therefore, these neurons may be affected by the increase in DA level in the brain triggered by ethanol administration which can cause receptor neuroadaptations that influence acetylcholine release. It is, therefore, of interest to investigate the effect of binge alcohol drinking on striatal cholinergic interneurons.

Ethanol and the Nucleus Accumbens

The NAc, also known as the ventral striatum, has been implicated in the reinforcing effects of drugs of abuse, including ethanol (Koob, 1999). The NAc is of particular interest

because of its association with reward and motivation (Robbins & Everitt, 1996). In addition, the NAc is implicated in mediating the compulsive nature of addiction (Everitt & Robbins, 2005). Ethanol is known to activate DA VTA neurons *in vitro* (Brodie et al., 1990) and *in vivo* (Gessa et al., 1985). This activation results in increased DA release into the NAc (Yim & Gonzales, 2000). Accumbal DA is important for mediating the development and maintenance of ethanol reinforcement (Gonzales et al., 2004). DA is said to promote plasticity during the development of ethanol reinforcement, and to modulate appetitive or intake behavior in order to maximize ethanol reinforcement (Gonzales et al., 2004).

The NAc is a heterogeneous structure. It is subdivided into two regions known as the core and the shell compartments (Figure 1A). The core of the NAc is located dorsomedially, and the core surrounds the anterior commisure. The shell of the NAc is located ventrolaterally. The core and shell of the NAc can be distinguished by immunohistochemical markers, afferent and efferent innervations, and functional differences associated with their projections (Usuda et al., 1998). Different levels of delta FosB expression between the shell and core in response to ethanol and other drugs of abuse further indicate the divergence between these subregions of the NAc (Perrotti et al., 2008).

The shell NAc receives inputs from the VTA, PFC, hippocampus, and amygdala (Gerfen, 1987; Zahm, 2000; Sesack & Grace, 2010). The shell NAc primarily innervates the VTA, extended amygdala, lateral hypothalamus, ventral medial VP, and mesencephalic tegmentum (Heimer et al. 1991; Berendse et al., 1992; Zahm, 2000; Sesack & Grace, 2010). The extended amygdala is a macrostructure that consists of the bed nucleus of stria terminalis, central nucleus of the amygdala, shell NAc, and sublenticular substantia innominata, (Koob, 1999). Because of its neural connections, the shell NAc is associated

with the limbic system. The shell NAc, a part of the extended amygdala, is considered to mediate the reinforcing effects of the drugs of abuse (Koob, 1999). In fact, the shell NAc is implicated in the functional property of reward (Di Chiara et al., 2004).

In addition to DA inputs from the VTA, the core NAc receives inputs from the PFC, amygdala, and substantia nigra (SN) (Gerfen, 1987; Zahm, 2000; Sesack & Grace, 2010). The core NAc sends its major efferent projections to the dorsolateral ventral pallidum (VP), SN, and entopeduncular nucleus (Berendse et al., 1992; Zahm, 2000; Sesack & Grace, 2010). The NAc core has been implicated in cue-induced alcohol seeking (Chaudhri et al., 2010). Drug-induced behavioral sensitization is associated with neural plasticity in the core NAc (Li et al., 2004; Ferrario et al., 2005; Alcantara et al., 2011). Synaptic connections in the core NAc might also underlie drug-induced compulsive stereotypic behaviors (Alcantara et al., 2011). The core NAc, therefore, may play an important role in the execution of compulsive ethanol intake behavior.

Cholinergic Interneurons of the Nucleus Accumbens

Cholinergic interneurons are the largest neuronal type in the striatum with a somatic diameter size of 17-23 μm in mice (Nakamura et al., 1993), 20-50 μm in rats (Kawaguchi et al., 1995), and 80-140 μm in human (Chan-Palay, 1988). They comprise 1-2% of the striatal cell population. Because of their large somata and extensive dendritic arborizations, striatal cholinergic interneurons integrate synaptic inputs across large regions of the striatum (Kawaguchi et al., 1995). They integrate a variety of cognitive, limbic, and motor information (Calabresi et al., 2000) and in turn synapse onto medium spiny projection neurons (MSNs), thereby influencing overall striatal signaling and behavioral outputs (Howe & Surmeier, 1995; Wang et al., 2006).

Cholinerigic neurons have been implicated in striatal reward-related associative learning (Aosaki et al., 1994) but are not associated with locomotor behavior (Zocchi & Pert, 1994). The associative learning implicated in addiction involves DA and glutamate (GLU) neurotransmission (Vanderschuren & Kalivas, 2000). Both neurotransmitter systems are pertinent to accumbal cholinergic interneurons since these cholinergic cells express DA D2 and D5 receptors (Alcantara et al., 2003; Berlanga et al., 2005) and metabotrophic GLU1a and GLU5 receptors (Tallaksen-Greene et al., 1998). These accumbal cholinergic neurons are influenced by DA VTA signaling (Newman & Winans, 1980; Redgrave & Gurney, 2006) and GLU inputs from the amygdala (Kita & Kitai, 1990) and hippocampus (Kelley & Domesick, 1982). Moreover, these neurons in turn can influence DA cell firing (Cragg, 2006) and GLU prefrontal-striatal signaling (Alcantara et al., 2001). It is conceivable, therefore, that in addition to a role in drug reward and relapse (Cragg, 2006), cholinergic cells in the accumbens may contribute to the development of compulsive behaviors such as binge alcohol drinking.

Nestby and colleagues (1997) reported that ethanol drinking enhances acetylcholine (ACh) release in the NAc. As indicated by Fos expression, accumbal cholinergic interneurons are activated by ethanol (Herring et al., 2004) and other drugs of abuse (Berlanga et al., 2003). In addition, ethanol drinking is correlated with Cdk5 expression, a marker of neural plasticity (Camp et al., 2006), and by DA D2 receptor downregulation in accumbal cholinergic interneurons, which may contribute to overall ACh signaling (Alcantara et al., in preparation). Consistent with those findings, Thanos and colleagues (2004) report that overexpression of DA D2 in the NAc results in a reduction in ethanol self administration. Importantly, ablation of accumbal cholinergic interneurons resulted in a

significant decrease in binge alcohol drinking in B6 mice (Camp & Alcantara, 2007), which further implies these neurons in the development of binge alcohol drinking.

Muscarinic M2-Positive Cholinergic Interneurons of the Nucleus Accumbens

Muscarinic M2 receptors act as autoreceptors on cholinergic axons, dendrites and cell somata (Levey et al., 1995; Baghdoyan et al., 1998). In the NAc, the M2 mRNA is present in cholinergic interneurons (Bernard et al., 1992). Muscarinic M2 receptors exert inhibitory effects on adenylate cyclase activity (Caulfield, 1993); hence, the activation of M2 receptors inhibits ACh release. Microinfusion of the muscarinic receptor antagonist, scopolamine, into the NAc altered lever pressing in operant conditioned animals, whereas microinfusion of the nicotinic receptor antagonist, mecamylamine, had little effect on lever pressing in these animals (Pratt & Kelly, 2004). This indicates that accumbal muscarinic receptors, but not nicotinic receptors, are associated with obtaining reward. Hence, muscarinic receptors may play a role in the rewarding effect of alcohol.

The muscarinic ACh M2 receptor gene (CHRM2) is associated with ethanol dependence and is considered a genetic risk for alcoholism (Wang et al., 2004; Luo et al., 2005). In addition, findings from Katner et al. (1997) suggest that muscarinic receptors are involved in the self-administration of ethanol. Receptors can be upregulated via *de novo* synthesis of new protein (Wonnacott, 1990) and/or reduction in turnover rate (Peng et al., 1994). Receptor downregulation may occur via internalization of the receptors (Katzmann et al., 2002). It has been shown that muscarinic M2 receptors in striatal cholinergic neurons are internalized after acute stimulation (Bernard et al., 1992). It is of interest, therefore, to study muscarinic M2 receptor neuroadaptations on cholinergic neurons in a model of alcohol abuse and dependence in order to better understand potential cell-specific receptor targets

for improved treatments.

Effects of ethanol on muscarinic receptors in the CNS are still inconclusive to date. Some findings support the increase in muscarinic receptors (Tabakoff et al., 1979; Rabin et al., 1980; Smith, 1983; Pietrzak et al., 1990), while others report a decrease (Hellstrom-Lindahl et al., 1993) or no change (Rothberg, 1993) in muscarinic receptor density with ethanol treatment. Some outcomes suggest that muscarinic density change is observed during abstinence (Nordberg & Wahlstrom, 1982) but not during the ethanol treatment. None of these studies utilized a binge alcohol dinking paradigm. The lack of availability of markers to recognize specific muscarinic receptor subtypes in these earlier studies, however, may contribute to the inconsistent findings. Several other factors are also likely to contribute to inconsistent findings. Such factors include the drinking paradigms utilized, the species and strain of animals, and the brain regions of interest. For example, down-regulation (Hellstrom-Lindahl et al., 1993) of the receptor was reported to occur in the thalamus of autopsy brain tissue specifically of age 59-84 of chronic alcoholics. Hence, it is difficult to reach a general consensus on the effect of binge alcohol drinking on muscarinic receptors. The effect that self-administration of ethanol has on muscarinic M2 receptors that are located on cholinergic interneurons in the NAc is still unknown.

Investigation of Binge Alcohol Drinking of the Nucleus Accumbens and Accumbal Cholinergic Interneurons

The present study examined the effect of binge alcohol drinking on muscarinic M2 autoreceptors located on cholinergic interneurons in the NAc of male B6 mice.

Accumbal sections immunolabeled for muscarinic M2 receptors were quantified to determine whether there is a change in the number of M2-positive accumbal cholinergic

interneurons following 4 weeks of binge alcohol drinking. Quantification procedures were performed using the optical fractionator probe in Microbrightfield (MBF) Bioscience Stereo Investigator software (version 9, Colchester, VT, USA). The combined use of B6 mice and the DID paradigm was implemented to study an animal model of binge alcohol drinking. It has been shown that increased ACh release in the NAc occurs during alcohol drinking behavior (Nestby et al., 1997). Upon activation, muscarinic M2 autoreceptors on cholinergic neurons inhibit ACh release. We tested the hypothesis that chronic binge alcohol drinking induces the upregulation of muscarinic ACh M2 receptors on cholinergic neurons of the NAc. The upregulation of M2 receptors on accumbal cholinergic interneurons may be a mechanism whereby an increase in M2 receptors is needed to compensate for the high levels of ACh release caused by ethanol drinking. These findings could provide insight into binge alcohol drinking-induced specific cell mechanisms that could be targeted by treatments for alcohol abuse and alcoholism.

In addition, this study examined the effect of binge alcohol drinking on the NAc volume. Although binge alcohol drinking has been suggested as a contributing factor for brain atrophy (Hunt, 1993) and necrotic neurodegeneration (Obernier et al., 2002), there has been no report on the effect of binge alcohol drinking on brain volume. However, several studies report that substance-dependent human subjects show reduced volume in PFC, cerebellum, hippocampus, amygdala and NAc (Shear et al., 1996; Nicolás et al., 2000; Agartz et al., 2004; Makris et al., 2004; De Bellis et al., 2005) and neuroimaging studies show decreased volume of the NAc among abstinent long-term alcoholics (Sullivan et al., 2005; Makris et al., 2008). Observed changes in brain volume may provide an indication of underlying cellular and molecular changes as well as interconnectivity and functional

changes in specific brain areas that may accompany binge alcohol drinking. In the present study, volume estimation of the NAc was performed using the Cavalieri probe in MBF Stereo Investigator. The Cavalieri probe was carried out on the same sections used to estimate the number of M2-positive cells in the NAc.

Furthermore, the effect of binge alcohol drinking on accumbal cholinergic cell volume was examined. Alcohol consumption increases extracellular ACh levels in NAc (Nestby et al., 1997). Andrew and MacVicar (1994) suggested that neuronal activation may contribute to cell swelling, in turn, contributing to an increase in cell volume. Due to an increased amount of ACh release, accumbal cholinergic neurons would have to produce greater amount of ACh. As a consequence, it can lead to an increase in accumbal cholinergic neuronal volume. The effect of binge alcohol drinking on an increase in accumbal cholinergic neuronal volume was examined with the use of the nucleator probe in MBF Stereo Investigator. The same sections used for optical fractionator and Cavalieri probes were used to obtain estimated soma volumes of the cholinergic interneurons of the NAc.

MATERIALS AND METHODS

Animals

All procedures were conducted in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Houston. All efforts were made to minimize the suffering of animals and the number of animals used in this study. Male B6 mice (Postnatal Day 35) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were individually housed in standard cages in a temperature and humidity controlled room under a 12:12 reversed light:dark cycle (light off at 7am). Food was available *ad libtium*.

Drinking in the Dark Paradigm

One week following arrival, animals were randomly assigned to the experimental or control group. Animals in the experimental group were given 20% (v/v) ethanol solution in the DID schedule. Animals in the control group received only water for the duration of the experiment. In the experimental group, for the first three days, each water bottle was replaced with an identical bottle containing ethanol solution at three hours into the dark cycle for 2 hours. On the 4th day of the experiment, the procedure was identical except that the bottle containing ethanol solution was left in the cage for 4 hours instead of 2 hours. This schedule lasted for 24 days. Fluid intake was recorded to the nearest 0.1g. In order to account for leakage and evaporation, a bottle containing either water or ethanol was placed in two empty cages. These bottles were weighed at the scheduled times. The changes in the weight of bottles left in empty cages were averaged. The calculated amount was subtracted from fluid intake weight from each animal as this amount was not a result of fluid consumption but rather reflected evaporation and/or leakage.

Tissue Preparation

Following the final drinking session, each animal was anesthetized with an intraperitoneal injection of ketamine and xylazine (0.1ml/10 mg). Animals were perfused transcardially with 15 ml of phosphate buffered saline (PBS; pH 7.4) followed by 0.8 ml/g body weight (approximately 20 ml per animal) of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. Brains were immediately removed and postfixed with 4% paraformaldehyde in PBS for 2 hours at 4 ˚C. The brains were then placed in 15ml of PBS containing 30% sucrose for 48 hr or until the brains sank in the solution. Brains were then OCT embedded. Coronal sections of 50 µm thickness were obtained using a cryostat (Leica,

CM 1850, Bannockburn, IL, USA). Sections were placed in a 24 well plate in the order retrieved (Corning Incorporated, Corning, NY, USA) containing cryoprotectant solution and stored at -20 ˚C until immunocytochemistry (ICC) procedures were performed.

Muscarinic M2 Immunocytochemistry

Brain sections were rinsed in 0.1M PBS (pH 7.4; 2 x 10min, 3 x 5min) and preincubated with 5% normal goat serum (NGS) and 0.1% H_2O_2 for one hour. Sections were then incubated with rat anti-M2 monoclonal antibody (1:500; Millipore, Billerica, MA, USA) in PBS containing 5% NGS for 2 hours at room temperature (RT) and 72 hours at 4 ˚C. The tissues were then rinsed with PBS (4 x 5 min), followed by incubation with secondary biotinylated goat anti-rat IgG (1:500; Millipore, Billerica, MA, USA) with 2% NGS in PBS for 1 hour at RT. The tissues were rinsed (6 x 5 min) with PBS, followed by incubation with an ABC Kit (Vector Laboratories) for 1 hour at RT. Muscarinic M2 receptor immunoreactivity samples were developed using DAB and 0.01% H_2O_2 in PBS, which resulted in a brown reaction product. The tissues were rinsed (3 x 10 min) in cold PBS to stop DAB reactions. Control sections were treated in the same manner with the omission of the primary antibody. The sections were mounted on gelatin-coated slides and air-dried overnight. Sections were dehydrated with a series of ethanol gradations followed by xylene and coverslipped.

Unbiased Quantitative Stereology

Design-based stereology is a method that utilizes random and systematic sampling for unbiased quantitative measures of the brain. Stereology is useful in extracting quantitative information about a three-dimensional volume of tissue from two-dimensional planar sections of tissue. Stereological quantifications were implemented with the use of an

Olympus BX51WI microscope and MBF CX9000 camera interfaced with the MBF Bioscience system. The MBF Stereo Investigator optical fractionator probe was used to estimate cell number in the NAc, the Cavalieri probe was used to estimate the volume of the NAc, and the nucleator probe was used to estimate the volume of cholinergic interneurons in the NAc in this study.

Optical Fractionator

The optical fractionator probe that is found in the MBF software system was used to estimate the number of M2 receptor immunolabeled cholinergic cells in the NAc of B6 mice following binge alcohol drinking and following water drinking in the case of the control animals. The optical fractionator method allows estimates of the total number of neurons in the region of interest (Oorschot, 1998) that is independent of tissue shrinkage that may occur during processing and dehydration. The slides were coded so that the group assignment was not revealed during the quantification. Every other section was included such that an average of 10 sections per animal was analyzed from left and right hemispheres. The contours of the NAc core and shell were delineated according to the atlas of Franklin and Paxinos (2007) (Figure 1A) using a 4x objective lens. Quantification of muscarinic M2 immunolabeled cholinergic interneurons in the NAc was performed (Figures 1B &1C). The contour delineations were obtained from Bregma +1.70 mm to +0.98mm for the shell NAc and from Bregma +1.70mm to +0.74 mm for the core NAc (Figure 2).

The optical fractionator probe program randomly generates and superimposes grids, which resemble a lattice (Figure 3A). Counting frames were superimposed onto the upper left corner of each grid. The counting frame was superimposed onto the image viewed under a 100x oil immersion lens. Cell counts in the NAc shell and core were performed with the

counting frame size of 80 µm x 60 µm, with the frame placed at the intersection of an 80 µm x 80 µm grid. In the present study, the counting frame size of 80µm x 60 µm was determined so that 0-3 muscarinic M2 labeled cells would fall in the counting frame. In addition, the grid size of 80 µm x 80 µm was considered optimal because this grid size resulted in the mean average coefficient of error (CE) value of 0.05. For each animal, the CE value was obtained. The CE values are indicative of the accuracy of estimation. The Gundersen CE was used because it assumed that sampling is performed systemically in a uniform manner (García-Fiñana et al., 2003). Data resulted in a Gundersen CE value of 0.05 or lower were used for statistical analysis. On average, 2476 and 2687 counting sites were visited per core and per shell, respectively for each animal.

The counting frame (Figure 3B) has the shape of a rectangle. Two adjacent red lines of the counting frame are exclusion lines. Muscarinic M2 receptor immunolabeled cells were counted if they were on the green inclusion line or inside the counting frame (Figure 3C). Cell counts were omitted if muscarinic M2 immunolabeled cells were outside of the counting frame or touched the red exclusion lines. The top and bottom regions where quantification is not performed are referred to as the guard zones. The quantification was not performed from 5 µm of the top and bottom of the section as these regions may be damaged during tissue sectioning and/or ICC procedures. The region that falls between the top and bottom guard zone is referred to as optical dissector height. The optical dissector height is the region in which quantification was performed. This region was focused through on the microscope and muscarinic M2 labeled cells were counted if cell somata came into focus within that dissector height. Approximately 50% tissue shrinkage occurred. The mounted sections were 24 µm thick on average. With the guard zone of 5 µm from the top and bottom

surface of the section, the optical dissector height was set at 15µm.

Cavalieri Principle

The Cavalieri principle provides unbiased estimates of the volume of arbitrary shape and size (Mayhew & Olsen, 1991; Oorschot, 1998). The Cavalieri method becomes useful when tissue sections are used to obtain estimated volume of the brain regions of interest. The volume of the region of interest on a tissue section can be measured reliably by utilizing a point counting technique (Roberts et al., 2000). The point counting technique requires superimposing test points onto an image of tissue section. The estimated volume of a brain region of interest can be obtained from the following equation: $estV = \sum P \times (a/p) \times T$ where $\sum P$ is the sum of the test points counted, a/p is the area per test point, and T is the length of an object of interest. The sum of the test points, $\sum P$, is obtained by simply adding the number of test points from all images. The area associated with each test point, denoted as a/p, requires knowledge of the magnification of the image and the size of the test points. When using tissue sections of known thickness, T can be obtained by multiplying the section thickness by the number of the images/sections that contain the region of interest.

The Cavalieri probe was used to obtain the estimated volume of the NAc. The same sections used to delineate the shell and core compartments of the NAc for the optical fractionator method were used for the Cavalieri method. The volume analysis was performed blind to group assignment. Test points were superimposed within the delineations of the regions of interest (Figure 4). Grid spacing of 80 µm was used as this grid spacing size resulted in a CE of 0.05 or below. Grid rotation angle was randomized. Using the formula above, based on the test points across the region, the software calculated the volume of core and shell NAc. Data that resulted in the CE value of 0.05 or below were used for

statistical analysis.

Nucleator

The nucleator principle is the most efficient method for particle volume estimation (Moller et al., 1990). The nucleator principle provides the unbiased estimates of the cell volume without the assumptions of cell shape (Gundersen, 1988). In the nucleator probe, the volume of a particle is estimated according to the equation $V = \frac{4\pi}{3} l_i^3$, where *li* is the mean average of the lengths of the intercepts (Gundersen, 1988). Each *l* value is obtained by measuring the distance in isotropic direction from an arbitrary point to the boundary of the particle of interest.

The same sections used to delineate the shell and core compartments of the NAc for the optical fractionator method were used for the nucleator method. The cell volume analysis was performed blind to group assignment. Sections were viewed under a 4x objective lens. Then the contours used for the optical fractionator probe were realigned. Using the optical fractionator workflow, counting sites were revisited under at a 100x oil immersion lens. Every 5^{th} counting site was revisited. This interval was chosen because it resulted in a CE of 0.05 or lower. The same counting criteria used for the optical fractionator probe were used to sample the cells. In this manner, the sampling of cells remained random and systematic. For cells that met the criteria for counting for the optical fractionator probe, a marker was placed in the middle of the cell (Figure 5A). Two perpendicular lines were then randomly generated by the software (Figure 5B) and the points at which the lines intersect the cell's membrane were marked (Figure 5C). Somal volume estimation was performed on average of 114 and 118 cells per core and per shell, respectively, for each animal**.**

Statistical Analyses

Daily ethanol intake was calculated in grams of ethanol per kilogram of body weight (g EtOH/kg body wt). All values were reported as group means ± standard error means (SEM). Statistical analyses of data were performed using Student's *t*-test between ethanol-treated and control groups.

For stereological quantifications of optical fractionator, Cavalieri principles, and nucleator probes, group means ± SEM were calculated. Three different group means were calculated: NAc (core and shell), core NAc, and shell NAc for each probe. The group means were compared using Student's *t*-test to detect whether statistical significant differences exist between ethanol treated and control groups. A value of $P<0.05$ was considered statistically significant for these analyses.

RESULTS

Drinking in the Dark Paradigm:

Animals in the experimental group self-administered an average of 7.3 ± 0.5 g/kg ethanol during daily ethanol access period (Figure 6). Immunolabeling of muscarinic M2 receptors was obtained from 16 animals (8 animals from control group, and 8 animals from alcohol group). The data presented from stereological analyses are from these 16 animals.

Muscarinic M2 Immunolabeling

Immunolabeling of muscarinic M2 receptors was obtained from 16 animals (8 animals from control group, and 8 animals from alcohol group). The data presented from stereological analyses are from these 16 animals.

Optical Fractionator

The average number of M2 positive cholinergic interneurons of the NAc is reported in Table 1. The average number of M2 positive cholinergic neurons in NAc (core and shell

combined) was 935.6 ±38.7 in the control group and 1087.9 ± 98.1 in the alcohol group (Figure 7A). This translates into an approximately 16.3% increase in the number of M2 positive cholinergic interneurons in the NAc in the alcohol group relative to the control group. There was no significant difference in the number of M2 positive cholinergic neurons in NAc (core and shell combined) [$t(14) = -1.44$, $P=0.09$, 1-tailed].

The average number of M2 positive cholinergic neurons in the core NAc was 416.6 ± 39.1 in the control group and 555.9 ± 62.5 in the alcohol group (Figure 7B). The difference translates into an approximately 33.4% increase in the number of M2 positive cholinergic interneurons in the core NAc in the alcohol group relative to the control group. Examples of M2 immunolabeled cells in the core NAc of the alcohol and control groups are shown in Figure 8. There was a significant difference in the number of M2 positive cholinergic neurons in the core NAc [$t(14) = -1.89$, $P=0.04$, 1-tailed].

The average number of M2 positive cholinergic neurons in the shell NAc was 519.0 ± 30.4 in the control group and 532.0 ± 57.3 in the alcohol group (Figure 7C). This translates into an approximately 2.5% increase in M2 positive cholinergic interneurons of the shell NAc in the alcohol group relative to the control group. There was no significant difference in the number of cholinergic neurons in the shell NAc [$t(14) = -0.20$, $P=0.42$, 1-tailed].

Cavalieri Principle

The average volume of the NAc is reported in Table 2. The average volume of the NAc (core and shell combined) was 2.5 ± 0.1 mm^3 in control group and 2.7 ± 0.1 mm^3 in the alcohol group (Figure 9A). This translates into an approximately 8.0% increase in the volume of the NAc in the alcohol group relative to the control group. There was no

significant difference between the control and the alcohol groups in the NAc volume [$t(14) = -1.11$, $P=0.29$, 2-tailed]. The average volume of the core NAc was 1.2 ± 0.1 mm³ in the control group and 1.3 ± 0.05 mm³ in the alcohol group (Figure 9B). This reflects an approximately 8.3% increase in the volume of the core NAc in the alcohol group relative to the control group. There was no significant difference between the control and the alcohol groups in the core NAc volume [$t(14) = -1.39$, $P=0.19$, 2-tailed].

The average volume of the shell NAc was 1.3 ± 0.1 mm³ in the control group and 1.4 ± 0.1 mm³ in the alcohol group (Figure 9C). This is an approximately 7.7% increase in the shell NAc in the alcohol group relative to the control group. There was no significant difference between the control and the alcohol groups in the shell NAc volume [$t(14) = -0.85$, $P=0.41$, 2-tailed].

Nucleator

The average volume of somal cholinergic interneurons of the NAc is reported in Table 3. The average volume of M2 positive cholinergic neurons in NAc (core and shell combined) was 1063.7 ± 116.3 µm³ in the control group and 1142.9 ± 103.2 µm³ in the alcohol group (Figure 10A). This translates into an approximately 7.4% increase in the somal volume of M2 positive cholinergic interneurons in the NAc in the alcohol group relative to the control group. There was no significant difference between the control and the alcohol groups in the cholinergic cell volume in the NAc [$t(14) = -0.51$, $P=0.62$, 2-tailed].

The average volume of M2 positive cholinergic neurons in the core NAc was 1115.6 ± 128.4 µm³ in the control group and 1264.2 ± 146.3 µm³ in the alcohol group (Figure 10B). This translates into an approximately 13.3% increase in the somal volume of M2 positive cholinergic interneurons of the core NAc in the alcohol group relative to the control group.

There was no significant difference between the control and alcohol groups in the cholinergic cell volume in the core NAc [$t(14) = -0.76$, $P=0.46$, 2-tailed].

The average volume of M2 positive cholinergic neurons in the shell NAc was 1011.7 ± 111.2 μm³ in the control group and 1021.7 ± 66.9 μm³ in the alcohol group (Figure 10C). This translates into an approximately 1.0% increase in somal volume of M2 positive cholinergic interneurons in the shell NAc in the alcohol group relative to the control group. There was no significant difference in the cholinergic cell volume in the shell NAc [$t(14) = -0.08$, $P=0.94$, 2-tailed].

DISCUSSION

It is reported that alcohol administration activates accumbal cholinergic interneurons (Herring et al., 2004) and elevates extracellular ACh concentration (Nestby et al., 1997). According to Camp et al. (2006), accumbal cholinergic interneurons express significantly more Cdk5, a marker for neuroplasticity, following alcohol self-administration. The present study examined the effects of binge alcohol drinking on muscarinic M2 receptor expression on cholinergic interneurons of the NAc in B6 mice.

The current study reports the upregulation of muscarinic M2 receptors on accumbal cholinergic cells in the core NAc following 4 weeks of binge alcohol drinking in B6 mice. The previous work has shown that B6 mice that underwent the DID paradigm showed a significant decrease in DA D2 receptors in the core NAc, but not in the shell NAc (Alcantara et al., in preparation). The current study is in agreement with previous work that cholinergic interneurons in the core NAc, but not the shell NAc, undergoes plasticity in a binge alcohol drinking model. The fact that cholinergic interneurons in the core NAc showed a greater

change in M2 receptor expression than in the shell NAc may be due to the involvement of the core NAc in drug seeking behavior (Kalivas & Volkow, 2005) and drug specific compulsive stereotypic behavior (Alcantara et al., 2011) while the shell NAc is associated with the reward and reinforcing effects of drugs of abuse (Koob, 1999; Di Chiara et al., 2004).

Alcohol self-administration activates cholinergic interneurons in the NAc (Herring et al., 2004; Camp et al., 2006), and elevates extracellular ACh levels in the NAc (Nestby et al., 1997). Therefore, the increase in muscarinic M2 receptors in cholinergic neurons of the core NAc may be a compensatory mechanism in order to maintain homeostatic extracellular ACh.

In the striatum, muscarinic M4 receptors are also located on cholinergic neurons and regulate ACh release via negative feedback (Bernard et al., 1999; Bonsi et al., 2008). Hence, the number of muscarinic M4 receptors may also be increasing as both M2 and M4 receptors act as autoreceptors. Thus, it would be worth investigating whether binge alcohol drinking leads to a change in the number of muscarinic M4 receptor expression on accumbal cholinergic neurons. Examining both muscarinic M2 and M4 receptors on accumbal cholinergic interneurons will allow better understanding of self-regulation of ACh release in accumbal cholinergic neurons. The M2 receptor was of focus in the present study because it is found exclusively on cholinergic neurons in the NAc whereas M4 receptors are also found in other cell types in the NAc.

A difference in the NAc volume was not detected following 4 weeks of binge ethanol drinking. This suggests that the volume of the NAc and its subregions, core and shell, remains the same following 4 weeks of binge ethanol drinking. This may be due to the fact that aging, chronic alcohol drinking, and abstinence seem to be contributing factors for brain

volume atrophy. The current study used relatively young animals in a 4-week DID paradigm while brain volume reports from human subjects usually use older individuals who are chronic long-term alcoholics (Rosenbloom & Sullivan, 1992).

Also, a significant difference was not detected in the somal volume of the cholinergic interneurons in the NAc. This suggested that neural plasticity of accumbal cholinergic interneurons in a 4-week binge alcohol model is likely to occur in terms of receptor expression rather than neuronal volume.

All of the non-significant data obtained with the optical fractionator, Cavalieri principle and nucleator showed increases, albeit not significant, in the alcohol groups compared to the control groups.

In summary, this study presents evidence for an upregulation of muscarinic ACh M2 receptors in cholinergic interneurons of the NAc in B6 mice following binge alcohol drinking. These findings may possibly reflect an autoreceptor compensatory mechanism that attempts to bring alcohol induced increases in ACh levels in the NAc to homeostatic levels. The volume of the NAc or neuronal volume of accumbal cholinergic interneurons was not observed after 4 weeks of the DID paradigm. It is possible, however, that these volume changes, could occur following longer periods of binge alcohol drinking given that a trend toward an increase in somal and NAc volumes were observed in the binge alcohol drinking group. The muscarinic M2 receptor findings may lead to novel receptor subtype targeted pharmacotherapeutic treatments for alcohol abuse and alcoholism.

Table 1. Number of Muscarinic M2 Positive Cholinergic Interneurons in the NAc.

	Water	Alcohol
Nucleus Accumbens	935.6 ±38.7	1087.9 ± 98.1
Core	416.6 ± 39.1	555.9 ± 62.5 *
Shell	519.0 ± 30.4	532.0 ± 57.3

Data are presented as mean number of M2 positive cholinergic interneurons per region ±SEM for 8 mice per group. (*indicates statistical significance [$P < 0.05$] relative to control group); Independent *t*-test, one-tailed comparison.

Table 2. Region Volume of the NAc (mm^3).

	Water	Alcohol
Nucleus Accumbens	2.5 ± 0.1	2.7 ± 0.1
Core	1.2 ± 0.1	1.3 ± 0.0
Shell	1.3 ± 0.1	1.4 ± 0.1

Data are presented as mean value of volume per region ±SEM for 8 mice per group. No significant difference was observed with independent t test, two-tailed comparison.

Table 3. Muscarinic M2 Positive Cholinergic Interneuron Somal Volumes in the NAc (μm^3).

	Water	Alcohol
Nucleus Accumbens	1063.7 ± 116.3	1142.9 ± 103.2
Core	1115.6 ± 128.4	1264.2 ± 146.3
Shell	1011.7 ± 111.2	1021.7 ± 66.9

Data are presented as mean value of somal volume per region ±SEM for 8 mice per group. No significant difference was observed with independent t test, two-tailed comparison.

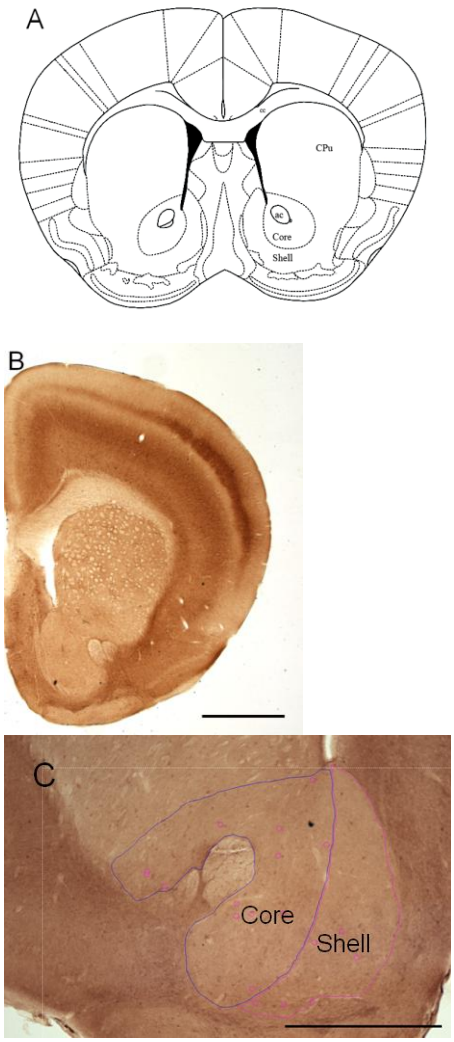


Figure 1. Muscarinic M2 Immunolabeled Coronal Sections of Male B6 Mice. Schematic representation of a coronal section of a mouse brain at Bregma +1.10mm **(A)**. The drawing is adopted from Franklin and Paxinos (2007). Ac, anterior commisure; CPu, Caudate putamen. Photomicrograph image of a muscarinic M2 immunolabeled section. The image is viewed under a 2x objective lens **(B)**. Muscarinic M2 immunolabeling patterns were consistent with Levey et al., (1991) in the NAc and other brain areas. For example an intense dark band of M2 immunolabel was consistently evident in the somatosensory cortex. The shown image corresponds to Bregma +1.18mm. The scale bar indicates 1mm. The contour of the shell (pink) and core (blue) were drawn using the MBF Bioscience Stereo Investigator optical fractionator probe using a 4x objective lens **(C)**. The contours are drawn according to the mouse brain atlas (Franklin & Paxinos, 2007). Counted cholinergic cells expressing M2 immunolabeling are marked with pink circles. The scale bar indicates 10µm.

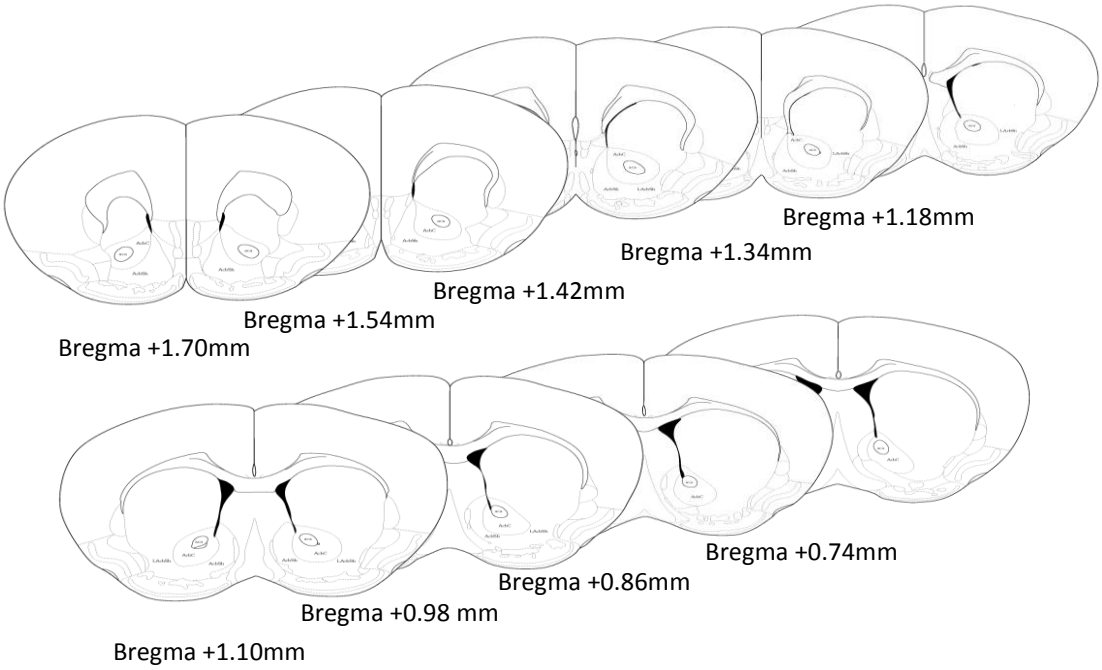


Figure 2. Schematic Representations of Coronal Sections of the Mouse NAc used for Stereological Quantifications. These schematic representations show the delineations of the core and shell compartments of the NAc that were used for the stereological studies. Approximately 9-10 sections were quantified per animal. The drawings (Bregma +1.70 mm to +0.74 mm) are adopted from Franklin and Paxinos (2007).

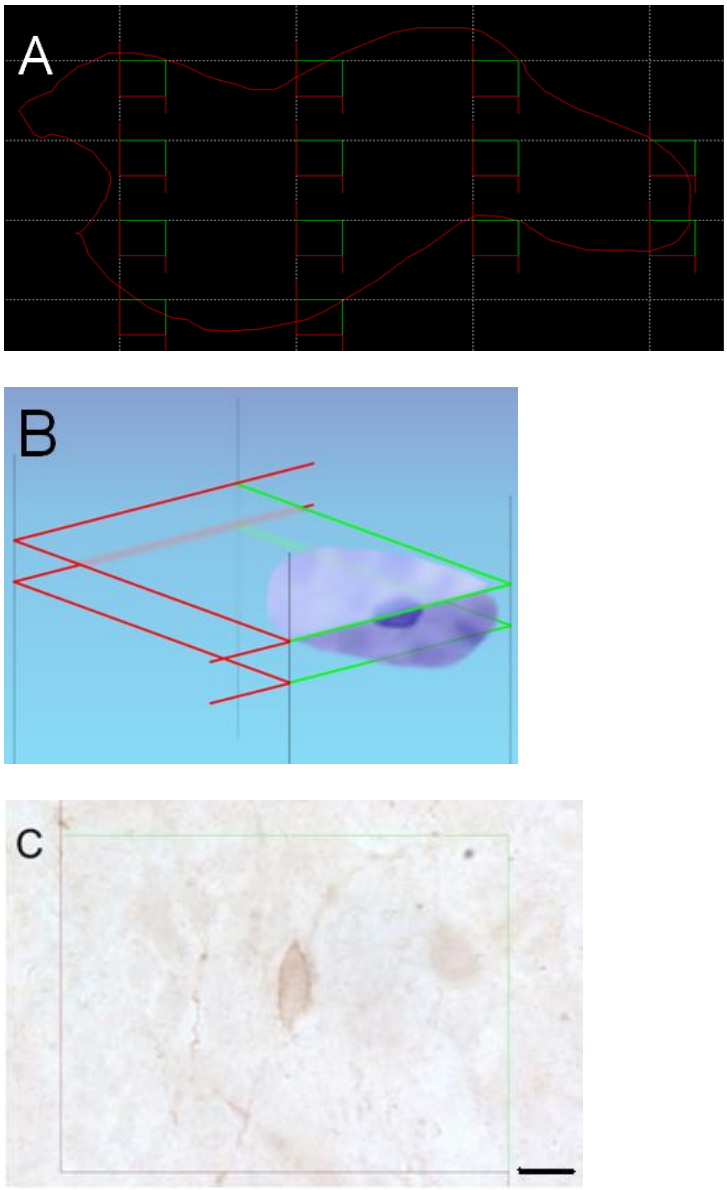


Figure 3. Optical Fractionator Steps for Quantification of m2-Positive Neurons in the NAc of B6 mice. MBF software randomly generated and superimposed grids (white dotted lines) are placed onto the drawn contour (red), of the NAc of the mouse **(A)**. The grids resemble a lattice. The counting frames are placed on the upper left corner of each grid by the software (Image adopted from mbfbioscience.com). The counting frame has exclusion lines (red) and inclusion lines (green) (**B)**. Cells that came into focus within the dissector height (region between the top and bottom counting frames) were counted. (Image adopted from mbfbioscience.com). Photomicrograph image of muscarinic m2 immunolabeled cells in the NAc of a male B6 mouse **(C)**. The image is viewed using a 100x objective lens. The scale bar indicates 10 µm.

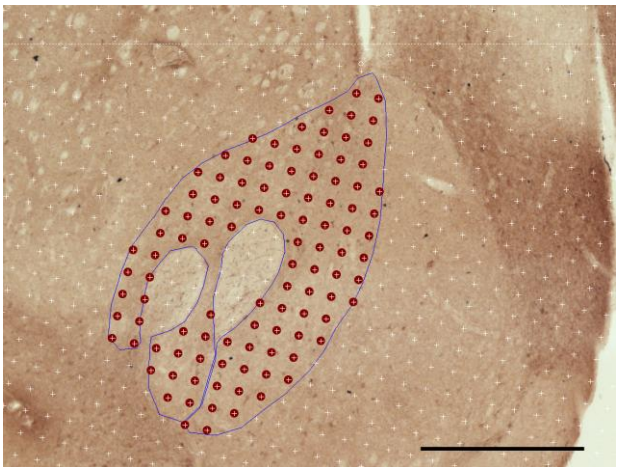


Figure 4. Photomicrograph Image of an M2 Immunolabeled Section of a Male B6 Mouse Used to Calculate the Volume of the NAc Using the Cavalieri Method. The image is viewed using a 4x objective lens. Test points are superimposed onto the microscopic image using the MBF Bioscience Stereo Investigator Cavalieri probe. Test points that fall within the core NAc are marked with red circles. The scale bar indicates 0.5mm.

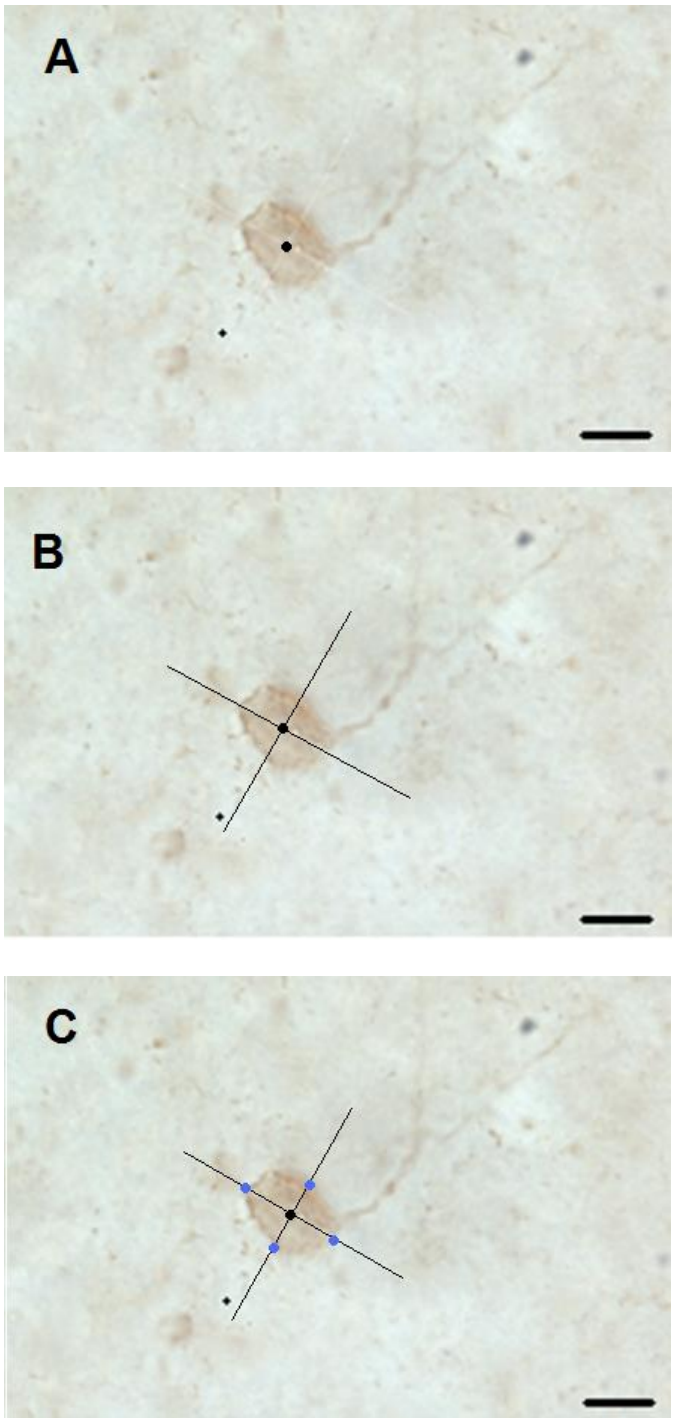


Figure 5. Photomicrograph Image of an M2 Immunolabeled Neuron of a Male B6 Mouse Used to Calculate the Volume of the Somal NAc Using the Nucleator Probe. The image is viewed using a 100x objective lens. The black dot indicates the center of the neuron that was marked by the observer **(A)**. The software generated 2 perpendicular lines that crosses at the black dot at random orientations **(B)**. The points at which the cell membrane intersects with the lines were marked by blue dots **(C)**. The scale bar indicates 10µm.

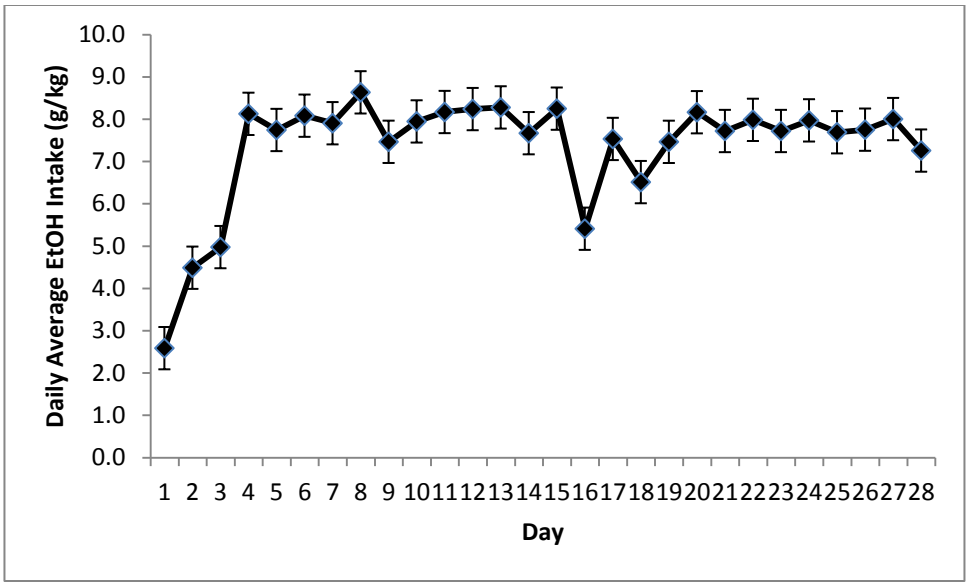
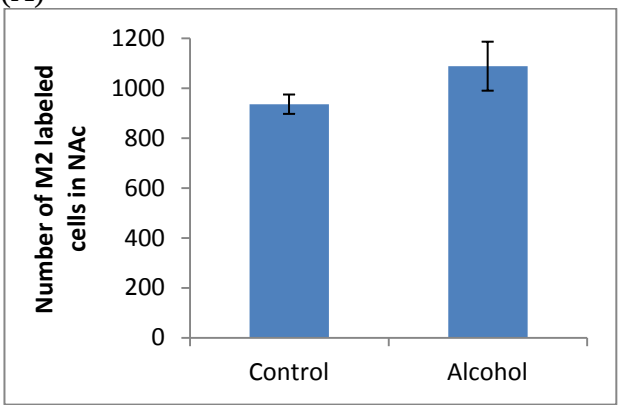
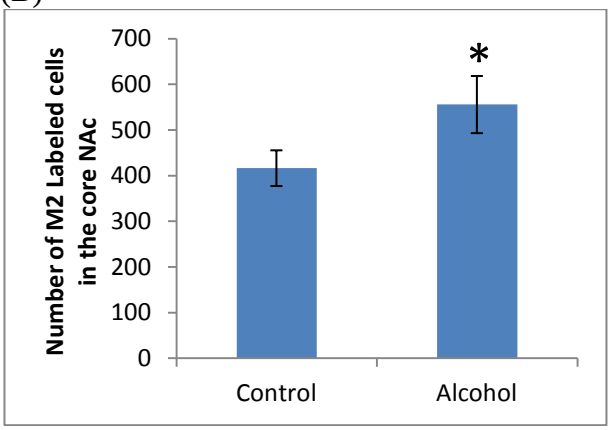


Figure 6. Daily Alcohol Intake (g/kg/day) of Male B6 Mice Under 28 days of the DID Paradigm. Animals in the experimental group from the present study consumed the mean alcohol intake of 7.6 ± 0.3 g/kg per day. Data are presented as mean value ±SEM.

(A)



(B)



(C)

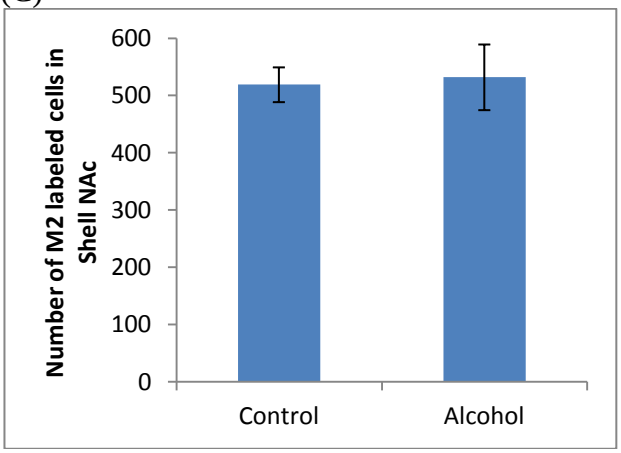
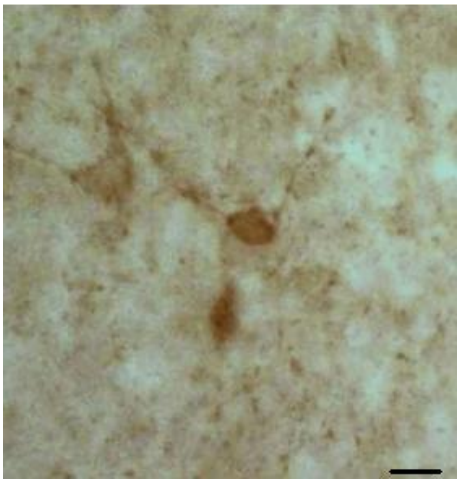


Figure 7. Number of Muscarinic M2 Receptor Positive Cholinergic Interneurons in the NAc. M2-positive cholinergic neurons in the NAc **(A)**, in the core NAc **(B)**, and in the shell NAc **(C)**. Data are presented as mean value of M2 receptor positive neurons per region ±SEM for 8 mice per group. (*indicates statistical significance [$P < 0.05$] relative to control group); Independent *t*-test, one-tailed comparison.

(A)



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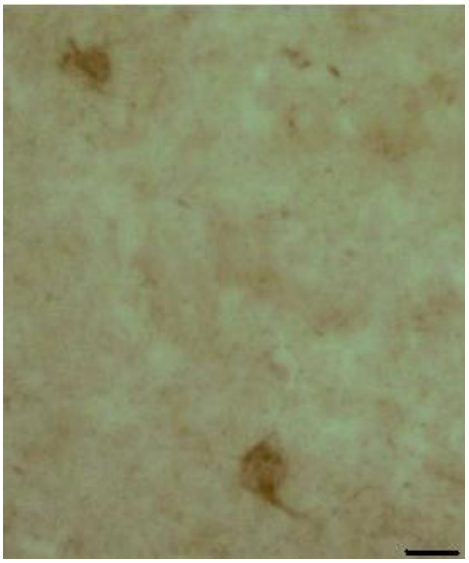
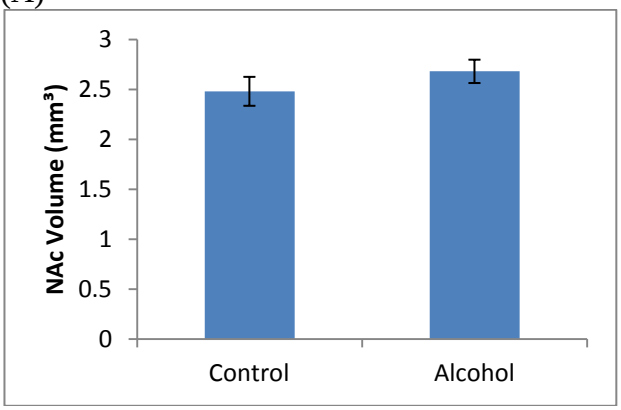
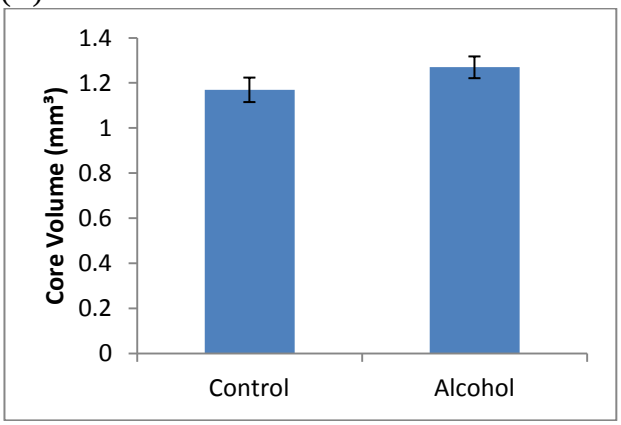


Figure 8. Photomicrograph Images of M2 Immunolabeled Tissue Used to Quantify M2-Positive Cells in the NAc Using the Optical Fractionator Method. The image was photographed using a 20x objective lens. Examples of M2-positive cholinergic interneurons in the core NAc of an animal from the experimental group **(A)** and of an animal from the control group **(B)**. The scale bar indicates 15µm.

(A)



(B)



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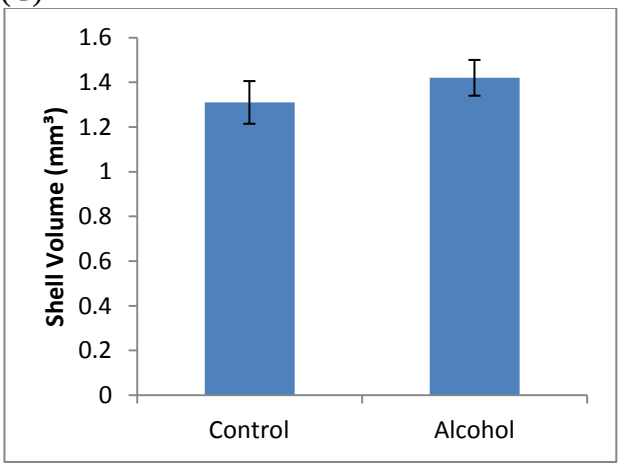
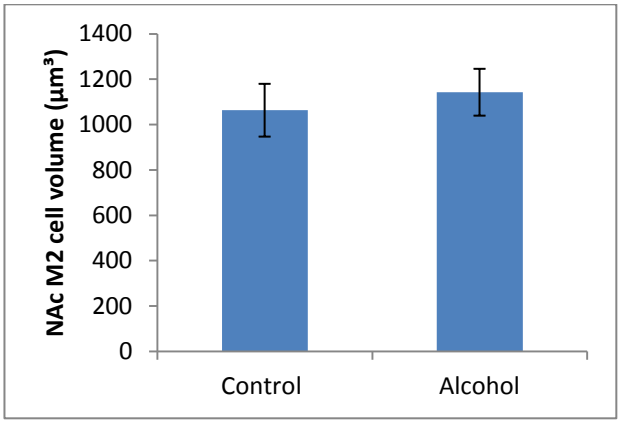
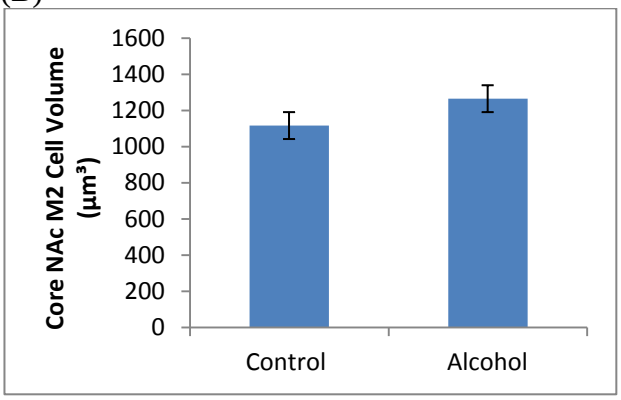


Figure 9. The Estimated Volume of the NAc Measured by Using Cavalieri Principle. Volumes in the NAC **(A)**, in the core NAC **(B)**, and in the shell NAc **(C)**. Data are presented as mean value of volume per region ±SEM for 8 mice per group. No significant difference was observed with independent *t*-test, two-tailed comparison.

(A)



(B)



(C)

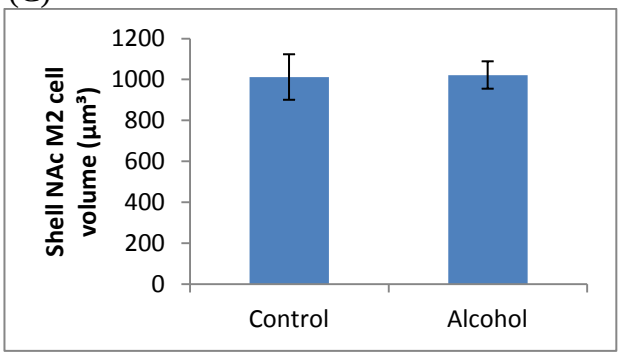


Figure 10. The somal volume of muscarinic M2 receptor-positive cholinergic interneurons. Somal volumes in the NAc **(A)**, in the core NAc **(B)**, and in the shell NAc **(C)**. The somal volumes were measured using nucleator principle. Data are presented as mean value of somal volume per region ±SEM for 8 mice per group. No significant difference was observed with independent *t*-test, two-tailed comparison.

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