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Role of BDNF in the Ability of Exercise to Attenuate Dependence-Related Escalated Alcohol Drinking in C57BL/6J Mice

by

Matthew Gustav Solomon

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Neuroscience

2019

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Abstract

MATTHEW GUSTAV SOLOMON. Role of BDNF in the Ability of Exercise to Attenuate Dependence-Related Escalated Alcohol Drinking in C57BL/6J Mice. (Under the direction of HOWARD BECKER)

Alcohol use disorder (AUD) continues to be a burden to society. Currently, few efficacious treatments exist. In addition to behavioral therapy and support groups (e.g. Alcoholics Anonymous), there are only three FDA approved pharmacotherapies. The lack of treatment options for alcohol addiction denotes the need to discover and develop new strategies and pharmacological targets to improve abstinence, prevent relapse, and inhibit the development of alcohol addiction. Chronic alcohol exposure reduces brain-derived neurotrophic factor (BDNF) in the medial prefrontal cortex (mPFC). Reductions of BDNF in the mPFC drive alcohol-dependent drinking in mice and conversely, elevating BDNF in this region blocks alcohol dependence-related drinking. Therefore, enhancing TrkB (BDNF primary receptor) activity in the mPFC, by pharmacological activation or increased expression of BDNF via exercise, may provide a new treatment strategy for AUD. To engender alcohol dependence mice are exposed to repeated cycles of chronic intermittent ethanol (CIE) vapor, producing escalated alcohol drinking compared to Baseline and control (Air) mice. Additionally, deficits in *Bdnf* mRNA and protein are seen in the mPFC after CIE exposure. Exercise (wheel running) noninvasively induces BDNF expression in the dentate gyrus (DG) of rodents and

in the blood of humans. This information led to the question of whether exercise could increase BDNF in the mPFC, reduce alcohol dependence-related drinking, and if this effect would occur through a BDNF-TrkB mediated mechanism. Studies tested the hypothesis that: Daily, limited (2-hr) voluntary wheel running would increase BDNF expression in the brain and through BDNF-TrkB signaling, attenuate CIE-induced escalated alcohol drinking. Following the Introduction, Chapter 2 demonstrates mice given limited access to a running wheel every day for several weeks, show increased *Bdnf* mRNA (qRT-PCR) and BDNF protein (ELISA) expression in the mPFC and DG. Building on these findings, Chapter 3 shows exercise attenuates CIE induced escalated alcohol drinking and mitigates reductions of BDNF mRNA in the mPFC caused by chronic alcohol exposure. Finally, in Chapter 4, using pharmacological inhibition of TrkB receptors, the ability of exercise to attenuate escalated alcohol intake is prevented. Taken together this study demonstrates exercise attenuates escalated alcohol intake in a model of dependence via BDNF-TrkB mediated signaling.

Chapter 1 - Introduction

Alcohol Use Disorder (AUD)

Background

Alcohol is the most commonly used drug in America, with close to half the world's population consuming alcohol (1) . Excessive alcohol use can lead to the development of alcohol use disorder (AUD), a chronic, relapsing brain disordered afflicting 16 million Americans (2). The occurrence of AUD in a year is approximately 14% of the population, with 20% of people meeting the clinical diagnostics in their lifetime for AUD (3, 4). To be diagnosed with AUD the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) requires at least 2 out of the 11 criteria to be met within a 12-month period. Alcohol abuse can lead to alcohol dependence (5). Dependence is characterized by compulsive alcohol intake, loss of control of drinking, preoccupation with alcohol, increased tolerance leading to the need for more alcohol to have the same effect, negative affective states during withdrawal, and continued used despite negative physical and social consequence (6).

Worldwide 3.3 million deaths are attributed to alcohol use, making AUD a leading cause of preventable death (7, 8). Alcohol use disorder places a large financial burden on the US economy through direct and indirect healthcare costs, crime, and loss of work productivity costing $249 billion in 2010 (9, 10). Social and physical health issues commonly co-occur with AUD, with psychiatric disorders such as anxiety and depression increasing with heavy alcohol use (11, 12). Alcohol use problems are common in patients with mental health disorders (13), where alcohol is commonly used to self-medicate in order to treat mental health disorders that often exist prior to the development of AUD (14, 15).

The risk for alcohol abuse and developing dependence is increased when drinking begins as an adolescence (16). Dangerous drinking behaviors are commonly associated with other risky behaviors including unprotected sex, driving under the influence, drug use, injuries, and criminal activity (17, 18), contributing to the adverse health and social consequences linked to AUD. Alcohol intoxication is commonly associated with domestic violence, deaths from homicide and suicide, child abuse, driving related injuries and death (19).

Binge drinking is a common, hazardous form of drinking, where greater than 4 drinks in females or 5 in males are consumed in a 2-hour period, resulting in blood ethanol concentration (BEC) levels of greater than 80 mg/dl (20, 21). Repeated cycles of heavy alcohol use or binge drinking followed by periods of abstinence can alter brain circuitry related to reward, motivation, and inhibitory control leading to the development of alcohol dependence, with alcohol use continuing despite negative physical and social consequences (2). This cycle leads to morphological, structural, and molecular changes in the brain (22, 23).

Despite the large number of people affected by, and the dangers associated with AUD only 8.3% of users seek and receive treatment (4). Treatments include behavioral therapy, group support (e.g. Alcoholics Anonymous), and pharmacological treatment ((24-28)). Current FDA approved pharmacotherapies are not adequately effective at the population level (29-31). Of those that do receive treatment, it often occurs years after the onset of the disorder (32, 33). Accessibility to treatment, due to cost or lack of health insurance coverage, is one limitation faced by those afflicted. Relapse occurs in up to 60% of treatment seekers mediated by interaction of multiple social, physiological, and psychological interactions (34-37). With the prevalence and magnitude of the AUD

problem, the high rate of relapse, and the dearth of treatment options available, it is critical that new targets and therapies are elucidated and developed to prevent the development of alcohol dependence and to improve treatment outcomes.

Stages of Alcohol Addiction

Alcohol addiction is a chronic, relapse brain disorder, comprised of multiple psychological and neurobiological factors. Multiple theories of addiction have been proposed, each addressing different aspects of the behavior, thoughts, and feelings that occurring at different stages throughout the cycle of addiction (for review see 38). While many of these theories address certain specific behaviors, the addiction cycle is more commonly viewed as being comprised of three stages, where within these stages more specific theories are found.

In the generally accepted 3-stage cycle of alcohol addiction the first stage involves alcohol binging and intoxication, which is followed by the second stage where an individual suffers negative affect caused by alcohol withdrawal. Finally, in the third stage craving, preoccupation, and anticipation for alcohol occurs, initially for the rewarding effects but transitioning to a need for alcohol to reduce the negative affect associated with withdrawal (2, 6). This cyclical pattern leads to dysfunction in the pathways controlling reward, compulsive-habit, and inhibitory control driving excess alcohol drinking (38, 39).

Binge/Intoxication

The first stage in the development of alcohol addiction involves binge drinking and intoxication. Binge drinking is defined as a pattern of dangerous, excessive drinking leading to BECs (40) of 80mg% or greater, resulting in intoxication (20, 41). In men this is commonly ≥5 drinks and in women ≥4 drinks in a 2-hr period (42). Repeated bouts of

binge drinking lead to a greater risk of alcohol dependence and neurodegeneration (43, 44). Neuronal degeneration can exacerbate alcohol dependence related symptoms, as degeneration in the circuitry connecting the amygdala and frontal cortex is associated with increased alcohol consumption and reduced behavioral control (45-47).

Changes in neurochemical release and signaling occur throughout the addiction process. In the early stages of drug and alcohol use the mesocorticostriatal dopamine system plays a key role in the rewarding effects and reinforcing properties of drugs (48). For example, alcohol use increases midbrain dopaminergic activity in humans and rats, mediating the positive-reinforcing properties of alcohol (2, 49, 50). Additionally, glutamate signaling is initially reduced by acute alcohol use and then transitions into enhanced signaling after chronic alcohol exposure (51). Furthermore, low doses of alcohol can reduce the release of excitatory neurotransmitters e.g. dopamine, through inhibiting the function of glutamate receptors (NMDA and mGluR5) (52-54).

Although glutamatergic signaling is decreased during acute intoxication, presynaptic release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (55) is enhanced, increasing postsynaptic GABA receptor activity (56). The duration of alcohol exposure differentially affects GABA receptor function. Exposure to alcohol at acute and sub-chronic levels results in increased GABA receptor functioning, with chronic exposure down-regulating GABAergic signaling (57-59). The role of GABA receptors in intoxication are further demonstrated by administration of the GABA receptor agonist baclofen, which reduces relapse in humans and decreases alcohol intake in rats (60, 61).

Not only is excitatory and inhibitory neurotransmitter signaling affected by alcohol use, but alcohol can dysregulate glucocorticoid signaling, affecting the motivational aspects of alcohol use (62). During intoxication the stress hormone corticosterone is

significantly elevated before displaying a reduction in expression where levels can remain depressed for several weeks after chronic alcohol exposure (63, 64).

During the binge and intoxication phase of the addiction cycle when alcohol is acutely administered, the reward threshold is reduced. As an individual transitions into dependence and chronically uses alcohol deficits in striatal dopamine (DA) emerge, disrupting normal reward processing and increasing the reward threshold, requiring higher levels of alcohol intake to produce the same effect (65).

Withdrawal/Negative Affect

When heavy drinking and intoxication is ceased, alcohol withdrawal syndrome occurs over the following hours to days. Withdrawal is a negative affective state consisting of stress, increased anxiety, CNS hyperexcitability, autonomic nervous system activation, and in severe cases delirium tremens (66, 67). Symptoms of alcohol withdrawal syndrome include anxiety, depressed mood, sleep disturbance, and anhedonia (68). Physical withdrawal symptoms usually subside within several days, but other symptoms can persist for weeks to months. (68). Physical symptoms mark acute withdrawal, while in prolonged abstinence a shift toward negative affect drives craving and relapse (69).

Multiple neuromolecular signaling pathways are involved in the negative affect of withdrawal. For example, alcohol withdrawal produces reductions in DA and serotonin (5-HT) activity in the nucleus accumbens (NAc) of rats (70, 71). These molecules are involved in behaviors such as irritability, aggression, and attention, commonly associated with negative symptoms of withdrawal (72, 73). Additionally, during withdrawal there is increased glutamatergic and reduced GABA signaling, which produces anxiety and dysphoria (57). Glucocorticoid signaling is increased in the mPFC during abstinence and is associated with depression, drug withdrawal and craving (74, 75). Glucocorticoid

receptor antagonism during abstinence decreases alcohol intake, with direct antagonism in the mPFC attenuating memory deficits seen during alcohol withdrawal (62, 76).

The opioid system has emerged as an important player in alcohol withdrawal. Chronic alcohol dysregulates the opioid system, altering affective states, leading to relapse. Treatment with the µ-opioid receptor antagonist naltrexone during withdrawal has been shown to decrease the urge to drink and when drinking did occur it reduced the number of drinks consumed (77, 78). Dynorphin (DYN) is an endogenous opioid that binds to the k-opioid receptor (KOR), a G-protein coupled receptor. Expression of DYN and KOR is throughout the reward and stress circuitry playing a role in affective states during withdrawal and alcohol upregulates DYN and KORs (79, 80). Withdrawal increases DYN/KOR signaling and activation of KOR producing dysphoria and reducing dopamine in the striatum (81, 82). In addition, antagonism of KOR increases DA release and reduces alcohol intake (83, 84). Demonstrating the role of the DYN/KOR system in negative affect of withdrawal, leading to relapse of alcohol drinking.

Preoccupation/Anticipation (aka craving)

The final stage in the cycle of addiction, which is enhanced by withdrawal symptoms, is craving for alcohol. Craving is the preoccupation and anticipation with obtaining drugs or alcohol, commonly described as a shift from goal-directed to compulsive "habit" behavior and the inability to control the desire and impulse to drink (85). Heavy alcohol use enhances sensitivity to cues and stress that can trigger craving and relapse, perpetuating alcoholism. Stress activates the HPA axis, releasing glucocorticoids that in turn modulate DA release in the striatum driving alcohol craving (86-88). Alcohol-associated cues, such as context or smell, or alcohol itself, are major risk factors that trigger alcohol craving and relapse (89, 90).

Withdrawal symptoms that can lead to relapse are a physical expression of CNS dysfunction. Chronic alcohol use results in neuroadaptations which affect reward, emotional response, and the regulation of alcohol intake. These adaptations can increase an individual's susceptibility to stress and alcohol-related cues, exacerbating the risk of relapse in abstinent alcoholics (85, 91). Even after withdrawal symptoms have subsided, abstinent alcoholics are more susceptible to stress and cues leading to relapse (92, 93).

In humans, hyperactivity in the vmPFC/ACC and ventral striatum predict alcohol craving and relapse in abstinent alcoholics (94). The vmPFC/ACC regulate emotional reward and decision making (95, 96) and the ventral striatum (97) is involved in reward and craving (2). These regions are connected through glutamatergic projections emanating from the PFC, controlling dopamine release (2, 98). Deficits in dopaminergic activity, as a result of chronic alcohol abuse contribute to the craving for alcohol leading to alcohol-seeking behaviors and relapse (99).

Relapsing alcoholics have been shown to have decreased volume in the amygdala and atrophy of the frontal cortex compared to healthy controls and nonrelapsing alcoholics (100, 101). Long-term alcohol abuse leads to reductions in cognitive control of the prefrontal cortical control over striatum (compulsive behavior and reinforcement) and amygdala (emotions), weakening the ability to resist craving and relapse to alcohol seeking and use (102).

The Prefrontal Cortex in Addiction

During the cycle of addiction, the transition from casual alcohol use to abuse and dependence involves changes in brain circuitry controlling behavior associated with reward and motivation (103). Not only is AUD a disorder affecting reward seeking and motivation, but deficits in inhibitory control circuitry are integral in the behavior.

The prefrontal cortex (PFC) is a key brain region in addiction (104). It controls executive function, working memory, top-down inhibitory control and behavioral flexibility, and plays a role in controlling motivational processes of the striatum (105-107). The PFC is comprised of three sub-regions including the medial prefrontal cortex (mPFC), the anterior cingulate cortex (ACC), and orbital frontal cortex (OFC). The mPFC can be further divided into the prelimbic and infralimbic regions (108), which have distinct roles in fear extinction and expression, inhibitory behavior, and drug-seeking (109, 110). Both the prelimbic and infralimbic regions of the mPFC control drug responses (111), responding in a go, no-go manner initiating and inhibiting drug related behaviors (112).

Multiple brain regions in addition to the PFC have been implicated in different aspects of alcohol use and dependence. Two subcortical regions involved are the striatum (alcohol reward and reinforcement, and compulsive alcohol use) (98, 113) and the amygdala (negative reinforcement, stress, and emotional response) (114). The PFC sends and receives projections to the amygdala and striatum creating circuits controlling executive function, negative emotional states, and motivated behavior respectively, which drive behaviors leading to alcohol use and dependence (115, 116). Heavy alcohol use and addiction decreases functional connectivity between frontal cortical and these subcortical regions (37). Glutamatergic afferent from the PFC to the ventral striatum (nucleus accumbens) regulate DA release (98). Exposure to alcohol causes changes in this pathway, which underlie the loss of control in addiction. In alcohol dependent rats, acute abstinence produces a functional disconnection between the PFC and central amygdala (CeA), through the recruitment of inhibitory neurons from the mPFC, causing working memory deficits (117). Neuroimaging studies have revealed activation of the PFC and corticolimbic circuitry in alcoholics responding to alcohol related cues (118, 119). Enhanced activation of the PFC and striatum to alcohol cues in abstinent alcoholics is a

predictor of relapse (120). Inactivation of afferents from PFC to NAc attenuate drug seeking, while inhibiting afferents to the amygdala blocks cue-induced reinstatement (121).

Prolonged alcohol abuse decreases brain volume, particularly in the frontal cortex and white matter regions of brain reward systems (122). Cortical thickness is positively associated with cognitive function and in alcoholics it is shown to be reduced in areas controlling reward (123). Postmortem and MRI studies with alcoholics show reductions in neuronal gray and white matter volume, in both males and females, particularly affecting the frontal lobe (124, 125). It has been observed, people with AUD have deficits in glucose metabolism leading to cortical atrophy and reductions in cognitive functioning (126). Loss of function in the PFC can affect control over emotions and behavior leading to craving and relapse. Relapsing alcoholics displayed greater atrophy in the PFC (OFC, ACC, and mPFC) relative to control subjects and alcoholics that remained abstinent (101). Reductions in the volume of mPFC (97) and in the amygdala (100) predicted quicker relapse in abstinent alcoholics. The structural alterations of the PFC caused by heavy alcohol use persist even during abstinence, affecting cognitive and inhibitory control over drug seeking behaviors (127).

Set-shifting and reversal learning are behavioral tasks that involve the PFC. When the mPFC and OFC are lesioned in rodents and humans impairments in these behaviors are detected (128, 129). Additionally, human alcoholics and mice exposed to chronic intermittent alcohol exposure demonstrate deficits in set-shifting and reversal learning, (130, 131). Indicating that chronic alcohol exposure causes dysfunction in the PFC leading to impaired decision making demonstrated by alcoholics.

Stress is a well-known trigger for alcohol use and relapse. Overlap between stress and reward behavior circuitry are thought to underlie how stress increases alcohol use and drives relapse (87, 91, 93). In response to stress, adrenocorticotropic hormone (ACTH) is released from the anterior pituitary and results in the secretion of glucocorticoids (GCs) from the anterior pituitary. Glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) and activated by binding of GCs (132) inducing signaling that leads to changes in genetic and epigenetic regulation, contributing to neurodegeneration and associated behaviors (132). Antagonism of GRs in the mPFC reduced memory deficits seen during alcohol withdrawal (76). Chronic alcohol exposure increases GC signaling in the mPFC in abstinence (74) and antagonism of the GR decreases alcohol intake during abstinence (62).

This information demonstrates the important role the PFC plays in addiction related behaviors. Thus, studying the effects of alcohol on the PFC can help elucidate the mechanisms that regulate addiction behavior to discover possible targets and treatment strategies for AUD.

Hippocampus

Studies have explored the impact of alcohol use and dependence on hippocampal structure and function evidencing reductions in hippocampal volume and deficits in memory and decision making (133-135). However, we are unaware of studies specifically addressing the role of the hippocampus in alcohol-dependent related drinking. The hippocampus is well established as a region which is sensitive to the effects of exercise, producing increases in BDNF expression (mRNA and protein) (136-139). In Chapter 2 the dentate gyrus subregion of the hippocampus was analyzed to serve as a control region to

determine if our model of daily, limited (2-hr/day) wheel running would be sufficient to induce elevated BDNF.

Modeling Alcohol Use and Addiction in Animals

Studying alcohol use and addiction in animal models has been critical to understanding the neurobiology and mechanisms which drive alcohol-seeking and drinking behavior. Animal models allow for the controlled testing of pharmacological agents to determine their efficacy in the treatment of excessive drinking. Several animal models have been developed to explore the biological and behavioral effects of alcohol use and exposure. These animal models include invertebrates such as Drosophila melanogaster (fruit flies), Caenorhabditis elegans (nematode), Danio rerio (zebrafish) which possess smaller and more simple nervous systems making them excellent tools for investigating the molecular and cellular basis for addiction (140, 141). Additionally, nonhuman primate models of substance abuse have been employed for decades (142). Similarities between the organization of the primate and human brain provide a model that can elucidate the effect of alcohol on brain structure and function. Furthermore, nonhuman primates grant researchers with an animal model that allows for the investigation of more complex behaviors. Finally, rodents including mice, rats, and prairie voles are used to examine molecular mechanism, neurocircuitry, and behavioral effects of alcohol use and addiction (143-147). In the following sections we briefly explore rodent models of alcohol use and dependence that are used in the experiments conducted in subsequent chapters.

Rodents

In the laboratory setting some strains of rodents (eg. C57BL/6J) will voluntarily consume alcohol (148), providing a useful model for examining alcohol-related behavior. Alcohol consumption is not limited to the laboratory setting as animals in the wild have

been found to eat fermented fruits, with some species have been found to eat to the point of being intoxicated (149).

Not all inbred strains of rodents consume the same level of alcohol. For example, DBA/2J mice consume significantly less alcohol than C57BL/6J mice, which are known to have a high preference for alcohol and are commonly used in alcohol related studies (67, 150). Utilizing these different strains of mice can help elucidate genetic and molecular components that influence alcohol intake. The BXD recombinant mouse line generated by cross breeding C57BL/6J and DBA/2J produced mice that consumed a range of alcohol at baseline and after alcohol dependence (151). Genome analysis of a BXD panel provides information about genes that correlate with drinking related behaviors and possible pharmacological targets (152, 153). Additionally, selective breeding of high and low drinking mice and rats from within a given line, such as the generation of HDID mice, HAD/LAD rats, or P- and NP-rats allow for exploration of genetic, molecular difference which lead to alcohol related behaviors.

Alcohol Drinking and Dependence Models

Multiple experimental procedures exist to model the different components of alcohol use in the cycle of alcohol intake and addiction (146, 147, 154-157). Several alcohol drinking and exposure paradigms have been developed to examine the effects of alcohol exposure on brain chemistry, neurocircuitry, and addiction related behaviors. When investigating the neural mechanism related to the motivational aspects of alcohol seeking operant self-administration paradigms are commonly used. This paradigm allows the experimenter to probe various aspects of alcohol seeking ad relapse and are sensitive to pharmacological treatment (158-160). The effects of alcohol use can also be determined by use of a liquid alcohol diet, known as the Lieber-DeCarli diet, which produces elevated

blood alcohol levels. It is commonly implemented to control for nutritional intake and to examine the effects of alcohol intake on alcoholic liver disease (161-163). Various voluntary drinking paradigms have been established that result in stable, nondependent levels of alcohol drinking, such as the two-bottle choice ethanol drinking procedure (described below) (144). Additionally, Drinking in the Dark (DID) is a voluntary drinking model used to examine binge-like alcohol consumption, as it elicits binge like alcohol intake (>80mg/dL) (68, 164-166).

In the paragraphs below the common two-bottle choice alcohol drinking and chronic intermittent ethanol exposure strategy used in the proceeding chapters are described.

Two-Bottle Choice Drinking

A standard, well-accepted model of alcohol intake is home-cage voluntary alcohol drinking, whereby a rodent has free access to one bottle of alcohol and one bottle of water over a 24-hr period, commonly referred to as two-bottle choice (2-BC) (144). This procedure allows the animal, like a human would, to decide whether or not they will consume alcohol. Multiple variations of the 2-BC procedure have been performed which employ the use of differing concentrations of alcohol (e.g. 10%, 15%, 20%) provided over varying amounts of time (e.g. 1-hr, 2-hr, 4-hr) and lasting days to months (83, 148, 154, 156, 167, 168). The use of 24-hr access to alcohol is a simple, easy model of alcohol intake that allows for assessment of alcohol consumption after a variety of pharmacological treatments or alcohol exposure paradigms and is a simple way to explore the effects of alcohol on a neurochemical and molecular level (146, 156, 168).

A drawback to unlimited 24-hr access to alcohol, is it can obscure effects of shorter acting pharmacological agents and when alcohol intake becomes physiologically relevant in regard to blood alcohol concentration. Restricting alcohol access to short periods of

time, e.g. 1- or 2-hr (83), is a way to get a more accurate measurement of alcohol intake and to determine the effects of drug treatments. The need for accurate BECs after alcohol access allows for confirmation that alcohol was consumed rather than spillage from the bottle as a result of the rodents playing with the bottles. Additionally, if the temporal pattern of alcohol drinking is of importance intake can be monitored using lickometers (167, 169). Interestingly, rodents with alternating days of 24-hr access to alcohol display greater alcohol intake (15% and 20% ethanol vol/vol) than rats and mice with continuous 24-hr access (165, 170).

Chronic Intermittent Ethanol (CIE) Exposure (Alcohol Dependence)

Rendering animals' alcohol (ethanol) dependent is a critical to the understanding of molecular, genetic, and epigenetic changes as well as the behavioral responses associated with alcohol dependence. Modeling dependence is commonly done using alcohol vapor exposure, whereby rodents are placed in an inhalation chamber and passively exposed to vaporized alcohol (171). This procedure provides the experimenter with control over the duration and level of alcohol exposure experienced by the animal. Repeated cycles of chronic, heavy alcohol vapor exposure to intoxication followed by withdrawal leads to alcohol dependence (171). A typical chronic intermittent alcohol exposure procedure is repeated for 4 cycles, where one cycle is comprised of a week of Chronic Intermittent Ethanol (CIE) exposure followed by a week of test drinking, typically 2-hr/day (172).

Prior to CIE or Air exposure a baseline alcohol drinking period occurs. Mice commonly have access to one bottle of 15% alcohol (v/v) and one bottle of water for 2-hr in the home-cage Monday-Friday. However, other durations of access, percent alcohol or adding sucrose or quinine to the alcohol can occur. Baseline typically lasts for 4 weeks to

establish stable levels of voluntary alcohol drinking. Each day immediately prior to the start of alcohol vapor exposure, mice are injected intraperitoneal with a loading dose of alcohol (1.6 g/kg; 8% w/v) with pyrazole, an alcohol dehydrogenase inhibitor (1 mmol/kg) in a volume of 20 ml/kg of the mouse's body weight. Air control mice are treated in a similar manner but receive an injection of saline with pyrazole. Mice are placed in alcohol vapor or air inhalation chambers for 16-hr/day for 4 days starting Monday and proceeding through Thursday. Mice are removed from chambers Friday morning and remain abstinent from alcohol until Monday (72-hr), where daily 2-hr access to 15% alcohol and water resumed as in baseline (172, 173).

Following alcohol vapor exposure mice significantly escalate their alcohol intake from baseline and compared to air exposed mice. This effect can be demonstrated after a single cycle of alcohol exposure and withdrawal, but with multiple cycles alcohol intake is enhanced even further (83, 157, 174). Additionally, CIE exposure has been demonstrated to increased drinking in a 24-hr access model (175). The CIE model of alcohol dependence not only increases alcohol intake in a free-access, home-cage drinking models but has been demonstrated to increase alcohol intake and lever responding in operant self-administration (176, 177). Beyond increasing alcohol intake, chronic vapor exposure produces anxiety-like behavior, increases blood alcohol concentrations (40), causes molecular and neurochemical changes, and is specific to alcohol-related reward (154, 176, 178). Thus, making the CIE model of alcohol dependence a useful paradigm to study the effects of AUD on the brain and behavior.

Current AUD Treatments

There is a lack of fully effective treatments for alcohol addiction. Briefly described below are the main treatment strategies for AUD.

Cognitive Behavioral Therapy (CBT)

Psychotherapy is a well-established, nonpharmacological approach to treat mental health disorders including addiction. Cognitive behavioral therapy (CBT) is a problem-focused, action-oriented form of psychotherapy, used to target and treat specific behavioral problems. Through CBT one recognizes cognitive distortions (biased, negative, irrational thoughts) that contribute to a cycle of negative thoughts, feelings and behaviors. Errors in thinking are identified and coping strategies are developed in order to challenge the cognitive distortions in thinking, to ultimately change the behaviors that lead to alcohol use. This is an effective treatment for substance use disorders where it has been known to improve abstinence in alcoholics (55, 179). Cognitive behavioral therapy is effective in treating mental health disorders that co-occur alongside AUD, such as depression and anxiety which contribute to excessive drinking (180).

Mutual Support Groups/12-Step Programs

Mutual-support groups like Alcoholics Anonymous (A.A.) and other 12-step programs are psychosocial support systems to improve abstinence. People meet to share experiences in a nonjudgmental, peer-support setting to gain self-awareness of the thoughts and behaviors that lead to substance use and the effect their use has on them and those around them. In-patients that attended regular A.A. meetings had reduced relapse and improved abstinence rates (181-183). Additionally, regular attendance to A.A. was associated with less drinking over an 8- and 16-year period (36).

Pharmacological Intervention

In the treatment of AUD pharmacotherapies are highly sought after by healthcare professionals to reduce craving, intake, and symptoms of withdrawal. Currently, only three

medications are Food and Drug Administration (FDA) approved. Two (naltrexone and Acamprosate) treat craving and intake of the of alcohol to improve abstinence and disulfiram (Antabuse) which creates avoidance to alcohol through aversion. These three compounds are briefly described below.

Disulfiram (Antibuse) has been around since 1949 and relies heavily on the psychological aspect of maintaining abstinence, because it requires compliance to the medication in a desire to avoid alcohol drinking. Alcohol is metabolized to acetaldehyde by alcohol dehydrogenase, acetaldehyde is then metabolized by aldehyde dehydrogenase to acetate. Disulfiram acts as an aldehyde dehydrogenase (ALDH) inhibitor, causing acetaldehyde to build up in the body (184). Unlike other pharmacological agents, Disufiram does not reduce craving or intake to maintain abstinence through a receptor mediated mechanism. It is through fear of the negative reaction between disulfiram and alcohol, resulting in unpleasant physical feelings (nausea, vomiting, flushing and sweating), that causes avoidance of alcohol in the maintenance of abstinence (185).

Naltrexone is a competitive μ-opioid receptor (MOR) antagonist, which blocks the effects of endogenous opioids which are increased during alcohol administration (186). Preclinical studies blocking MOR in the VTA prevent DA release in the NAc (187) . Alcohol activates the endogenous opioid system increasing DA in the NAc (188). By blocking MORs, naltrexone reduces the rewarding properties of alcohol by blunting opioid receptor mediated dopamine release within the nucleus accumbens (77, 189). Clinical studies have revealed naltrexone decreases the urge to drink, prevented heavy drinking, reduced the number of drinks consumed and improved abstinence (55, 77).

Acamprosate is the third FDA approved medication for AUD and was originally believed to act by antagonizing NDMA receptors modulating their activity (190, 191). Chronic alcohol use enhances glutamate signaling in the brain and targeting the glutamate system by NMDA receptor antagonists can reduce the reinforcing effects of alcohol and treat AUD (51, 192). Evidence further supporting the role of reducing glutamate signaling in the treatment of excessive alcohol consumption comes from studies demonstrating reductions in mGluR5 receptors reduces the development of alcohol dependence. Furthermore, Acamprosate had no effect in mice lacking mGluR5 (52). Acamprosate was thought to target NMDA receptors to reduce glutamate signaling, improve abstinence, block excessive alcohol intake (51, 190, 193). However, while the calcium salt of Acamprosate was effective in reducing alcohol administration in rats the sodium salt of Acamprosate was ineffective (194). This evidence indicates that the important active moiety of Acamprosate is the calcium salt.

New, Exploratory Medications

In search for new efficacious drugs for treating AUD other brain signaling pathways which have been shown to be involved in drug and alcohol use are being explored as potential targets. Researchers are turning to pharmacological agents that are currently FDA approved and established as treatments for other disorders to determine their efficacy in the treatment AUD.

Agents targeting the noradrenergic system are being investigated as potential medications for alcohol addiction. Prazosin and doxazosin are alpha-1 antagonists which have been demonstrated to reduce the number of drinks per week and craving induced by stress and cues (195, 196). The alph-2A receptor agonist guanfacine decreased alcohol intake in high drinking rats as well as attenuating alcohol seeking and cue-induced

reinstatement and normalized glutamatergic transmission in the mPFC that is dysregulated by alcohol use (197).

Some alcohol related behaviors have been demonstrated to be controlled by the opioidergic system. New compounds targeting opioid receptors have been generated and show promise in efficacy. One such agent being investigated is nalmefene a μ- and δ-opioid receptor antagonist, with partial κ-opioid receptor agonism. A clinical study in Europe revealed nalmefene was able to reduce binge drinking and alcohol intake in dependent individuals (198).

Reducing hyperexcitability through inhibiting glutamate activity, can reduce alcohol craving and obsessive drinking behavior (51). Potentiating GABA activity through GABA receptor activation is another promising mechanism. Compared to placebo, the anticonvulsant topiramate acting through agonistic action at the GABA receptor, reduced glutamate hyperexcitability reducing alcohol craving and obsessive drinking behavior compared to those given placebo (199).

The stimulant methylphenidate used to treat children with attention deficit hyperactivity disorder (ADHD) increases hippocampal neurogenesis and elevates BDNF in blood plasma (200, 201). When children with ADHD were treated for six weeks with methylphenidate BDNF in the plasma increased significantly (200). Another study did not find significant differences of BDNF in the plasma between healthy control children and those with ADHD but did see a significant increase in BDNF after 8 weeks of methylphenidate administration (202). Due to the common abuse of stimulants like methylphenidate (203, 204) and the concern of overdoses if combined with alcohol, these pharmacotherapies may not be suitable for the treatment of AUD.

Described above is a sampling of medications being investigated as potential AUD therapeutics. Although, the list of approved medications for AUD is limited and unfortunately not efficacious in all individuals, requiring the development of new medications and treatment strategies to improve abstinence and control alcohol intake.

Exercise

Voluntary exercise is a natural method being explored to treat neuropsychiatric, neurodegenerative, and addiction-related disorders. The sections below describe some of the information related to the description and benefits of exercise and the evidence currently available that relates to exercise in the treatment of addiction.

Health Benefits

Physical activity is the movement of the body by skeletal muscles which increases energy expenditure above basal metabolic rate (calories required for survival), measured in kilocalories to survive (205). Exercise is a sub-category of physical activity, and the terms are often used interchangeably (205). Although, exercise is more accurately defined as highly structured, planned, repetitive physical activity aimed at increasing energy expenditure, improving physical fitness, and health (205, 206). Sedentary behavior is defined as any waking behaviors that uses ≤1.5 metabolic equivalents (MET), defined as energy expenditure when sitting, or lying down (207).

A host of health benefits are associated with exercise. Moderate to intense exercise is associated with reductions premature death, cardiovascular risk, cancer, type II diabetes, and increases energy expenditure, inducing weight loss and reducing obesity (208). Resistance exercise increases bone density, preventing bone loss and osteoporosis (209). Exercise improves immune and anti-inflammatory function (210-212),

reducing low-grade inflammation and increasing anti-inflammatory cytokines (213). Improvements in cardiovascular health are noted including lowered resting pulse, improvement of blood vessel condition, and increasing blood flow to the brain (213, 214).

Of relevance to neuropsychiatric disorders, exercise elevates brain-derived neurotrophic factor (BDNF) (136, 137) increasing neurogenesis and the growth of dendritic spines, reducing the loss of gray and white matter of the brain (215, 216). The improvements to blood flow, immunity, inflammation, and neurogenesis contribute to improved memory and cognition, and decreasing the risk of developing Alzheimer's and Parkinson's disease (217, 218). Additionally, exercise improves sleep and reduces stress, depression and anxiety, all of which exacerbate neuropsychiatric disorders, and it has been shown to reduce drug and alcohol use and improve abstinence (219-222).

Exercise is Rewarding

Exercise is naturally rewarding to humans and animals and it is voluntarily performed by rodents (137, 213, 223, 224). In addition, rodents will operantly respond for a wheel (224, 225) and develop conditioned place preference (CPP) for the wheel-paired side (226-228). Not only do mice in laboratory conditions partake in running behaviors, even in the wild mice have been shown to voluntarily run on wheels (229). The rewarding properties of exercise, like alcohol and other drugs of abuse, are mediated via the mesolimbic reward pathway (226). This makes rodent models for investigating the use of exercise in the treatment of excessive alcohol intake feasible.

Exercise as a Treatment for Alcohol Use Disorder

Exercise intervention is being used and investigated as a natural adjunct in the treatment of drug and alcohol use, as it targets brain systems implicated throughout the stages of addiction and has demonstrated the ability to assist in the prevention of drug

and alcohol abuse and to improve abstinence (230, 231). The naturally rewarding nature of exercise makes it an option as an alternative, nonpharmacological reinforcer to alcohol, reducing the potential of abuse and addiction (223, 232-234).

Unfortunately, there is dearth of literature surrounding the effects of exercise on alcohol intake in preclinical animal models. The existing studies have not focused on the effect of exercise in dependence states, but rather on the effects of exercise on nondependent alcohol drinking. The majority of these studies allowed unlimited wheel running in the home cage for 2 to 3 weeks with concurrent 24-hr access to 10% alcohol. They demonstrated a reduction in alcohol consumption only in female mice with no change in alcohol metabolism or food consumption (136, 138, 139, 223). One caveat to these studies is the concurrent unlimited access to both the running wheel and alcohol, these are competing behaviors which cannot be performed simultaneously. When looking at the studies where alcohol drinking and wheel running were performed at different times the results vary.

Alternating weeks of wheel running in the presence or absence of alcohol (10% ethanol) did not affect subsequent alcohol consumption in male C57BL/6J mice (235). Additionally, a study conducted using 11 days of running did not alter binge-like alcohol drinking in the drinking in the dark paradigm in mice (136). Complicating matters further, when wheel access was provided during a 1- or 2-week alcohol withdrawal period, subsequent alcohol consumption was increased in rats that exercised during withdrawal (236). The variability of these results is most likely due to the different methodologies used. Although all of the studies allowed unlimited running wheel access and alcohol was provided 24-hr/day, the animal's sex and species, the number of days of running, and the

history of behavioral experience are different across the different results. Additionally, as mentioned previously these studies do not address alcohol dependence related drinking.

The efficacy of exercise in human alcohol abuse and addiction has been demonstrated in the literature. When adolescents and young adults participated in team sports of regular physical activity, they were shown to be less likely to smoke, drink alcohol, or use drugs (231, 237-239). In detoxified alcoholics, acute exercise at a low and moderate intensity reduced the urge to drink alcohol and reduced the amount of alcohol consumed (239, 240). Abstinence outcomes were improved when patients participated in treatment programs that involved exercise (241-244). Interestingly, in nonalcoholic, healthy adults multiple form of exercise have been demonstrated to cause increases in alcohol intake (245-247). Although an increase in nondependent drinking is seen, exercise blunts the development of alcohol abuse and dependence (231, 243).

Exercise has been demonstrated to reduce the administration of a variety of drugs and therefore by examining these studies we can further verify the ability of exercise to modulate excessive drug intake and provide insight into the neurological mechanisms for this effect. For example, wheel running attenuated acquisition of nicotine self-administration (248) and methamphetamine self-administration was reduced when rats had concurrent access to a running wheel (249). Nonconcurrent treadmill and wheel running reduced the self-administration of cocaine, heroin, and morphine in male and female rats (250-254). The ability of exercise to attenuate drug administration is not limited to aerobic exercise. Weighted vests were attached to rats to simulate resistance training revealed a reduction in self-administration of i.v. cocaine and heroin (255, 256).

Brain-Derived Neurotrophic Factor (BDNF)

Background

The focus of the work provided in the proceeding chapters involves the role of brain-derived neurotrophic factor (BDNF) in alcohol addiction and exercise. As a member of the neurotrophin family along with nerve growth factor (193), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), BDNF plays an important role in the brain. It is highly involved in synaptic plasticity, differentiation and survival of neurons, and the growth and maintenance of dendrites and dendritic spines (257). Multiple lines of research have revealed a critical role for BDNF in learning, memory (258), addiction, neuropsychiatric disorders, and neurodegenerative diseases (257).

Mitochondrial dysfunction is a common pathology associated with degenerative brain disease and can be suppressed by BDNF in models of neurodegeneration (259). The neuroprotective function of BDNF is demonstrated in animal models of Huntington's disease, where BDNF reductions lead to cell death and recovery of BDNF levels protects against neuronal loss (260).

Expressed throughout the brain, BDNF is found in all cell types including neurons, astrocytes, oligodendrocytes, and microglia, with the greatest expression being in the hippocampus and cortex (257, 261). Transcription of *Bdnf* mRNA commonly occurs in an activity dependent fashion through an influx of calcium (Ca2+) through voltage gated calcium channels or NMDA receptors (262). While the hippocampus is a major producer of BDNF the majority of striatal BDNF protein originates in the cortex, with the remainder coming from the substantia nigra (263, 264). However, the hippocampus does project to the infralimbic mPFC where hippocampal BDNF facilitates fear extinction (46).

Trafficking of BDNF in neurons is extremely important to neuronal survival. For example, in Huntington's disease polyglutamate (ployQ) expansions in huntingtin (Htt) causes protein misfolding, leading to aggregation of the protein (260). Transport of BDNF-containing vesicles from the frontal cortex to the striatum is mediated by Htt interacting with molecular machinery that transport vesicles along microtubules to the synapse for release (265, 266). The polyQ mutation in Htt causes aggregation of Htt, preventing proper interaction with microtubule trafficking machinery inhibiting BDNF transport (260). Lack of BDNF in corticostratial synapses results in a loss of LTP and the protective effects of the striatum on regulating alcohol intake (267-269).

Mature BDNF protein is translated from mRNA into the precursor protein, preproBDNF, in the endoplasmic reticulum. This form of BDNF is cleaved into proBDNF which is packaged into secretory vesicles in the Golgi where it is either released from the synapse or further processed to mature BDNF (mBDNF) before being released (270). Experiments fusing green fluorescence protein (GFP) to BDNF illustrate the packaging of BDNF into vesicles at the Golgi, which are then transported throughout the neuron, frequently co-localized to PSD-95, which is commonly associated with NMDA receptors at excitatory synapses (271).

When BDNF is released from synapses it binds to its main receptor tropomyosin receptor kinase B (TrkB), where it causes the receptor to dimerize and autophosphorylate the intracellular tyrosine domain activating one of three signaling cascades (272). Phosphorylation of TrkB recruits Shc which can activate signaling through either the PI3K pathway or the MAP kinase pathway, inducing pro-survival and pro-differentiation genes (272). Activation of TrkB by BDNF can stimulate PLCgamma, increasing IP3, Ca2+, and PKC activity increasing synaptic plasticity and gene transcription (272) and can induce

expression of BDNF itself in a positive feedback mechanism (273). The BDNF-TrkB complex is internalized through endocytotic vesicles, forming a signaling endosome that is transported through the neuron to the needed location inducing gene transcription and other cellular responses (272). Differential splicing of exons that encode parts of the intracellular catalytic domain of TrKB have been shown to produce a truncated TrkB isoform that does not possess the kinase domain, where it controls neurotrophin signaling and regulation of intracellular Ca2+ release through IP3 pathway (272, 274).

In addition to activating TrkB, mBDNF and proBDNF can bind to p75NTR, a member of the tumor necrosis receptor family, with low- and high-affinity respectively. Signaling of p74NTR leads to activation of pro-apoptotic effects via Jun kinase or conversely pro-survival via NF-kB pathways (272, 275).

Exon Variants

Located on chromosome 11 the *Bdnf* gene is complicated in its structure. Originally thought to be comprised of four exon variants (276) it was later determined that the rat gene contained 9 unique exons and the human 11 (257). Eight of the exons are 5' upstream noncoding, promoter sequences. They are each spliced to *Bdnf* exon IX, which encodes the BDNF protein, generating 11 variants all capable of producing the functional protein (257). Adding further complexity, a short- and long-3'UTR exist which additionally aid in mRNA trafficking and expressional control through microRNA regulation (257).

The function of multiple exon variants is two-fold: 1. They act as unique promoter regions requiring specific transcription factors (257), and 2. They are involved in the trafficking of *Bdnf* mRNA to specific neuronal regions for localized protein translation (277, 278). The majority of *Bdnf* mRNA found in the brain are variants containing exons I, II, IV, and VI (257, 279). Variants are expressed to different degrees in different regions of the

brain. For example, *Bdnf* exon I, IV, and VI are highly expressed in the amygdala. In the cerebellum and medulla all variants apart from exons V and VII were detected with exon II being expressed the most here than in other regions (279). In the hypothalamus exon I and II contributed the most to total BDNF protein, where in the PFC and HPC exon IV and VI were the dominant sources (261).

In cultured neurons *Bdnf* mRNA I, II, IV, and VI were found in the soma and proximal dendrites. When the neurons were stimulated exons II and VI had been trafficked to the distal dendrites with exon I and IV staying in the soma an proximal dendrites (277, 280). To confirm a functional, morphological role of the *Bdnf* mRNA exon variants, siRNA technology was used to silence the different *Bdnf* mRNA variants (278). Knocking down *Bdnf* exons I and IV resulted in reduced dendritic crossings at 30µm but had no effect beyond 60µm. Conversely, silencing exons II and IV with siRNA reduced dendritic crossings at 90µm (278). In addition, they also found neurons overexpressing exons I and IV increased TrkB phosphorylation, a sign of BDNF-TrkB activation, at receptors proximal to the soma, and exons II and IV activated receptors at much more remote distances. These data provide evidence that trafficked exon variants have a functional role in TrkB receptor activation and dendritic morphology.

Exon variants appear to play a role in the expression of other *Bdnf* exons. For instance, when exon I was genetically deleted exons II, IV and VI, along with BDNF protein were reduced in the hypothalamus (261). Similarly, when exon IV was removed exon II, exon VI, and protein levels decreased. Conversely, knocking out exon I in the PFC caused an increase in exon II and IV expression, with no reduction in protein. However, knockout of exon IV in the PFC did not affect other transcript variants, but protein was reduced.

Interestingly, in the HPC exon I removal increased exon IV expression and showed no reduction of protein. When exon IV is eliminated both exon I mRNA and total protein are reduced [250]. This work demonstrates a region specific, complex relationship between exon variants and protein. It provides evidence that a redundancy mechanism may exist to mitigate loss in BDNF protein. Furthermore, loss of BDNF protein from knockout of exon IV and VI, but not I and II, caused significant impairments in PFC GABAergic interneuron markers (261). Mice with genetic knockout of exon IV have impaired inhibitory transmission from GABAergic neurons to layer V pyramidal neurons of the PFC (281).

Mice with either *Bdnf* exon I or II genetically disrupted exhibit increased serotonin transporter expression in the PFC, HPC, and hypothalamus (HYP) [250], enhancing serotonin reuptake from the synapse. These mice display increased aggression associated with serotonin deficits in the PFC (282). Interestingly, mice deficient in exon IV or VI did not exhibit behavioral abnormalities. This demonstrates a role of specific *Bdnf* mRNA exon variants in behavior.

In human *Bdnf* exon II, a valine (Val) to methionine (Met) polymorphism at residue 66 is known to occur. The Met variant impairs the transport and vesicular secretion of BDNF protein, restricting BDNF to the cells body and lowering BDNF in blood plasma (283, 284). The polymorphism is associated with increased risk of neuropsychiatric disorders such as major depressive disorder [274], bipolar (285, 286), increased cognitive impairment in Parkinson’s disease (287), Alzheimer’s disease (288), memory impairment (289), and is linked to early onset of alcohol abuse (290-293).

Additional Regulatory Control of BDNF Expression

Epigenetic modifications regulating BDNF protein expression are implicated in neuropsychiatric disorders (294). Acetylation and deacetylation of histones affect ability of chromatin to compact or relax compacting, reducing or enhancing gene transcription respectively, is one such epigenetic modification (295). Deficits in histone H3 acetylation, specifically at Bdnf I and IV, are associated with reduced BDNF protein expression and blocking histone deacetylation by treatment with HDAC inhibitor trichostatin A (TSA) elevates BDNF protein levels (296, 297). Additionally, it has been proven that the endogenous HDAC inhibitor beta-hydroxybutyrate, like its synthetic analogue sodium butyrate, act to inhibit histone deacetylation increasing BDNF expression (298, 299).

Another component to mRNA transcription control is binding of transcription factors to promoter sequences on the DNA, whereby additional factors can be recruited and complexed together allowing for the activation of RNA polymerase for transcription (191). As previously stated, one role of the various *Bdnf* mRNA exon transcripts is to serve as unique promoter sequences for the transcription of *Bdnf* mRNA, ultimately to produce active BDNF protein encoded by exon IX (257). Most of the investigation into the transcription factor binding sites regulating *Bdnf* mRNA expression has focused on exon IV, with several studies evidencing transcription factors which bind to exon I (300-302).

The various *Bdnf* mRNA exons have been demonstrated to be under unique transcriptional control. For example, while both exon I and IV are expressed in an activity dependent manner the factors that are critical for their expression are different (303). Luciferase assay showed mutation of the CREB site did not significantly affect activity-dependent transcription of exon I, whereas it is critical for exon IV expression (303). While USF1/2 can bind to sites on exon I and exon IV it appears to only play a role in activity

dependent transcription of exon IV as induced by KCl depolarization (303). A dominant-negative form of AP-1 was demonstrated to be critical in the expression of Bdnf I but not for exon IV mRNA (273). The same transcription factor can be involved in the transcription of several *Bdnf* mRNA exon variants. For example, the ARNT2-NPAS4 dimer was confirmed to be involved in the transcription of both exon I and IV (300, 303). For example, MEF2 was initially reported to activate transcription of exon I (304), later it was revealed to bind to a site on exon IV (305). In addition to transcriptional activation, it has been shown that exons I and II possess a neuronal restrictive silencer element (NRSE) that is repressed by repressor element-1 silencing transcription factor (REST) (302). Another difference in transcriptional control is seen in exon VI where a glucocorticoid-responsive element is influenced by corticosterone (306) and two forms of CaMKII specifically activate exon IV (307).

Methylation of DNA is another mechanism regulating *Bdnf* mRNA transcription. DNA methyltransferase (DNMT) methylates the CpG islands in Bdnf IV (308). Methylation allows for the binding of the methyl binding domain (MBD) protein MeCP2, to the methylated region of the DNA recruiting repressor proteins (309, 310).

The BDNF single nucleotide polymorphism (SNP) at position 196 of *Bdnf* II, a valine to methionine mutation at codon 66 (Val66Met) occurs is associated with impaired trafficking and release of BDNF protein and dysregulated dopaminergic functioning (311, 312). Healthy human adults with the Val66Met variant consumed higher levels of alcohol, had earlier onset of alcohol dependence, and had accelerated relapse to alcohol drinking than Val66Val (290, 293, 313). In the monetary incentive delay task Met carriers had greater BOLD response than Val in the striatum during reward anticipation, which was a predictor of greater alcohol intake (40). A mutant mouse was generated containing a

human Val66Met variant exhibited compulsive, excessive alcohol drinking. When the wild type Val variant was expressed in the mPFC of these mice, alcohol intake was dramatically reduced (314).

BDNF in Alcohol Use

A strong inverse relationship exists between alcohol use and BDNF protein levels throughout the brain and human alcoholics have demonstrated low BDNF protein levels in blood (315, 316). The use of preclinical animal models has directly linked reductions in BDNF protein have to escalated alcohol intake, and by increasing or replenishing BDNF protein in the brain escalated alcohol intake can be prevented or reduced (174, 317). An overall reduction of BDNF in the brain is associated with increased alcohol intake, demonstrated by transgenic mice with BDNF protein deficiencies (317-319). As well, systemic inhibition of BDNF-TrkB signaling caused increased alcohol intake and globally upregulating BDNF-TrkB activity reduced alcohol consumption (317, 320).

Although global upregulation of BDNF is associated with reduced alcohol intake and reductions with increased intake, further examination illustrates brain regional differences. For example, in alcohol preferring P-rats, that exhibit high levels of voluntary alcohol intake, *Bdnf* mRNA and protein were found to be reduced in the CeA and MeA but not in the BLA (321). Moreover, P-rats display higher HDAC2 activity which corresponds to reduced acetylated histone H3 and has been demonstrated to reduce *Bdnf* mRNA transcription. Acute administration of alcohol decreased HDAC activity in the CeA, increasing *Bdnf* mRNA expression and attenuating anxiety-like behavior (298, 322). Further demonstrating the role of reduced BDNF in the CeA to increase alcohol intake, infusion of *Bdnf* mRNA antisense oligodeoxynucleotides (ODNs) into the CeA caused rats to consume more alcohol (323). These data may allude to a potential mechanism in some

individuals that drives increased drinking, as a way to alleviate anxiety by increasing alcohol intake to elevate BDNF within the CeA and MeA.

For BDNF to affect downstream signaling it must bind and activate the TrkB receptor. It was found that following 7 weeks of CIE exposure the TrkB receptors in the hippocampus but not mPFC demonstrated increased phosphorylation compared to controls 3-hr into acute withdrawal which returned to control levels after 3 weeks of abstinence (324). Total TrkB expression was found to not be altered by CIE exposure in the hippocampus, whereas the mPFC demonstrated increased total TrkB protein expression during acute withdrawal but not protracted abstinence (324). Thus, these data reveal a potential role of the BDNF-TrkB system in the hippocampus during early alcohol withdrawal.

BDNF in the Brain (mPFC and Striatum)

The medial prefrontal cortex (mPFC) is critical in top-down cognitive control of executive behavior and in addiction (104, 105). Several studies exposing rodents to alcohol have demonstrated a reduction in *Bdnf* mRNA and protein within the mPFC. Chronic alcohol vapor exposure reduced *Bdnf* mRNA and protein in rodents measured proximal to alcohol vapor exposure and after prolonged alcohol withdrawal (178, 325, 326). Even in models of voluntary drinking, including 14 days of operant self-administration or 6 weeks of home-cage drinking, *Bdnf* mRNA was diminished in the mPFC (327, 328). This reduction of BDNF in the mPFC appears to be involved with alcohol drinking behavior. This effect was demonstrated by direct infusion of BDNF protein into the mPFC of mice rendered alcohol dependent by chronic vapor exposure that attenuated the escalated alcohol intake (174). Moreover, using viral-mediated overexpression of BDNF in the mPFC, CIE-induced escalated alcohol intake was prevented (174). One mechanism for

the reduction of BDNF seen in the mPFC after alcohol exposure is through enhanced miR-206 expression. MiR-206 is a negative repressor of *Bdnf* mRNA and was revealed to be elevated in the mPFC of rats even after 3 weeks of alcohol withdrawal (325, 326). Operant self-administration for alcohol was enhanced when miR-206 was virally overexpressed in the mPFC of nondependent rats (325).

Alcohol has opposite effects on BDNF protein expression in the striatum versus the mPFC. In the cortex alcohol causes a reduction in the expression of BDNF, whereas in the striatum BDNF is elevated. Further complicating matters, the striatum is divided into multiple sub-regions, in which BDNF demonstrates opposing functions. The sub-regions include dorsolateral striatum (DLS), dorsomedial striatum (DMS), and ventral striatum (329) which contains the nucleus accumbens (97). Majority of the work looking at the role of BDNF in the striatum has focused on the DLS and NAc and has revealed opposing functions of the dorsal and ventral striatum.

The DMS is involved in goal-directed behaviors that may influence alcohol seeking (330). Alcohol intake was not affected when BDNF protein was infused in this region (331). However, the NAc, also implicated in goal-directed behavior, shows an increase in BDNF levels after alcohol exposure, an opposite effect to the mPFC. This was demonstrated in rats 3 weeks after chronic alcohol exposure (325). This effect is not restricted to prolonged, heavy alcohol exposure. Both voluntary 2-bottle choice and operant self-administration increased BDNF protein expression in the NAc (327, 332). Pharmacologically inactivating the TrkB receptor, with ANA-12, prevented increased alcohol intake in both 2-bottle choice and a model of binge drinking (332).

One mechanism through which BDNF in the NAc may enhance alcohol intake is through increasing dopamine receptor 3 (D3R) expression. Pharmacological inhibition and

genetic knockout of D3R decreased alcohol intake in mice (332). The release of BDNF protein from cortical neurons has been shown to increase the expression of D3R in the NAc and when TrkB is pharmacologically inactivated a reduction in D3R expression was detected (332, 333).

The DLS takes on a distinct role to that of the DMS and VS, being involved in controlling compulsive, habit-like behavior (334, 335). In rodents a single administration of low-dose alcohol, either voluntary 24-hr access two-bottle choice drinking paradigm or acute injection, increased BDNF expression in the DLS (268, 317, 336). Further supporting the positive association between alcohol and BDNF in the striatum, striatal primary neurons treated with acute alcohol increased BDNF expression and release (268). Operant self-administration of alcohol resulted in increased *Bdnf* mRNA in the DLS (331). Regulation of dorsal striatal BDNF may be regulated in part by reductions in miR-124a, a negative regulator of *Bdnf* mRNA in the dorsal lateral striatum (336). Alcohol elevates BDNF in the DLS which activates MAP kinase signaling, resulting in increased transcription of DRD3 and dynorphin, which is required to reduce alcohol intake (268, 317, 320). Repeated bouts of alcohol intoxication reduced levels of cortical BDNF, which is trafficked to the DLS, additionally BDNF in the DLS becomes less effective at preventing excessive alcohol drinking (328). The DLS plays a key role in the formation of compulsive, habit-like behaviors (337) and as prefrontal BDNF decreases and BDNF generated within the dorsal striatum increases, a shift from recreational to uncontrollable, compulsive use may occur.

BDNF and Exercise

It is well known from research in humans and animals that wheel running (exercise) is able to increase BDNF (mRNA and protein) levels throughout the brain. Much of the

research has focused on the effect of aerobic exercise to increase BDNF in the hippocampus, primarily the dentate gyrus sub-region.

Several weeks of voluntary wheel running will increase BDNF in the HPC and PFC of mice and rats (136, 137, 298, 338). Even a single session of wheel running in rats has been shown to increase hippocampal BDNF levels (339, 340). Rats which were trained to run for 3 days had increased *Bdnf* mRNA levels in the HPC after a 6- or 12-hr running session 10 days following the training period (341). In a study exploring how often exercise must be performed in rats to elevate BDNF protein in the brain, 4 weeks of continuous 24-hr wheel access significantly elevated BDNF protein in the HPC, and increases were maintained even after 90 days of running (137). This study further demonstrated that BDNF protein was elevated in the HPC when running occurred on alternating days, however it took 21 days to see significant increases. It took 2 weeks of no running to see BDNF protein levels return to baseline in mice with continuous access to a running wheel, those with alternating days of running saw BDNF levels decay in 1 week. Interestingly, exercise appears to prime the BDNF system. Where it takes 2 weeks to see increased BDNF expression in the brain, rats with a history of running were able to robustly increase BDNF levels with a single bout of running after 1 and 2 weeks of sedentary behavior (137).

Another form of aerobic exercise training in rodents is forced treadmill running. This paradigm allows for enhanced control over the time and intensity of the aerobic exercise and will increase BDNF in the brain (342). One consideration is the intensity of the exercise and duration must be carefully chosen, as too fast of speed or too long of a training session can induce stress in the rodent and suppress BDNF expression and elevate corticosterone, which could confound the interpretation of the data (342, 343). It has been

found that 3 weeks of forced treadmill running, 30-min/day, was sufficient to increase BDNF immunopositive cells in the cortex and striatum of rats (344).

The ability of exercise to increase BDNF is not restricted to rodents. Both resistance training and endurance exercise increases BDNF levels in humans (345, 346). Peripheral BDNF, measured in the blood, comes primarily from the brain and can cross the blood brain barrier (BBB) in both directions (347). It has been found that humans who performed 4-hr of rowing exercise had increased blood BDNF, with approximately 75% of the BDNF coming from the brain determined by calculating the concentration difference between BDNF concentrations measured from blood collected from the jugular vein and brachial artery (347-349). Acute, moderate exercise (30 min/day) increased BDNF in the blood of humans, an effect that declined 60 minutes after exercise (350, 351). Three months of cardiovascular endurance exercise, performed for 60 min/day, elevated BDNF levels in the blood of humans (349). Even in trained athletes, serum BDNF was elevated following a brief exercise session to exhaustion (352). Overall, exercise in general increases BDNF levels, with the intensity or duration of exercise not increasing the intensity or duration of exercise does not further increase BDNF levels in the blood of healthy men (353).

Mechanisms for Exercise Induced BDNF Expression

Beta-hydroxybutyrate

Ketone bodies such as beta-hydroxybutryate (βOHB) and acetoacetate are produced primarily in the liver during intermittent fasting, starvation, high fat diets e.g. ketogenic diet, and exercise (298, 354). In hepatic cells mitochondrial β-oxidation metabolizes fatty acids to acetyl-CoA, which is then converted to βOHB by β-hydroxybutyrate dehydrogenase (BDH1). The liver shuttles βOHB in to the blood stream

through SLC16A6 channels and crosses the blood brain barrier (BBB) via monocarboxylic acid transporters (MCT1/MCT2) (354, 355).

Ketone bodies have neuroprotective effects in models of neurodegenerative diseases, which are suggested to be the result of epigenetic modifications of histones and receptor signaling (354, 356). Histone acetylation is one mechanism which controls the expression of *Bdnf* mRNA (297, 357). Histone deacetylases (HDACs) remove acetyl groups from histones decreasing gene transcription and can be inhibited by βOHB (358). It is established that HDAC inhibitors such as SAHA and trichostatin A (TSA), can induce BDNF expression (297, 298). Exercise has been demonstrated to increase βOHB which inhibits HDAC activity and increase *Bdnf* mRNA and protein expression (298, 359).

In addition to endogenous HDAC inhibition, βOHB can enhance histone acetylation by histone acetyltransferase (HAT). The lysine residue on the N-terminal tail of histones can be modified by the addition of βOHB which recruits HATs to enhance gene transcription (360). Furthermore, βOHB once in the brain can be converted to acetyl-CoA, increasing the amount of substrate which can be used by HATs to acetylate histones (354).

FNDC5/Irisin

The transcription co-activator PPAR-γ coactivator-1-alpha (PGC-1α) is derived in areas of high oxidative metabolism e.g. skeletal muscle, brain, cardiac muscle, and liver and reductions in the brain are associated with neurodegeneration (361). The interaction of PGC-1α with PPAR-γ mediates gene transcription and increases in fibronectin type III domain containing 5 (FNDC5) in muscle and brain (329, 362). Exercise induces cleavage of FNDC5 into irisin, which is secreted into the blood, increasing *Bdnf* mRNA and protein expression (329, 363). Irisin is not only generated in the brain but peripherally generated

irisin can cross the blood brain barrier (213). Rats exposed to 8 weeks of treadmill running increased PGC-1α mRNA expression in the frontal lobe and hippocampus (364). Even as little as one 3-hr endurance swim session increased PGC-1α in rat muscle (365). Correlating with increased PGC-1α levels, exercise increases FNDC5 mRNA in the brain of rodents (213, 329). In human plasma elevated levels of PGC1A mRNA was detected after acute or prolonged exercise (366). Studies involving humans have confirmed the correlation between an increase in FNDC5 and irisin in the blood after exercise (367, 368).

Other Neurobiological Molecules Affected by Exercise and Alcohol

Exercise and alcohol use modulate the expression multiple molecules including catecholamines and endogenous opioids. In the next section a selection of these molecules is briefly discussed relating the effect of alcohol and exercise on their expression and the role they have in BDNF expression.

Dopamine

Both drugs of abuse and exercise activate the reward pathway to affect the release of dopamine. The dopaminergic system is comprised of the nigrostriatal and mesolimbic pathways, which include the substantia nigra (SN) and ventral tegmental area (VTA), regions in which dopamine (DA) is mainly synthesized from the amino acids phenylalanine and tyrosine. The dopaminergic system is highly involved in reward, aversion, and the regulation of mood. Dopaminergic afferents project from the VTA and SN to brain regions including the dorsal striatum, nucleus accumbens and prefrontal cortex (75, 369-371).

Drugs of abuse have been revealed to cause adaptations of dopaminergic function in the brain. Acute alcohol exposure enhances dopamine and chronic exposure leads to hypofunction of the mesolimbic system with decreased DA release, driving craving and relapse (2, 70, 75). The role of DA in the alcohol drinking behavior is illustrated by studies

showing administration of alcohol increases the release of DA in rodents and humans (50, 372, 373). Administration of DA receptor agonists and antagonists modulate alcohol intake and preference (374).

In contrast, exercise produces a hyperdopaminergic state, increasing DA concentrations and DA receptor binding (226, 370). The enhancement of DA transmission by exercise, is demonstrated to be a mechanism in which exercise can improve sleep and reduce stress (375). Stress vulnerability in rodents and humans is associated with reduced expression of dopamine receptor 2 (DRD2) (376, 377). Exercise may be able to reduce stress through the enhancement of striatal DRD2 expression, as exercise has been demonstrated to increases DRD2 (inhibitory) in medium spiny neurons in the striatum (378, 379).

Not only has exercise been shown to regulate dopamine receptor expression, but it increases tyrosine hydroxylase, an important enzyme in the synthesis of DA (226, 379). Dopaminergic activity may be further modulated by exercise through the reductions in adenosine receptors which act antagonistically to DA receptors (378). Furthermore, exercise can increase dopamine through increasing delta opioid receptor mRNA in the NAc, which has been demonstrated to increase DA release in this region (226).

Dopamine signaling is implicated in elevating BDNF levels in the brain. Incubation of striatal and hippocampal tissue slices with dopamine increased BDNF expression in a time and dose-dependent manner (380). Further confirming the role of dopamine in BDNF expression, striatal tissue slices treated with D1 receptor agonist SKF38393 increased BDNF protein and TrkB surface expression in the striatum (380-382). This effect was also seen in the striatum, where SKF38393 injections in rat pups increased phosphorylated TrkB receptors (383). In vivo treatment for 7 days with rotigotine, a dopamine agonist that

targets all dopamine receptors, increased BDNF protein in the hippocampus and cortex (384). Dopamine induced increases of BDNF protein are not restricted to neuron. Administration of DA increased the production of BDNF protein in astrocytes (385, 386). Dopamine signaling via D1DR activation may mediate the increased production of BDNF through a cAMP/PKA/CREB signaling mechanism (387).

Not only does DA influence BDNF expression, but data exist revealing a role of BDNF to regulate dopamine receptor expression and therefore dopaminergic activity. For example, cultured cells treated with BDNF increased D1DR mRNA and protein, mediated via the PI3K signaling pathway (388). In cultured astrocytes, application of BDNF increased D5 receptor mRNA expression (389). Furthermore, when the BDNF scavenger TrkB-IgG was administered in vivo in rats reduced D3 receptor mRNA (333).

Glutamate

Functioning as the major CNS excitatory neurotransmitter, glutamate is a nonessential amino acid which can be synthesized from α-ketoglutarate and glutamine (390). It is released from the presynaptic neuron and binds to its target receptors on the postsynaptic neuron inducing excitation (390). Glutamate binds to the ion channel receptors N-methyl-D-aspartate (NMDA), AMPA and kainate, as well as the metabotropic family of glutamate receptors (mGluR) (391).

Glutamate is well-known to be involved in the reinforcing properties of alcohol, and administration of alcohol reduces glutamate release and suppresses glutamatergic activity in the nucleus accumbens (192, 392). In the CeA, acute alcohol suppresses glutamate signaling, but chronic alcohol exposure enhances glutamatergic signaling (51, 392). Likewise, in the striatum, chronic alcohol exposure increases glutamate, contributing to striatal dysregulation seen in addiction (393). The role of glutamate in alcohol intake can

be further demonstrated in rat strains which consume different amounts of alcohol. For example, Lewis rats have lower baseline levels of glutamate in the PFC at baseline and self-administer more alcohol than Fisher rats (394).

Acute alcohol application to rat PFC neurons inhibits NMDA receptor activity (105). In response to NMDA receptor inhibition by alcohol, NMDA receptor mRNA expression is increased (395). The increase in NMDA receptor expression from chronic alcohol abuse contributes to glutamatergic hyperexcitability during withdrawal, which contributes to alcohol withdrawal syndrome. The effects of acute alcohol have been mimicked by using NMDA receptor antagonists (192). Alcohol alters function of NMDA receptors by directly inhibiting the flow of ions through the receptor (396), as well as affecting the function of mGluR5 (52). The role of glutamate receptors in alcohol drinking, seeking, relapse, and reward have been further confirmed using the NMDA receptor antagonist Acamprosate (185) and mGluR5 antagonist MPEP (52), where they blocked increased glutamatergic signaling, reducing drug seeking and relapse.

Exercise has been determined to modulate glutamatergic transmission through altering glutamate and glutamate receptor expression and function. In addition, changes in glutamatergic signaling can regulate expression of BDNF. For example, in humans, magnetic resonance spectroscopy (MRS) revealed an increase in glutamate in the frontal and visual cortex following exercise (397). Exercise increases phosphorylation of Ser845 on the C-terminal tail of GluR1, which induces peri-synaptic insertion of AMPA receptors, contributing to increased glutamate signaling and down-stream activation of kinases (398). In mice, exercise increased AMPA GluR1 and GluR2/3 receptor subunits in the frontal cortex and increased phosphorylation of NR1 and NR2B subunits of the NDMA receptor. Treatment with MK801 revealed increased binding to NMDA receptor channels, indicating

an increase in open NMDA receptors and therefore, enhanced glutamatergic signaling (399). Treadmill running exerts a neuroprotective effect by reducing the expression of mGluR5 and NR2B receptors and the release of glutamate in the striatum of rats (400). In rats that underwent daily bouts of endurance exercise, in this case swimming, an increase in muscle glucose transporter isoform GLUT4 was detected (365). The reduction in glutamate may be as a result of increased clearance from the synapse, through increased transporter expression and increased mitochondrial oxitdation of glutamate (401). Increased glutamate clearance and decreased receptor expression would reduce hyper-glutamatergic signaling reducing excitatory neurotoxicity and may assist in prolonging abstinence.

Positive modulation of AMPA receptors increased *Bdnf* mRNA and protein expression in vivo and ex vivo in HPC slices and in primary cortical neurons (402, 403). Additionally, it has been demonstrated that blocking NDMA receptors in the hippocampus attenuated elevations in *Bdnf* mRNA engendered by voluntary exercise in rats (404). It is proposed that AMPA receptor activation by glutamate increases cAMP response element binding (CREB) protein activity via MAPK and CaM-kinase phosphorylation (405). As previously described in the Introduction, CREB activation can drive expression of BDNF. Therefore, glutamate-mediated enhancement of CREB activity can lead to an increase in BDNF expression (303, 340).

Norepinephrine

Norepinephrine (NE) is synthesized from dopamine in synaptic vesicles of the locus coeruleus, which innervate cortical and limbic regions such as the prefrontal cortex and amygdala modulating attention, executive function and which become hyperactive during withdrawal and stress (75, 406). Excessive activation of these neurons is linked to alcohol-

drinking behaviors and relapse (407, 408). For example, inhibiting NE synthesis reduced voluntary alcohol drinking (409) and increasing NE release with yohimbine causes reinstatement of alcohol drinking in human and animal models (410, 411). Data obtained from rats provides evidence that physical exercise increases the expression of NE in multiple brain regions, including HPC, LC, CeA and MeA (412, 413). Rats forced to exercise using a treadmill had elevated levels of NE in the hypothalamus and prevented immobilization stress-induced reductions of NE in the AMG and LC (414). Voluntarily wheel running or forced running on a treadmill for 60 min/day for 8 weeks increased expression of NE in noradrenergic cells bodies in the brain of rats (415).

Following acute and chronic aerobic exercise there is activation of noradrenergic neurons, increasing NE in the brain and blood of humans (416, 417). Resistance exercise has also been demonstrated to increase NE levels. Resistance trained humans at rest were not found to have elevated NE levels compared to nontrained people. However, in preparation for exercise plasma levels of NE where determined to be higher 30 minutes prior to exercise, suggesting the anticipation of the exercise increases NE levels (418).

There exists a strong causal link between elevated NE expression and increased BDNF expression. Stimulation of the vagus nerve results in an increase of NE and BDNF in the mPFC of rats (419). Cultured hippocampal cells treated with NE increased the release of BDNF (420, 421). Further, inhibiting the beta-adrenoreceptor with the antagonist propranolol or DSP-4 blocked increased *Bdnf* mRNA transcription in exercised rats (412, 413). Inhibiting the reuptake of NE by blocking NE reuptake transporter with reboxetine, increased *Bdnf* mRNA in rats that did not exercise, this response was further enhanced in exercising rats (422). Reboxetine treatment increased TrkB phosphorylation, an indicator of increased TrkB activation (423). However, a NCBI PubMed search found

no experimental studies examining the effect of reboxetine on alcohol intake in humans or rodents. It is proposed that exercise increases NE levels, thereby enhancing activation the beta-adrenoreceptor which, in turn, increases the phosphorylation of CREB (a BDNF transcription activator) through PKA (303, 340). Activation of beta-adrenoreceptors can directly phosphorylate TrkB, inducing signaling cascades that leads to the enhanced transcription of BDNF and other genes (424).

Serotonin

Serotonin neurons located in the ventral tegmental area (VTA) and dorsal raphe (DR) project to the nucleus accumbens (NAc) (97), amygdala (AMG), and frontal cortex, regions that are part of the reward circuitry and development of alcohol addiction (425). Increased alcohol intake and vulnerability to dependence has been positively correlated with a decrease in serotonin (426, 427). Acute alcohol exposure increases serotonin release, whereas chronic alcohol exposure decreases serotonin in humans (425-427). Rat strains that demonstrate high levels of alcohol intake, such as the alcohol preferring P-rat, deficits in serotonin signaling are seen (428). Additionally, to demonstrate the role of serotonin in alcohol intake, serotonergic neurons in rats were lesioned with 5,6- and 5,7-dihydroxytryptamine, resulting in increased alcohol drinking (425-427). Furthermore, targeting serotonin 5-HT1A and 5-HT1B receptors with agonists reduced alcohol intake in animals (425, 429). Blocking the serotonin transporter by genetic deletion or with selective serotonin reuptake inhibitors (SSRIs), increased serotonin in the synapse and reduce alcohol intake in rodent models of alcohol use (430). The effect of SSRI treatment to reduce alcohol intake in humans is less well elucidated and the use of SSIR treatments may be affective in a subset of individuals who develop alcoholism later in life (431).

Exercise has been demonstrated to increase the availability of tryptophan, an essential amino acid for the production of serotonin (5-HT) in the brain (432). The leucine-preferring (L-type) amino acid transporter traffics tryptophan into the brain, where it can be converted to serotonin via the intermediate molecule 5-hydroxytryptophan (5-HTP). Levels of tryptophan and serotonin were increased in the plasma and brain of rats that exercised (433, 434). In humans, acute aerobic exercise increased serotonin in urine, serum, and whole blood (435, 436). In rodents peripheral serotonin has been demonstrated to cross into the brain from the blood via serotonin transporters located in the blood brain barrier (437, 438).

Treatment with SSRIs reduce symptoms of depression and increases BDNF protein levels (439-441). Both fluoxetine and citalopram, increased TrkB phosphorylation at the PLC-γ1 site and not Shc binding sites (423, 442). Direct application of serotonin to hippocampal neurons increased expression of BDNF in a dose dependent manner (443). Serotonin signaling can upregulate BDNF expression through 5-HT2A/C, as antagonism of the receptor using ketanserin attenuated exercise induced BDNF expression in the hippocampus (412). This effect is further demonstrated by selective agonism of 5-HT1A and 5-HT2A elevating *Bdnf* mRNA and protein in the HPC, DG, PFC, CeA, and NAc shell of rats (444). The increase in BDNF may be mediated through enhanced CREB function, as SSRI treatment increased phosphorylation of CREB, a well-known mediator of *Bdnf* mRNA transcription (423).

The role of serotonin in the expression of BDNF may not be as clear as it seems. Studies performed have elicited findings contrary to those previous stated. In one study 3 weeks of fluoxetine treatment did not increased hippocampal BDNF protein (445). Mice genetically deficient of tryptophan hydroxylase, displayed elevated BDNF levels in the

HPC (446). Furthermore, 5-HT1A antagonist WAY100635, did not attenuate BDNF expression but enhanced it (412). However, this effect should be taken cautiously because of the compound's dopamine D4 receptor agonist properties (447, 448).

Endogenous Opioids

The opioidergic system, comprised of endogenous opioids (endorphins, enkephalins and dynorphin) and their receptors mu (MOR), delta (DOR), and kappa (KOR) are implicated in alcohol drinking behavior. For example, in rodents studies show β-endorphin, an endogenous MOR agonist, was increased in the NAc and VTA after exposure to alcohol (449, 450). After exposure to alcohol, alcohol preferring C57BL/6J mice present higher β-endorphin levels than DBA/2 mice, an alcohol nonpreferring strain (451). Furthermore, rodents with MOR and KOR knocked out or pharmacologically inhibited with naltrexone (MOR antagonist) or nor-binaltorphimine (KOR antagonist) reduced voluntary alcohol self-administration (78, 452-454)

Targeting opioid receptors is a current strategy for treating alcohol addiction. Compared to control humans, alcoholics who underwent withdrawal presented increased MOR expression in the ventral striatum, correlating with alcohol craving [443]. Positron emission tomography (PET) imaging studies using carfentanil (synthetic opioid) showed reduced MOR binding in the NAc after alcohol drinking (455, 456). In humans, naltrexone treatment decreased the urge to drink alcohol and reduced the number of drinks consumed (77, 457, 458). Activation of the MORs has been suggested to result in the increase in dopamine release in the NAc (187) and blocking the receptor blunts release, thereby reducing drug and alcohol intake (459, 460).

The positive rewarding effects and the feeling of euphoria, commonly referred to as the "runners high" are a result of increased endogenous opioid release in the frontal cortex

(459, 461). Adolescents that engaged in exercise used less prescription opioids and heroin during their lifetime (462). Rats that performed resistance exercise displayed reduced heroin self-administration (255, 256). Further, the exercise intensity (aerobic and resistance) is positively correlated with beta-endorphin levels (459, 460). The induction of endogenous opioids can elicit an analgesic response, which can be blocked by naloxone administration (460). This provides an indication that the effect of exercise on drug may reduce drug and alcohol intake by increasing endogenous opioids (186, 459).

Evidence exists indicating the ability of the opioid system to increase expression of BDNF. Antagonism of the delta opioid receptor with BW373U86 increased BDNF in the HPC, BLA and FC, with the ability of BW373U86 to increase BDNF being blocked by the antagonist NTI (463). The delta-opioid receptor (DOR) agonist DPDPE dose dependently increased *Bdnf* mRNA in the FC of rats (464). Administration of endogenous opioid enkephalin (binds MOR and DOR), i.c.v. into the brain increased *Bdnf* mRNA in the hippocampus (465). Where conversely, antagonism of MORs with naltrexone did not affect the ability of BW373U86 to increase *Bdnf* mRNA (465). Kappa-opioid receptor antagonism, with nor-BNI, increased BDNF protein expression in the HPC and AMG 24-hr after administration i.c.v., while it did not significantly increase BDNF in the frontal cortex BDNF did appear to be trending toward an upregulation (466).

The information above leads to realization that multiple, complex interaction and potential mechanisms exist, by which exercise can induce the expression of BDNF in the brain.

Study Objective

Information presented on the previous pages demonstrates a role of BDNF in the brain to reduce alcohol intake and that exercise can increase BDNF and reduce alcohol

intake. To date, it is unclear how exercise reduces alcohol intake, and there are no studies that have looked at using exercise to elevate BDNF in the brain in the context of an alcohol dependence (CIE) model. This dissertation aims to address the important research question: can daily exercise increase BDNF expression in the brain and, through BDNF-TrkB signaling, attenuate CIE-induced escalated alcohol drinking? Over the next three chapters studies aim to show limited (2-hr/day) exercise (wheel running), prior to chronic alcohol exposure (CIE vapor), can attenuate escalated alcohol drinking associated with dependence through a BDNF-TrkB mediated mechanism.

Chapter 2: Effects of Limited Wheel Running on *Bdnf* mRNA Expression in the Brain.

In Chapter 2 the main hypothesis that exercise under daily limited access conditions (2-hr/day) will increase *Bdnf* mRNA and BDNF protein expression in target brain regions (mPFC and DG) is tested. It is also hypothesized that daily limited access to alcohol will not alter activity or exercise-induced elevated BDNF levels in brain.

Chapter 3: Effects of Limited Running on CIE-Dependent Drinking.

Chapter 3 examines the hypothesis that daily limited access to running wheels will reduce CIE-induced escalated drinking in male mice, and this effect will be associated with activity-related elevation of BDNF mRNA and protein expression in target brain regions.

Chapter 4: Role of BDNF-TrkB Signaling in the Effects of Exercise (Wheel-Running) on Alcohol Drinking in Dependent and Nondependent Mice.

In Chapter 4 studies test the hypothesis that systemic administration of the TrkB antagonist ANA-12 will prevent/block the ability of running wheel activity to attenuate CIE-induced escalated drinking in male mice.

Chapter 2 - Effects of Limited Wheel Running *on Bdnf* mRNA Expression in the mPFC

Introduction

Alcohol use disorder (AUD) is a leading cause of preventable death and illness (467). Chronic heavy alcohol (ethanol) use can lead to the development of alcohol dependence, a chronic relapse disorder characterized by loss of control and compulsive alcohol consumption, and negative symptoms associated with withdrawal (468, 469). Currently there are only three FDA approved medications for the treatment of AUD and, unfortunately, they are not fully efficacious in all treatment seeking individuals (30, 31). Thus, there is a great need for discovery of new and more effective therapeutic interventions.

A potential therapeutic target for the treatment of AUD is brain-derived neurotrophic factor (BDNF). BDNF has been demonstrated to be involved in a number of alcohol-related behaviors, including drinking and dependence (467, 468). For example, in humans, a valine (Val) to methionine (Met) substitution at codon 66 confers a BDNF polymorphism that results in the impairment of vesicular transport and secretion of BDNF (272, 273). Several studies have demonstrated individuals with the Met variant have lower serum BDNF levels (470, 471). However, other studies have provided evidence showing people with the Met variant also display both no changes and elevated serum BDNF (469, 472, 473). In addition to being associated with anxiety, depression, and impaired memory, the polymorphism is linked to early onset of alcohol abuse in humans (474, 475). Further supporting the role of BDNF in alcohol addiction, human alcoholics display reduced blood BDNF levels compared to healthy controls (321, 450, 476).

In rodent models, BDNF expression has been shown to regulate alcohol drinking. For example, mutant mice with the val66met polymorphism display compulsive, excessive

alcohol drinking (326). Additionally, transgenic mice generated with BDNF deficiencies or those with global brain-inhibition of BDNF-TrkB signaling display increased alcohol intake (299-301). Further supporting the role of BDNF in alcohol drinking behavior, when BDNF-TrkB activity is increased in the striatum and mPFC, alcohol intake is decreased (154, 299, 302, 330, 332).

The P-rat is a selectively bred strain of rat that will consume high levels of alcohol. These rats have reduced *Bdnf* mRNA and protein levels in the CeA and display heightened anxiety (303). When P-rats consumed alcohol BDNF levels in the CeA increased and anxiety decreased (304, 305). Infusion of BDNF antisense oligodeoxynucleotides in to the CeA of rats increased alcohol drinking (306), further confirming the role of the BDNF expression in alcohol drinking, specifically in the CeA.

The striatum is another region of the brain that can regulate alcohol drinking behavior through BDNF-TrkB signaling. Unlike the CeA, BDNF in the striatum tends to increase alcohol consumption. For example, chronic alcohol vapor exposure and voluntary alcohol drinking increased BDNF expression in the ventral striatum (VS) of rats (307, 309). Binge drinking was blocked when BDNF's primary receptor, TrkB, was inhibited with the antagonist ANA-12 (317). The dorsal lateral striatum (DLS) is a brain region involved with the regulation of habit behavior, whereby subjects will continue to perform a behavior even when value is no longer associated with the action. Following alcohol administration, the DLS shows elevated BDNF expression (299, 316, 319, 320).

The medial prefrontal cortex (mPFC) regulates top-down control of executive function (110-112). It is also responsive to alcohol cues (125, 126) and is involved in alcohol seeking behaviors (26-28, 108, 109). Expression of BDNF in the medial prefrontal cortex (mPFC) has been directly linked to alcohol drinking behavior in rodent models of dependence. Repeated cycles of alcohol vapor exposure have been demonstrated to

increase alcohol intake in rats and mice (166, 170, 172, 477-479) and cause reduced *Bdnf* mRNA and protein expression in the mPFC of rats and mice [152, 311, 312, 455]. MicroRNA-206 (miR-206) is a negative repressor of *Bdnf* mRNA, that is over expressed as a result of alcohol dependence (330, 332). Over-expression of miR-206 in the mPFC enhanced alcohol intake in nondependent rats (307). The importance of BDNF in the mPFC in regard to alcohol dependence is illustrated in studies where alcohol dependent mice had BDNF protein infused directly into the mPFC, blocking escalated alcohol drinking (169). Additionally, over-expression of BDNF in the mPFC prevented the development of alcohol dependent-like drinking (169). These results indicate a significant role of BDNF in the mPFC in the regulation and treatment of alcohol dependence drinking.

The BDNF gene is a complex gene comprised of 9 exons that act as unique promoter sequences for the regulation of BDNF transcription (244, 260, 284) and aid in trafficking of BDNF throughout neurons (263, 264). The greatest expression of BDNF is in the hippocampus and cortex of the brain, with expression occurring in all cell types (243, 244). All exon variants are individually spliced to exon IX which encodes the functional BDNF protein [245]. Of the variants, exons I and IV are 2 of the most abundant versions in the brain and are expressed in an activity-dependent manner (244, 265, 284). Over-expressing *Bdnf* exons I and IV in neurons increased TrkB phosphorylation, a sign of increased BDNF protein expression and TrkB signaling (278). Mice rendered alcohol dependent by vapor exposure showed reductions of *Bdnf* exon I, IV, and IX mRNA in the mPFC and HPC of mice (173, 460). As described above, manipulating the expression of BDNF in the mPFC can impact the consumption of alcohol. However, there is limited clinical ability to elevate BDNF in the mPFC through traditional modern medical approaches. Hence, an alternative, non-invasive approach to increasing BDNF expression in the brain is of high treatment value.

Exercise is a non-pharmacological approach being investigated for the treatment of AUD. Exercise is naturally rewarding to humans and animals and it is voluntarily performed by rodents (137, 213, 223, 224). Both aerobic and resistance exercise will increase BDNF levels in the DG of rodents (217, 218, 258, 259, 299) and in plasma and serum of humans (346-348, 350-354). Exercise has been shown to reduce alcohol intake in adolescent and adult humans as well as improve abstinence (24, 25, 231, 238-245). Additionally, exercise has been shown to reduce alcohol drinking in rodent models (217, 224, 237). While the mechanism by which exercise influences motivation to drink alcohol in unclear at present, several possibilities have been advanced. For example, exercise may be useful as a treatment for AUD due to its general health benefits, including improving physical and mental health, providing an alternative to alcohol use, and reduces stress (136, 138, 208, 213, 219, 221, 223). Thus, exercise may prevent or reverse deficits in BDNF caused by chronic alcohol exposure, thereby reducing motivation to drink.

In exploring the effects of exercise on alcohol intake, current published work is limited and has mainly focused on voluntary levels of alcohol intake over 24 hours. For example, when mice were given 24-hr concurrent access to a running wheel and 10% alcohol for 2-3 weeks, a reduction in alcohol intake was seen in female but not male C57BL/6 mice (223, 232). Neither sex of mice displayed altered binge-like alcohol intake in the "Drinking in the Dark" paradigm after 11 days of continuous access to wheel-running (90). In another study, alternating weeks of 24-hr wheel running either in the presence or absence of 10% alcohol did not alter intake in male C57BL/6J mice (226). A study conducted using Lewis rats with unlimited access to a running wheel during a 1- or 2-week alcohol withdrawal period increased subsequent alcohol intake (225). The different methodologies, species, sex, and the varying number of days wheel access was provided limit our ability to fully interpret the effects of running on alcohol intake.

A common methodological feature of published studies described above is that rodents had continuous and concurrent access to both wheel-running and alcohol drinking. Drinking and running are competing behaviors that cannot be performed simultaneously in rodents and can obscure the possible effect one behavior may have on the other. Additionally, humans typically schedule periods of time in which they exercise. Therefore, providing unlimited wheel access in rodent models does not optimally translate to human behavior. To our knowledge, the effects of daily limited access to exercise (wheel-running) on alcohol consumption has not been explored. Further, to examine the potential for BDNF to play a role in the effects of exercise on alcohol drinking, it is critical to determine whether scheduled access to wheel-running alters BDNF expression in brain.

Accordingly, the aim of this study was to determine whether daily limited access to an activity wheel in the home cage (2-hr/day) for six weeks alters alcohol consumption and whether this scheduled exercise regimen results in elevated *Bdnf* mRNA expression in the mPFC. The DG was also analyzed as a positive control, as it is a region well known to demonstrate exercise induced increases in BDNF (217, 218, 299). In Experiment 1 mice were provided 2-hr/day home-cage running wheel access to determine if limited wheel access would be sufficient to increase *Bdnf* mRNA and protein levels in the mPFC and DG. Experiment 2 introduced 2-hr alcohol drinking 1-hr prior to wheel access. This experiment examines the effects of alcohol drinking and exercise have on each other and *Bdnf* mRNA expression. Wheel running was provided after alcohol access to avoid any influence of exercise on general thirst and/or the need for more calories.

Methods

Subjects

Adult male C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME) were individually housed under a 12-hr light/dark cycle (lights on at 8:00 AM). Mice were maintained in a temperature and humidity controlled AAALAC-accredited facility at the Medical University of South Carolina, with food (Teklad rodent diet) and water available ad libitum. Mice were weighed weekly during the course of the experiments. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Wheel-Running

Mice were given home-cage access to an activity wheel (Bioserve cat #S3174 and S3175) 2-hr/day beginning 6 hours in the dark cycle. The distance mice ran was tracked and calculated using bike computers (Enkeeo Model BKV-1527) attached to the running wheels. The No-Wheel groups were left undisturbed in their home cages.

Brain Tissue Collection

Mice were sacrificed by decapitation and brains rapidly removed. The medial prefrontal cortex (mPFC) and dentate gyrus region of the hippocampus (HPC) were dissected on ice using 1-mm microdissection punches, with the mouse brain atlas serving as a guide (480). Tissue samples were placed into RNA Later (Thermo Fisher; cat no. AM7021). Total RNA was extracted from the brain tissue samples ReliaPrep RNA extraction kit (Promega; cat no. Z6012) according to the manufacturer’s instructions. Total RNA was quantified on a Denovix DS-11FX spectrophotometer. Tissue collected for BDNF ELISA assay was frozen on dry ice and stored in at -80°C until use, described below.

qRT-PCR

Quantitative real time-polymerase chain reaction (qRT-PCR) assays were performed using similar procedures to those previously published by our group (178). For *Bdnf* exon I, IV, and IX mRNA measurements, cDNA was created using QuantiTect Reverse Transcription Kit (Qiagen; cat no 205314) according to manufacturer's instructions. Custom designed TaqMan qRT-PCR primers were used to specifically target and amplify BDNF I mRNA, were designed using Integrated DNA Technologies (IDT) primer designer online software and manufactured by Life Technologies (BDNF I, Forward: GATGCCAGTTGCTTTGTCTTC; Reverse: CCACCACTTGGTGTGACTTAT; Probe: TCGCCAAGGTGGATGAGAGTTGAA; BDNF IV, Forward: GCCTAGATCAAATGGAGCTTCT; Reverse: GCCGATATGTACTCCTGTTCTG; Probe: ACCTCCGCCATGCAATTTCCACTA; BDNF IX, Forward: GTGACAGTATTAGCGAGTGGG; Reverse: GGGATTACACTTGGTCTCGTAG; Probe: CAGTTGGCCTTTGGATACCGGGA)s. Cyclophilin (Ppia) (Life Technologies) was used as the reference gene, for data normalization. For the reactions, 10 µl of sample cDNA and TaqMan Universal Master Mix II, with UNG (Applied Biosystems; cat no 4440042), were loaded in triplicate into a 384-well optical PCR plate and analyzed on a BioRad CFX384 Real Time PCR system. Cycling parameters: 50° C for 2 min, 95° C for 10 min, followed by 40 amplification cycles with melting at 95° C for 15 sec, and annealing/extending at 60° C for 60 sec. Fluorescence readings are obtained after each cycle. The 2^-DDCT method (481) was used to calculated fold change in expression of the target gene (BDNF I) relative to the reference gene (Ppia) using the CTL group as the reference condition.

ELISA

Brain-derived neurotrophic factor (BDNF) was assayed using BDNF Duo ELISA (R & D Systems) as per manufacturer's instructions. Briefly, brain tissue was lysed in 1% BSA in PBS pH 7.4 via sonication. Standards (0-1500 pg/mL) and samples were added in duplicate to a 96-well plate, coated with BDNF-capture antibody and blocked with 1% bovine serum albumin (BSA). Plates were incubated at room temperature for 2-hr and washed with Wash buffer (0.1% Tween in PBS). Detection antibody was added to a 96-well microplate and incubated at room temperature for 2-hr and then washed with Wash buffer. Strepavidin-HRP was added to each well and incubated 20-min. 50-μL Stop solution (Thermo) was added to well and plate was washed with Wash buffer.

Statistical analysis

All statistical analyses were performed using SPSS Statistics version 25. Statistical significance for all analyses was set at $p<0.05$. In Experiment 1, average running distance (km) over the last 7 days was analyzed by ANOVA, with Group as the main factor. Weekly body weight (grams) was analyzed by Repeated Measures ANOVA, with Group as the between-subjects factor and Week as a within-subjects factor. Pairwise comparisons with Bonferonni corrections were performed when appropriate. BDNF qRT-PCR (fold change) and ELISA (percent of Control) data were analyzed by ANOVA with Group as the factor with mRNA or protein levels as the dependent variable. When appropriate pairwise comparisons with Bonferonni corrections were performed. With average weekly alcohol intake (g/kg) as the independent variable, Repeated Measures ANOVA was performed with running access as the dependent variable. Pairwise comparisons with Bonferonni corrections were performed when appropriate.

Experimental Design and Procedure

Experiment 1: Effects of 2-hr/day Wheel Access on Running, *Bdnf* mRNA and Protein Expression in the mPFC and DG of C57BL/6J Mice.

This study included 5 groups (N= 8-14/group). Four groups of mice were given access to wheel-running for 4 weeks. Separate groups of mice were sacrificed 24-hr, 1-wk, or 2-wk after the final day of running. An additional group was given 2-days access to wheel-running after the 2-wk time point and sacrificed 24-hr later. The fifth group of mice did not receive access to activity wheels (NWH). These mice (NWH group) were sacrificed at each of these time points. Brains were extracted and dissected on ice for BDNF protein and mRNA analysis.

Experiment 2: Effects of Alcohol Drinking on Limited Access Wheel-Running and *Bdnf* mRNA Expression in the mPFC and DG of C57BL/6J Mice.

This study involved a 2 (Group: Wheel, No-Wheel) x 2 (Alcohol: EtOH, water) design, yielding 4 groups (N=9-12/group). Mice in the EtOH groups received 1 bottle of alcohol (15% v/v ethanol) and 1 bottle of water in their home cage for 2 hours, starting 3-hr into the dark cycle. The remaining mice received 2 bottles of water. One hour following removal of bottles, mice in the running groups (WH-EtOH, WH-water) were given home cage access to a running wheel for 2 hours. Wheel access occurred 7 days a week and drinking sessions occurred Monday to Friday (5 days). The experiment lasted 6 weeks and mice were sacrificed 24-hr after the final day, brains extracted and dissected on ice. Total RNA was isolated from select brain regions (mPFC, DG) for mRNA analysis by qRT-PCR.

A

B

2-hr EtOH drinking   2-hr Wheel Access   Wheel Removed   Sacrifice

Figure 2.1 - Schematic representation of Experiment 1 and 2.
(**A**) Experiment 1: 5 groups (N= 8-14/group). 4 groups of mice were given access to wheel-running (2-hr/day) for 4 consecutive weeks. Groups were sacrificed 24-hr, 1-wk, or 2-wk after the final day of wheel-running with one group receiving 2-days access to a running wheel after the 2-wk time point and sacrificed 24-hr later. Control mice (NWH group) were sacrificed at each of these time points. (**B**) Experiment 2: 2 (Exercise: Wheel, No-Wheel) x 2 (Alcohol Access: EtOH, water), 4 groups (N= 9-12/group). Mice received 1 bottle of alcohol (15% v/v ethanol) and 1 bottle of water (EtOH groups) or 2 bottles of water (water groups) for 2-hr. Mice in wheel-running groups (WH-EtOH, WH-water) had 2-hr home cage wheel access 1-hr after the drinking session. Mice were sacrificed at 24-hr after the final day of the study.

Results

Experiment 1: Limited Exercise (2-hr/day) Increases BDNF in the mPFC and DG

During the 4 weeks of daily limited access (2-hr/day) to activity wheels in the home cage all mice reached stable levels of wheel-running (Figure 2.2). One-way ANOVA of average distance ran during the last 7 days revealed no differences between the groups [$F(3,47) = 0.11$, $p = 0.953$].

Mice with daily limited access to wheel-running (WH groups) gained less weight over the 4-week test period in comparison to NWH mice (Figure 2.3). This was confirmed by ANOVA, which revealed significant main effects of Group [$F(4, 55) = 4.59$, $p < 0.003$], Week [$F(4,220) = 56.94$, $p < 0.005$], and the Group x Week interaction [$F(16,220) = 4.10$, $p < 0.005$]. While all mice gained weight over time, at Week 4 all WH groups weighed significantly less than NWH mice ($p<0.05$). Further, reduced body weight persisted for 2 weeks following cessation of wheel-running access in the 2-wk and 2-Wk+2-day groups [$F(2,29) = 6.03$, $p < 0.007$] (Figure 2.3).

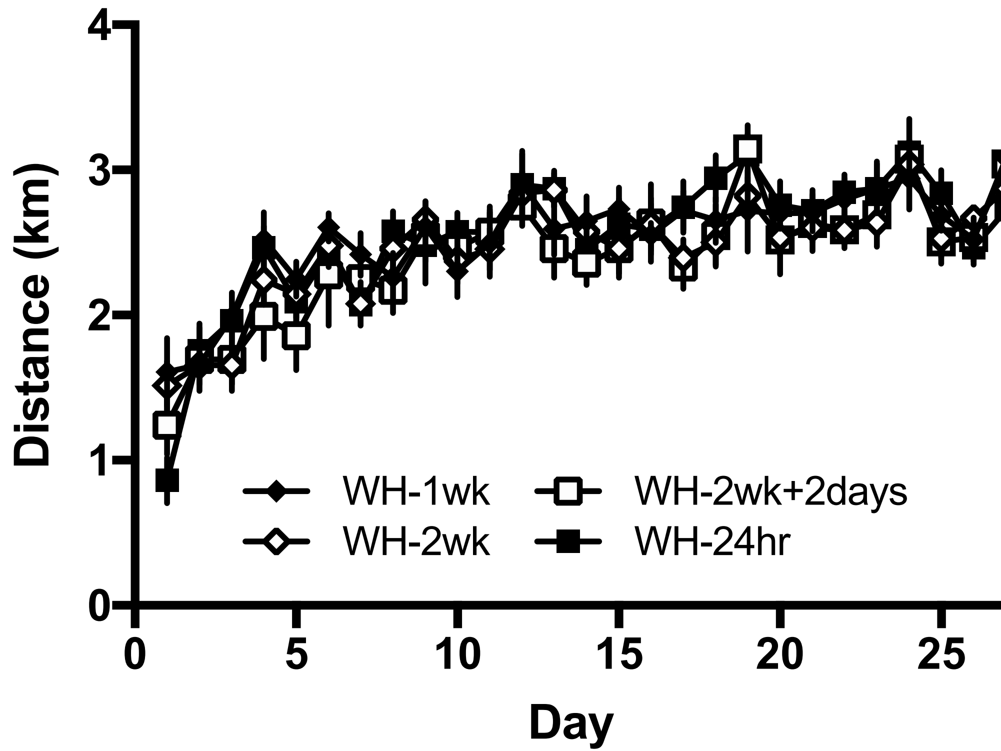


Figure 2.2 – Experiment 1: Average Daily Wheel Running.
No difference in Group average daily wheel running (km) over the last 7 days of running. Values are mean ± s.e.m. (N= 8-14/group)

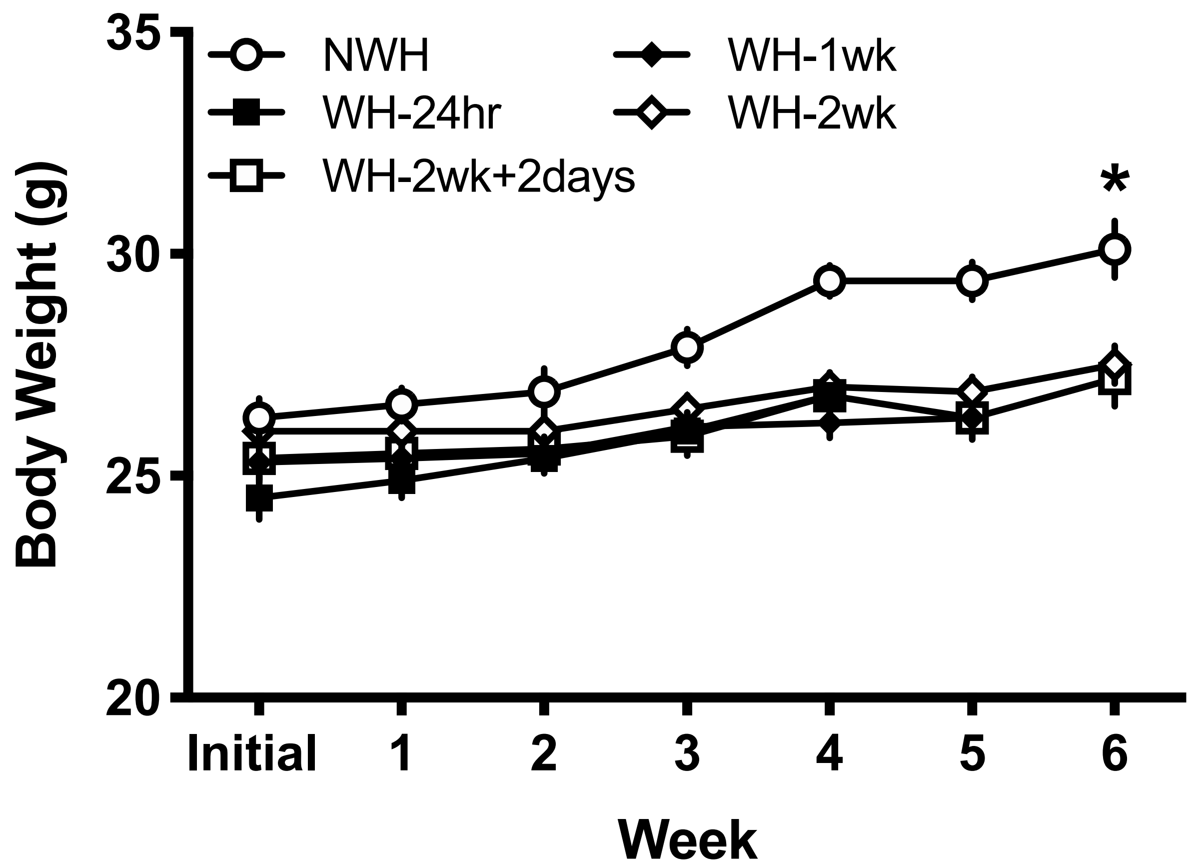


Figure 2.3 – Experiment 1: Weekly Body Weights
Exercise (WH) resulted in lower body weights compared to sedentary group (NWH) at the end of 4 weeks, main effect of Group ($p<0.003$). Values are mean ± s.e.m. (N=8-14/group). Significantly differs from NWH (*$p<0.05$).

Analyses of *Bdnf* mRNA exons I, IV, and IX and BDNF protein were performed on tissue from the mPFC and DG, with the latter brain region selected because it is a region where exercise is well known to increase BDNF expression (136, 137). In the mPFC, exercise significantly increased *Bdnf* exon I mRNA (Figure 2.4 A), as indicated by a main effect of Group [$F(5,52) = 10.26$, $p < 0.0005$]. Post -hoc comparisons indicated that 24-hr after 4 weeks of daily running (WH-24-hr), *Bdnf* exon I mRNA was significantly elevated relative to the NWH condition ($p < 0.05$). This increase normalized to control (NWH) levels 1-week and 2-weeks after activity wheels were removed. However, two days of 2-hr running following 2 weeks of inactivity (2-wk+2-day) resulted in significantly increased *Bdnf* exon I mRNA expression compared to the NWH group ($p < 0.05$). Analyses of exercise effects on *Bdnf* exon IV mRNA expression in mPFC revealed a similar profile of results (Group [$F(4,53) = 4.03$, $p < 0.007$]). Post-hoc comparisons indicated that only the 24-hr and 2-wk+2-day groups displayed significantly increased *Bdnf* exon IV mRNA compared to NWH, 1-wk, and 2-wk groups (ps< 0.05). Analysis of *Bdnf* exon IX mRNA indicated a main effect of Group [$F(4,54) = 3.38$, $p < 0.02$]. Further analysis demonstrated a significant increase in expression for 24-hr and 2-wk+2-day groups compared to the 1-wk and 2-wk groups (ps< 0.05) and a trend for increased expression compared to NWH (ps< 0.1).

In the DG, exercise significantly increased *Bdnf* exon I mRNA expression relative to the NWH condition, as indicated by a significant main effect of Group [$F(4,48) = 8.48$, $p < 0.0005$]. Post-hoc comparisons indicated that this effect was evident at 24-hr and 1-week following the 4-week scheduled wheel-running access period (ps< 0.05). While *Bdnf* exon I mRNA expression in DG returned to control (NWH) levels after 2-weeks, only two days of 2-hr wheel-running was required to significantly elevate *Bdnf* exon I levels ($p < 0.0005$) (Figure 2.4B). Analysis of *Bdnf* exon IV mRNA expression in DG indicated a significant

main effect of Group [$F(4, 55)= 5.16$, $p< 0.001$]. Interestingly, unlike exon I, 24-hr and 1-wk WH groups did not show changes in mRNA expression compared to the NWH condition, but 2 weeks after the 4-week wheel-running access period there was a significant reduction in exon IV expression ($p< 0.05$). When wheel-running access resumed for two daily 2-hr sessions (2-wk+2-day group), there was only a trend toward an increased in mRNA expression compared to the NWH condition ($p= 0.096$) (Figure 2.4B). Analysis of *Bdnf* exon IX expression in DG revealed a main effect of Group [$F(4,54)= 3.18$, $p< 0.03$]. Significant increases in exon IX mRNA expression were evident in the 24-hr and 1-wk WH groups compared to the NWH group ($ps< 0.05$). This effect normalized after 2 weeks and an additional 2 days of wheel-running did not significantly elevate *Bdnf* exon IX expression in DG (Figure 2.4B).

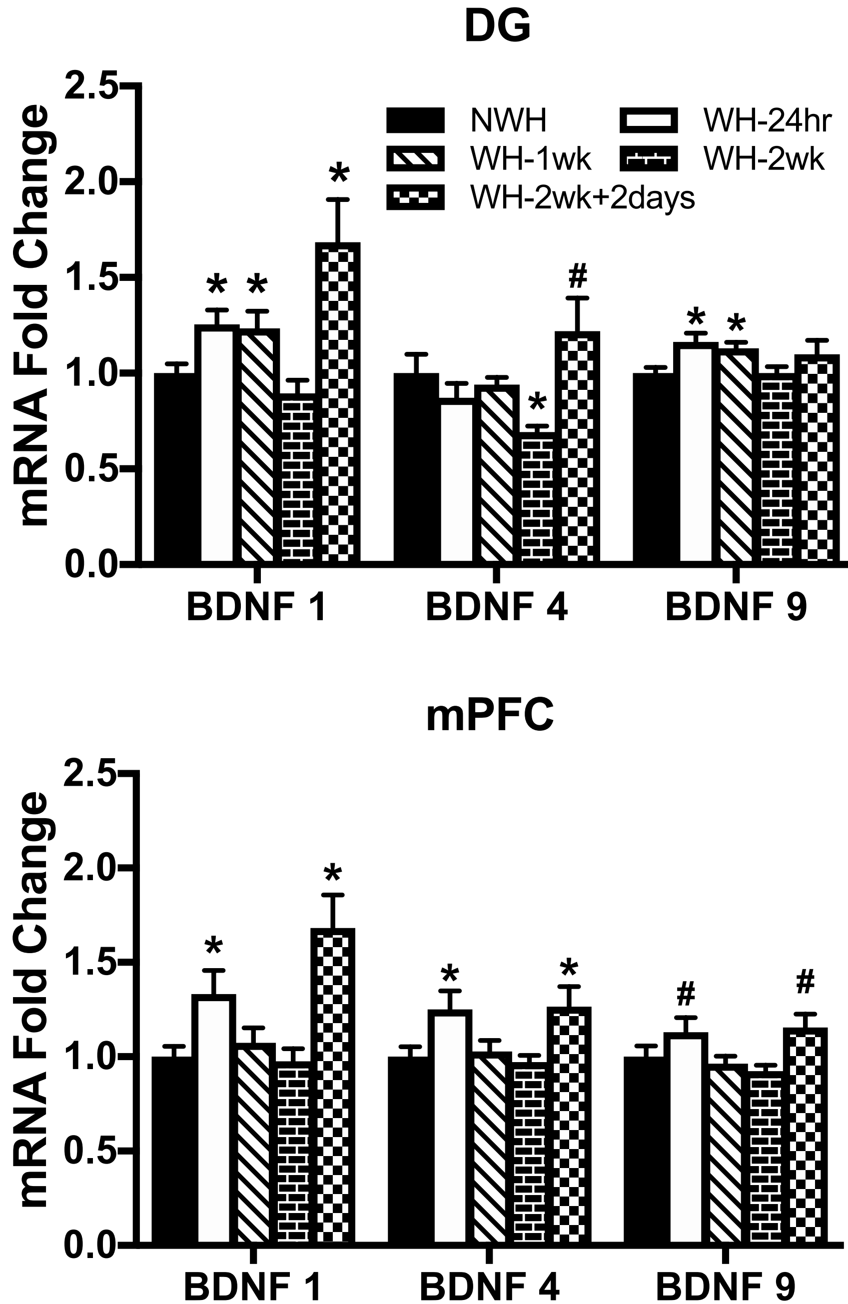


Figure 2.4 – Experiment 1: *Bdnf* mRNA Expression in DG and mPFC
(**A**) DG (**B**) mPFC relative to sedentary (NWH) control group. (**A**) Exon I: main effect of Group ($p<0.0005$); exon IV: main effect of Group ($p<0.001$); exon IX: main effect of Group ($p<0.03$). (**B**) Exon I: main effect of Group ($p<0.0005$); exon IV: main effect of Group ($p<0.007$); exon IX: main effect of Group ($p<0.1$). Values are mean ± s.e.m. (N=8-14/group). Differs from NWH (*$p<0.05$; #$p<0.1$).

In tissue collected from the same animals, BDNF protein was assayed by ELISA (Figure 2.5 A & B). Analysis of BDNF protein levels in the mPFC (Figure 2.5A) indicated a significant main effect of Group [$F(4,41) = 6.61$, $p < 0.0005$]. Protein was significantly increased in the 24-hr and 1-wk WH groups relative to NWH condition ($ps < 0.05$) and then returned to NWH levels after two weeks of no running (2-wk WH group). Similar to mRNA expression, 2 days of 2-hr running (2-wk+2-day group) was sufficient to significantly elevate protein levels in mPFC compared to the NWH group ($p < 0.05$). ANOVA of BDNF protein isolated from the DG (Figure 2.5B) revealed a main effect of Group [$F(4,49)=3.47$, $p<0.015$], with all wheel-running groups displaying significantly elevated levels compared to the NWH group ($ps<0.05$).

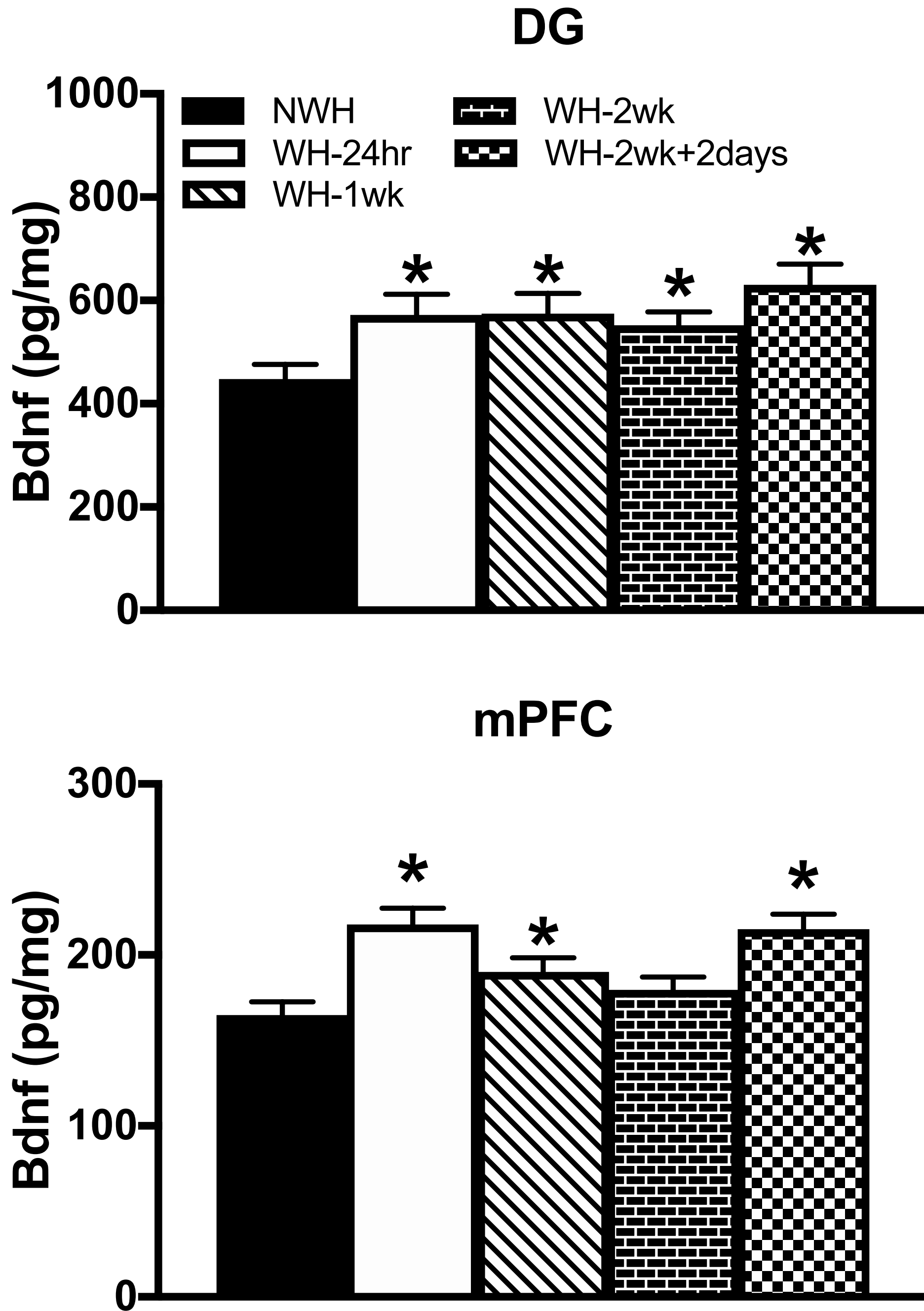


Figure 2.5 – Experiment 1: BDNF Protein Expression
BDNF protein expression measured by ELISA (**A**) DG, main effect of Group ($p<0.015$). (**B**) mPFC, main effect of Group ($p<0.0005$). Values are mean (pg Bdnf/mg total protein) ± s.e.m. (N=6-10/group). Differs from NWH (*$p<0.05$).

Experiment 2: Voluntary Alcohol Drinking Does Not Affect Exercise Induced *Bdnf* mRNA expression in the mPFC or DG

This experiment examined whether alcohol drinking and exercise (wheel-running) interact to influence *Bdnf* mRNA expression in the mPFC and DG. As in Experiment 1, wheel-running (2-hr/day) was relatively consistent and this was true regardless of whether mice were given access to drink alcohol (15% ethanol) (Figure 2.6). Analysis of daily activity expressed as distance (km) during the last 7 days of the 6-week experiment supported this observation in that the main factor Alcohol Access was not significant [$F(1,19)= 0.15$, $p= 0.7$].

While all groups gained weight over the course of the 6-week study, ANOVA indicated a significant main effect of Exercise [$F(1,40)= 5.20$, $p<0.03$] and the Exercise x Week interaction [$F(5,200)= 6.88$, $p< 0.005$]. Post-hoc comparisons indicated that wheel-running mice weighed less than NWH mice at Weeks 2-6 ($ps< 0.05$). This effect was evident in mice with or without access to alcohol (Exercise x Alcohol Access x Week interaction: [$F(5,200)= 1.28$, $p=0.27$] (Figure 2.7).

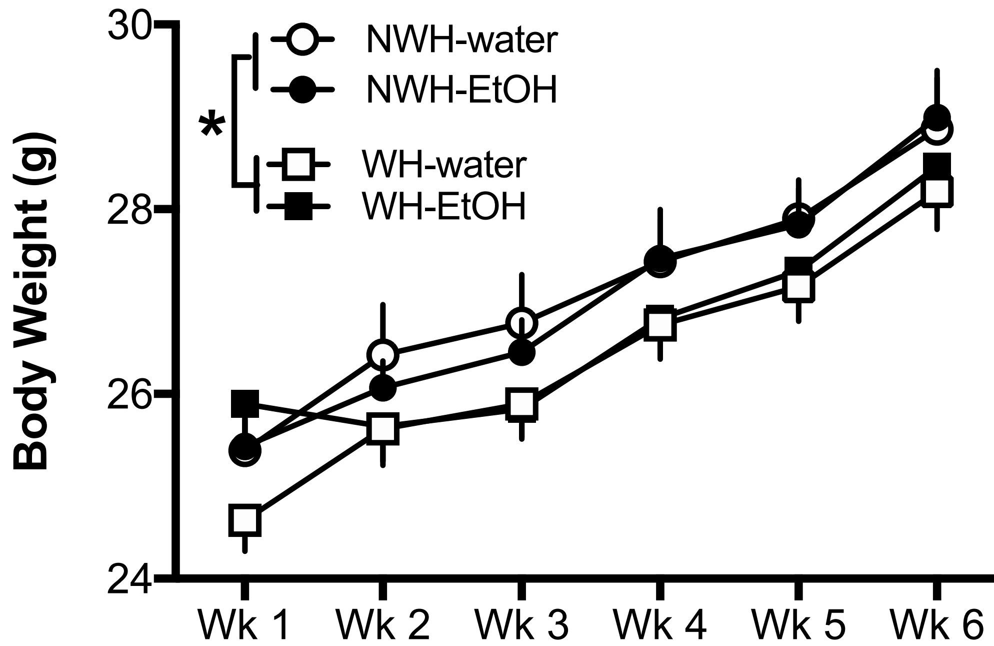


Figure 2.6 - Experiment 2: Weekly body weights.
Alcohol (15% v/v ethanol) drinking did not affect body weight. Exercise (WH) running caused lower body weights compared to sedentary (NWH) mice, main effect of Group ($p<0.03$). Values are mean ± s.e.m. (N=9-12/group). Significantly differs (*$p<0.05$).

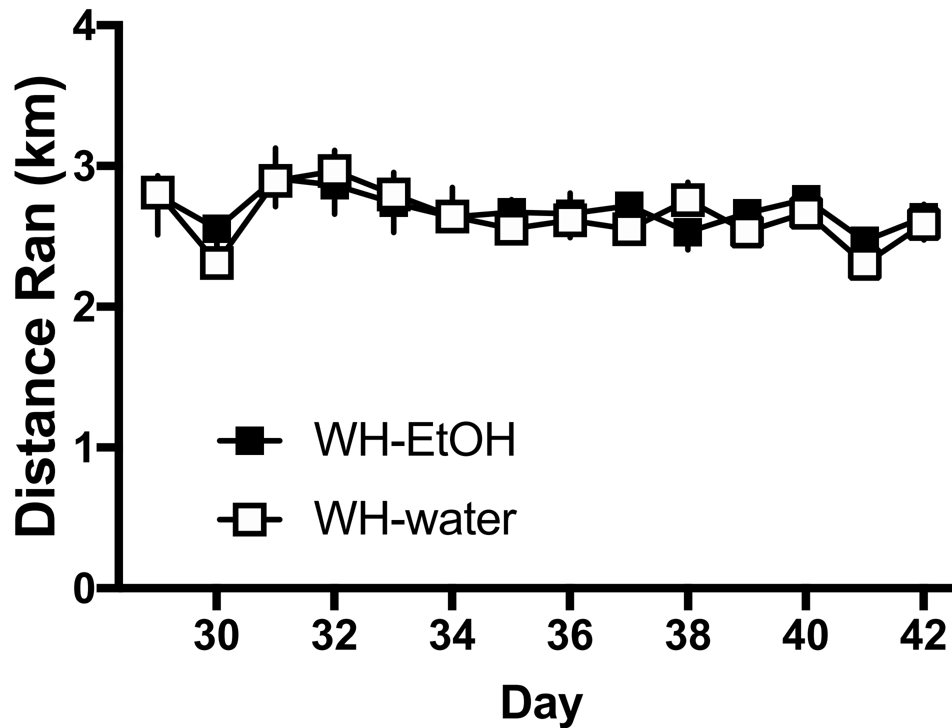


Figure 2.7 - Experiment 2: Average Daily Wheel Running.
Average daily running (km) over the last 14 days of Baseline. No effect of alcohol to alter running. Main effect of Group over the last 7 days of running (p=0.7). Values are mean ± s.e.m. (N=9-12/group).

The effects of exercise on alcohol consumption (2-hr wheel-running occurring 22-hr prior to the beginning of the alcohol access periods) are presented in Figure 2.8A and 2.8B. While mice without access to activity wheels (NWH group) maintained relatively stable levels of alcohol intake (g/kg) over the 6-week experiment, WH mice consumed more alcohol beginning the third week of the study (Figure 2.8A). This was supported by ANOVA, which indicated a significant main effect of Exercise [$F(1,24)= 4.20$, $p<0.005$] and an Exercise x Week interaction [$F(5,120)= 3.50$, $p<0.005$]. Post-hoc comparisons indicated that alcohol intake was significantly greater in WH mice compared to the NWH group during Weeks 4-6 (ps< 0.05). Additionally, given that mice with access to activity wheels evidenced reduced weight over the course of the study, alcohol intake expressed as volume (ml) was analyzed as well. Similar to results with data expressed as g/kg, increased alcohol consumption was observed in wheel-running mice (Figure 2.8B). Although ANOVA indicated a trend for a significant main effect of Exercise [$F(1,24)= 2.92$, $p<0.1$], the Exercise x Week interaction was significant [$F(5,120)= 2.8$, $p<0.05$]. Post-hoc tests revealed that wheel-running significantly increased volume of alcohol consumed during Week 6 ($p< 0.002$) and a trend for increased intake during Week 4 ($p< 0.08$).

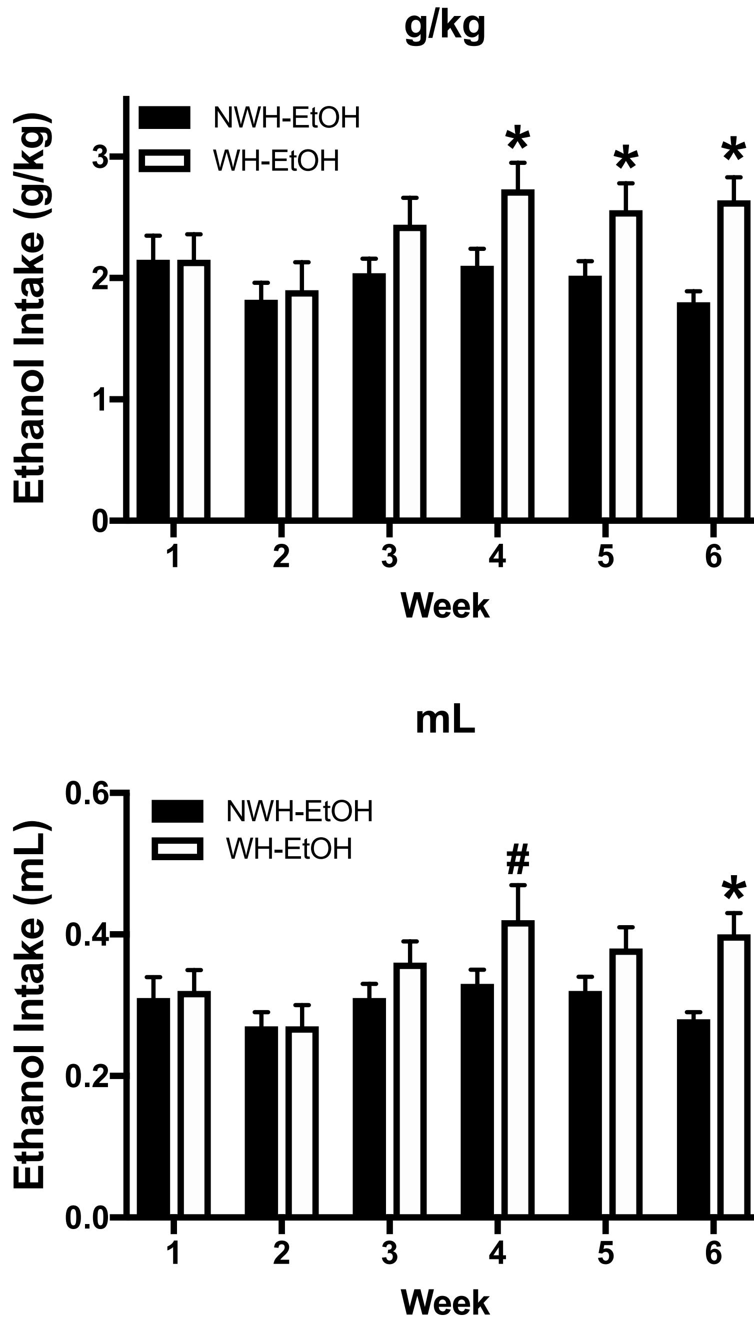


Figure 2.8 - Experiment 2: Baseline Alcohol Intake.
(**A**) g/kg (**B**) mL. (**A**) Exercise x Week interaction ($p<0.005$), main effect of Exercise (WH) ($p<0.005$) (**B**) Exercise x Week interaction ($p<0.005$), main effect of Exercise ($p<0.1$). Values are mean ± s.e.m. (N=9-12/group). Differs from NWH-EtOH group (*$p<0.05$; #$p<0.1$).

Examination of brain tissue at the end of the study indicated that exercise significantly increased *Bdnf* mRNA expression in the mPFC and DG, and this was true whether mice had access to alcohol during the study. Analysis by ANOVA indicated a significant main effect of Exercise, with increased expression of *Bdnf* mRNA exon I [$F(1,35)= 16.09$ $p< 0.0005$], exon IV [$F(1,34)= 13.27$, $p< 0.001$], and exon IX [$F(1,36)= 9.81$, $p< 0.004$] in the mPFC (Figure 2.9A). In the DG (Figure 2.9B) *Bdnf* mRNA displayed a similar profile of results, with a main effect of Exercise for of exon I [$F(1,34)= 27.25$, $p< 0.0005$], exon IV [$F(1,33)= 6.19$, $p< 0.02$], and exon IX [$F(1,39)= 12.57$, $p< 0.001$].

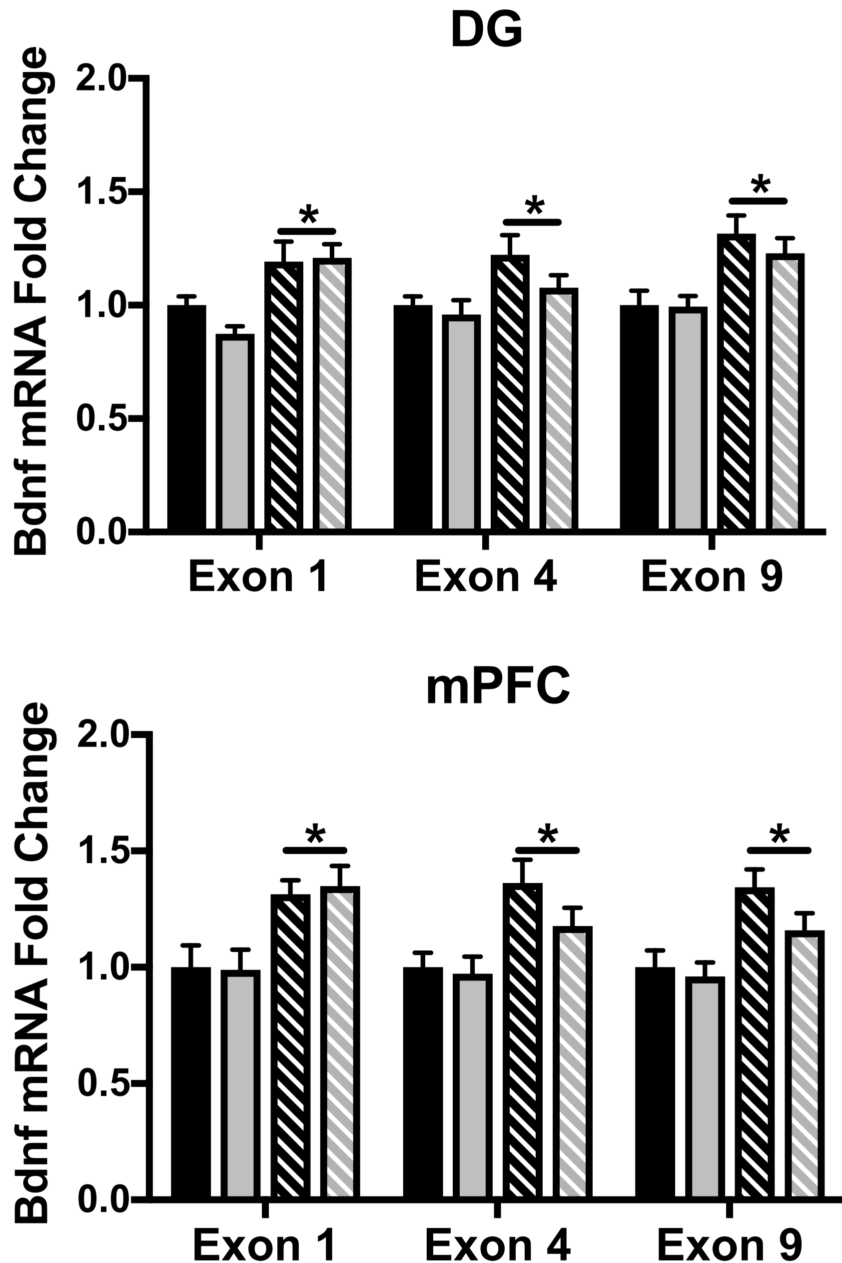


Figure 2.9 – Experiment 2: *Bdnf* mRNA Expression in DG and mPFC
(**A**) DG (**B**) mPFC relative to Control (NWH). (**A**) Main effect of Exercise (WH) exon I ($p<0.0005$), exon IV ($p<0.02$), exon IX ($p<0.001$). (**B**) Main effect of Exercise exon I ($p<0.0005$), exon IV ($p<0.001$), exon IX ($p<0.004$). Values are mean ± s.e.m. (N=7-10/group). Differs from NWH groups (*$p<0.05$).

Discussion

The aim of these studies was to determine if daily limited access (2 hr/day) to exercise (wheel-running) over multiple weeks increased *Bdnf* mRNA and protein expression in the mPFC, a region implicated in contributing to escalated alcohol drinking associated with dependence (174). Results indicated that this regimen of exercise significantly elevated *Bdnf* mRNA and BDNF protein levels in the mPFC as well as the DG subregion of the hippocampus. Additionally, results demonstrate the ability of exercise to rapidly elevate *Bdnf* mRNA and BDNF protein in the mPFC and DG of mice with a history of wheel running, replicating similar findings previously reported in the DG of rats (137).

Continuous (unlimited) daily access to wheel-running is well-known to increase BDNF (mRNA and protein) expression in the DG (136, 137, 213). Complementing these findings, our results show that a daily limited schedule of exercise (2-hr/day wheel-running) over several weeks significantly increased BDNF expression in the DG and in the mPFC. Similar to mice with unlimited access to an activity wheel (136-139, 235, 236), mice provided wheel access in the home-cage 2-hr/day maintained consistent levels of running over the course of several weeks. Further, daily alcohol intake did not affect wheel-running behavior or influence the ability of exercise to elevate BDNF expression in the target brain regions. Interestingly, wheel-running produced a modest increase in alcohol consumption even though the scheduled 2-hr wheel access occurred 21-hr prior to alcohol access.

Chronic alcohol exposure has been shown to reduce *Bdnf* mRNA expression in multiple brain regions, including the cortex (325, 328, 482, 483) and hippocampus (484, 485). Using a well-established mouse model of alcohol dependence that involves repeated cycles of CIE exposure, our laboratory has demonstrated similar effects (174, 178, 486).

Further, increasing BDNF activity in the mPFC via direct microinjection of the neurotrophic factor or viral-mediated over-exppression of BDNF significntly reduced escalated alcohol drinking associated with dependence (174). Collectively, these data support the notion that BDNF activity in the mPFC contributes to the regulation of alcohol consumption, making the BDNF-TrkB system a potential target in the treatment of alcohol addiction. Unfortunately, no pharmacological treatments are availble that specifically target BDNF-TrkB system for use in the treatment of alcohol addiction.

Exercise has been shown to reduce the development of dependence (231, 243), improve abstinence (241-244), and reduce alcohol intake (241, 474, 475). Both resistance and aerobic exercise increase BDNF levels in humans and rodents (136, 221, 255, 256, 339, 340). This suggests that exercise may be a viable non-pharmacological strategy to increase BDNF levels and thereby exert therapeutic effects in the treatment of alcohol addiction.

Few studies have examined the effects of exercise on alcohol drinking behavior. In those published studies, mice had concurrent, unlimited access to alcohol and an activity wheel in the home-cage (138, 139, 235, 236). This approach may confound drinking results because these are competing behaviors that cannot be performed simultaneously. Additionally, unlimited access to alcohol in the home-cage may obscure subtleties in drinking behavior (e.g., temporal patterns of alcohol intake in relation to exercise). As well, humans do not typically exercise throughout the course of their day, but rather perform exercise during designated time periods. Therefore, the present studies were designed to temporally separate access to alcohol and exercise.

In the present studies, activity wheels were provided in the home-cage after the alcohol drinking session was terminated to avoid an influence of exercise on a need for

more calories or from an overall increase in thirst. Interestingly, exercise had a modest effect of increasing alcohol consumption, an effect that emerged around the 4th week of the study (Experiment 2). Although body weights of wheel-running mice were lower than sedentary mice, this effect was observed at the end of the study (Week 6) for volume of alcohol consumed, even though the drinking sessions occurred 21-hr after the daily scheduled exercise period. An explanation for this effect is not clear at present. Of note, in similar studies, exercise (wheel-running) did not alter alcohol consumption when access was restricted to 1-hr rather than 2-hr each day.

In contrast to our results, studies have shown access to a running wheel produced a reduction in alcohol intake, however, this effect was only observed in female mice given concurrent, unlimited activity wheel and alcohol (10% ethanol) access (136, 138, 139). Other studies have shown increased alcohol intake, but only when exercise was restricted to periods of alcohol deprivation in rats (236) or after 12 days of wheel-running in C57/BL6cr mice (487). Taken together, published work indicates that the relationship between exercise and alcohol consumption is complicated and outcomes may differ due to differences in methodology (e.g., limited vs. unlimited and concurrent vs. nonconcurrent access to exercise and alcohol drinking; species; sex).

A number of limitations should be noted in the present experiments. While unlimited daily wheel-running has been shown to not affect food intake in mice (136), we did not measure food consumption in our limited access model. Further, although exercise is known to alter neurotransmission of endogenous opioid peptides and catecholamines (399, 400, 414-416). Such changes in neurotransmitters can influence alcohol intake (192, 488), these effects were not examined in the present experiments. Also, since only the daily scheduled exercise periods were 2-hr in duration, it is not clear whether longer

access to activity wheels (e.g., 4- or 6-hr) would produce more robust effects on BDNF expression and alcohol consumption. Finally, as only males were evaluated in these experiments, it is unclear whether study outcomes may differ between male and female mice.

In summary, results from the present studies indicate that daily 2-hr access to wheel-running over several weeks significantly increased *Bdnf* mRNA and BDNF protein expression in both the mPFC and DG brain regions. Wheel-running behavior was relatively stable throughout the experiments, regardless of whether mice had access to alcohol 1-hr following the exercise period. Additionally, this scheduled exercise regimen produced a modest increase in alcohol consumption that emerged after 3-4 weeks. This effect was observed even though alcohol was presented to the mice (2-hr/day) starting 21-hr after the previous exercise session. These data provide the foundation for follow-up studies aimed at examining the effects of exercise on escalated drinking associated with dependence and whether such effects are related to exercise-induced elevation of BDNF expression in brain.

Chapter 3 - Effects of Limited Running on CIE-Dependent Drinking

Introduction

An overarching conceptualization of alcohol addiction is commonly considered to entail a cycle of 3 main stages: 1) preoccupation/anticipation (craving) where an individual has a compulsory need to seek out and consume alcohol, 2) loss of control over alcohol use (binge drinking and intoxication), 3) withdrawal and negative affective state (eg. Dysphoria) (2). Repeated cycles of heavy alcohol use to intoxication followed by withdrawal and abstinence leads to the development of alcohol dependence (154, 167, 168). Alcohol dependence leads to dysregulation in cortical and sub-cortical pathways which regulate behavioral responses to reward and that govern behaviors related to compulsive, repetitive alcohol use (38, 39).

Top-down control of executive function involved in addiction-related behavior is mediated by the medial prefrontal cortex (mPFC) (104, 105). Altered brain function is mediated by disruptions in neurotransmitter and neuropeptide production, release, and signaling, leading to enhanced alcohol seeking and consumption (6, 51, 71, 192, 489). Additionally, neurotrophic factors have been shown to play a role in contributing to the addiction process. Specifically, brain-derived neurotrophic factor (BDNF) has been implicated by multiple studies to be a key player in addiction-related behavior (174, 313, 325, 328, 490).

In alcohol dependent humans (alcoholics), BDNF in blood plasma and serum is shown to be reduced compared to healthy control individuals (315, 316). Rodent models of alcohol dependence reveal *Bdnf* mRNA and BDNF protein is decreased in the mPFC of the brain (178, 326, 486). Other regions of the brain including the striatum and amygdala

have shown changes in BDNF expression as a result of alcohol use (323, 468). Strains of rats bred to consume greater amounts of alcohol, such as the P-rat, have been found to have reduced BDNF (mRNA and protein) in the brain (321, 476). The role of BDNF in alcohol drinking behavior is further demonstrated in transgenic mice transgenic mice that express lower levels of BDNF, whereby these mice consume more alcohol than wild-type controls (317-319). When BDNF is directly infused in the mPFC of alcohol dependent mice it prevents the escalation in alcohol intake demonstrated during dependence (174). This latter finding provides more direct evidence of the role of BDNF in the regulation of alcohol consumption.

Chronic intermittent ethanol (CIE) exposure, is a well-established paradigm for inducing alcohol dependence in rodents. When C57BL/6J mice are exposed to repeated cycles of alcohol vapor followed by withdrawal they develop dependence-like behavior, demonstrated by increased alcohol intake in 2-hr test drinking sessions (157, 172) and in operant self-administration procedures (177, 491). The increase in alcohol intake induced by CIE exposure increases blood alcohol concentration and is specific to alcohol-related reward as sucrose intake is not altered in CIE exposed mice (154). Chronic alcohol exposure results in neurochemical and molecular changes, including changes in BDNF expression in the mPFC (174, 178, 326). Viral-mediated over-expression of BDNF in the mPFC of mice prior to repeated cycles of CIE exposure blocked the development of dependence-related escalated alcohol drinking (174).

It has been well established that aerobic exercise increases *Bdnf* mRNA and BDNF protein expression in the hippocampus (dentate gyrus; DG) of rodents (136, 137, 342). In Chapter 2 we demonstrated limited daily voluntary wheel running was able to increase *Bdnf* mRNA in the mPFC in addition to the DG of C57BL/6J mice. Exercise, both

aerobic and resistance, has been shown to reduce drug and alcohol intake in rodents and humans (221, 255, 256, 338). To the best of our knowledge, the effects of exercise on drinking in the context of alcohol dependence has not been explored. Additionally, it is unknown whether exercise increases BDNF in human alcoholics.

Based on the ability of exercise to increase BDNF (mRNA and protein) in the mPFC (demonstrated in Chapter 2), and the fact that elevating BDNF in the mPFC via microinjection or viral-mediated overexpression in mPFC blocks and/or prevents CIE-induced escalated alcohol intake (174), we tested the hypothesis that voluntary wheel running would attenuate CIE-induced escalated alcohol intake. It was also hypothesized that exercise would prevent CIE-induced reductions in *Bdnf* mRNA in the mPFC.

Methods

Subjects

Adult male C57BL/B6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were individually housed under a 12-hr light/dark cycle (lights on at 8:00 AM) in a temperature and humidity controlled AAALAC-accredited facility at the Medical University of South Carolina, with food (Teklad rodent diet) and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Study Design and General Procedure

The study was conducted as a 2 [Exercise: wheel, no-wheel] x 2 [Group: CIE, Air] factorial. All mice received 2-bottle choice drinking in the home-cage (15% v/v ethanol vs. tap water) for 2-hr, starting 3-hr into the dark cycle. Alcohol access occurred Monday through Friday (5 days) (Figure 3.1). Alcohol intake in g/kg was calculated by weighing

bottles before and after the drinking session. Body weights were collected weekly to calculate alcohol intake.

During the 6-week Baseline period, mice in the wheel running group (WH) were given home-cage access to a running wheel for 2-hr, 7-days/week, 1-hr after the 2-hr drinking sessions. Mice in the no-wheel condition (NWH) were left undisturbed in the home-cage. At the end of the Baseline phase of the study, WH and NWH mice were further divided into groups for chronic intermittent alcohol vapor (CIE) or air (AIR) exposure, generating four groups (NWH-Air, NWH-CIE, WH-Air, WH-CIE). Mice were exposed to repeated cycles of CIE or AIR, 16-hr/day for 4 days, followed by a 72-hr alcohol withdrawal/abstinence period. Exercise (wheel access) was suspended during CIE/AIR exposure and resumed 24-hr after mice were removed from inhalation chambers. Repeated weekly cycles of CIE/AIR exposure were alternated with Test drinking weeks when alcohol access was resumed as during Baseline.

In a separate experiment, independent cohorts of mice were similarly treated, but sacrificed for *Bdnf* mRNA exon I, IV, and IX expression analysis at the end of baseline, immediately following the 4th CIE/Air exposure cycle, and 24-hr after a 4th Test drinking session (Figure 3.1).

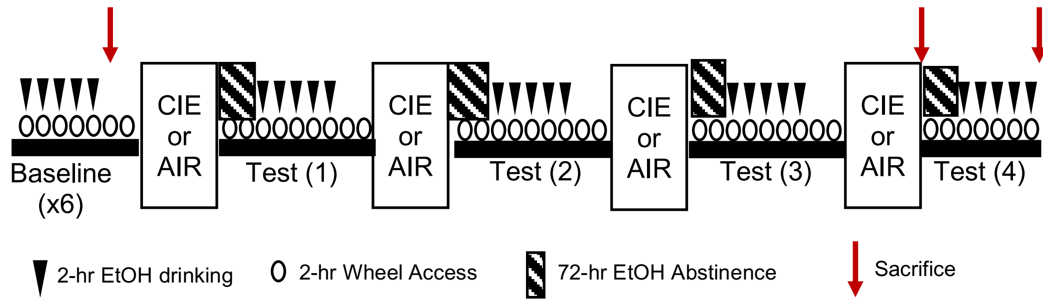


Figure 3.1 - Schematic of Experiment 3.
All mice had 6 weeks baseline access to alcohol (15% ethanol v/v) and water for 2-hr. Mice in the exercise Group received 2-hr home-cage wheel access, 1-hr after alcohol drinking. Groups were further divided and mice were placed in alcohol vapor or air inhalation chambers (16-hr/day for 4 days) during each weekly exposure cycle. Exercising mice resumed wheel access (2-hr/day) during Test weeks.

Chronic Intermittent Ethanol (CIE) Exposure

Mice were exposed to chronic intermittent alcohol (ethanol) vapor or air in inhalation chambers, as previously described (154, 157, 167, 168). Prior to each day of alcohol vapor exposure, mice were administered 1.6 g/kg alcohol with 1 mmol/kg pyrazole in saline, to stabilize blood ethanol concentration (BEC). Air exposed mice received only pyrazole in saline. Alcohol and pyrazole were administered intraperitoneally (ip.) in a volume of 0.02 mL/g body weight. Mice were placed in alcohol vapor or air inhalation chambers (60x36x60 cm Plexiglas boxes) 16-hr/day for 4 days during each weekly exposure cycle.

Using an air stone, alcohol (95% ethanol) was vaporized, mixed with fresh air and delivered to the alcohol chamber. Air alone was delivered to the air chambers. The concentration of alcohol in the chambers were monitored daily, and air and alcohol flow rates were adjusted to maintain BECs in the 200-250 mg/dl range throughout cycles of exposure. BECs were determined as previously described (154, 157, 168). Briefly, blood was collected from the retro-orbital sinus using heparinized capillary tubes and assayed using an Analox Instrument analyzer.

Brain Tissue Collection

Mice were sacrificed by decapitation and brains rapidly removed. The medial prefrontal cortex (mPFC) and dentate gyrus region of the hippocampus (HPC) were dissected on ice using 1-mm microdissection punches, with the mouse brain atlas serving as a guide (480). Tissue samples were placed into RNA Later (Thermo Fisher; cat no. AM7021). Total RNA was extracted from the brain tissue samples using ReliaPrep RNA extraction kit (Promega; cat no. Z6012) according to the manufacturer’s instructions. Total RNA was quantified on a Denovix DS-11FX spectrophotometer.

qRT-PCR

Quantitative real time-polymerase chain reaction (qRT-PCR) assays were performed using similar procedures to those previously published by our group (178). For *Bdnf* exon I, IV, and IX mRNA measurements, cDNA was created using QuantiTect Reverse Transcription Kit (Qiagen; cat no 205314) according to manufacturer's instructions. Custom designed TaqMan qRT-PCR primers were used to specifically target and amplify *Bdnf* exon (I, IV, IX) mRNA, were designed using Integrated DNA Technologies (IDT) primer designer online software and manufactured by Life Technologies (BDNF I, Forward: GATGCCAGTTGCTTTGTCTTC; Reverse: CCACCACTTGGTGTGACTTAT; Probe: TCGCCAAGGTGGATGAGAGTTGAA; BDNF IV, Forward: GCCTAGATCAAATGGAGCTTCT; Reverse: GCCGATATGTACTCCTGTTCTG; Probe: ACCTCCGCCATGCAATTTCCACTA; BDNF IX, Forward: GTGACAGTATTAGCGAGTGGG; Reverse: GGGATTACACTTGGTCTCGTAG; Probe: CAGTTGGCCTTTGGATACCGGGA). Cyclophilin (Ppia) (Life Technologies) was used as the reference gene, for data normalization. For the reactions, 10 µl of sample cDNA and TaqMan Universal Master Mix II, with UNG (Applied Biosystems; cat no 4440042), were loaded in triplicate into a 384-well optical PCR plate and analyzed on a BioRad CFX384 Real Time PCR system. Cycling parameters: 50° C for 2-min, 95° C for 10-min, followed by 40 amplification cycles with melting at 95° C for 15-sec, and annealing/extending at 60° C for 60 sec. Fluorescence readings are obtained after each cycle. The 2^-DDCT method (481) was used to calculated fold change in expression of the target gene relative to the reference gene (Ppia) using the CTL group as the reference condition.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 25 with significance set at $p<0.05$. Exercise measured as running distance (km) was averaged over the last seven days of Baseline and analyzed by one-way ANOVA with Group as the factor. Weekly body weight (gm), was analyzed by Repeated Measures ANOVA, using Exercise and Group as between-subjects factors and Week as a within-subjects factor. Repeated measures ANOVA was performed on weekly average alcohol intake (g/kg), with Exercise and Group as between-subjects factors and Week as a repeated measure. *Bdnf* mRNA (fold change) was analyzed by two-way ANOVA (Exercise x Group factorial). Separate analyses were conducted for *Bdnf* mRNA for exon I, IV, and IX. Pairwise comparisons with Bonferonni corrections were performed when appropriate.

Results

Exercise (Wheel-Running) Attenuates CIE-induced Escalated Alcohol Intake

The effects of exercise (wheel-running) on alcohol drinking was determined in dependent (CIE-exposed) and nondependent (Air-exposed) mice. During the Baseline phase of the study, stable levels of running were established (Figure 3.2), with no differences seen between groups (WH-Air: 2.11 ± 0.20 km, WH-CIE: 2.39 ± 0.13 km). This was confirmed by repeated measures ANOVA, which indicated that wheel-running during the last seven days of Baseline did not differ between groups [Group x Day interaction: $F(1,18) = 1.535$, $p=0.232$]. Although Group was a pseudo-variable at this point in the experiment, these results indicate that the groups had similar exercise history prior to CIE/Air exposure.

Mice with access to a running wheel evidenced lower body weights (Figure 3.3), with a significant difference between exercise and sedentary mice being detected by repeated measures ANOVA [Exercise: $F(1, 37) = 9.034$]. Within-subjects effect of Week [$F(5, 185) = 139.593$, $p<0.005$] and a Week x Exercise interaction [$F(5, 185) = 37.713$, $p<0.005$] indicate mice gain weight over time and Weeks 3-6 sedentary mice weigh more than exericse mice (ps<0.02). Main effect of Group [$F(1,39)= 8.77$, $p<0.005$].

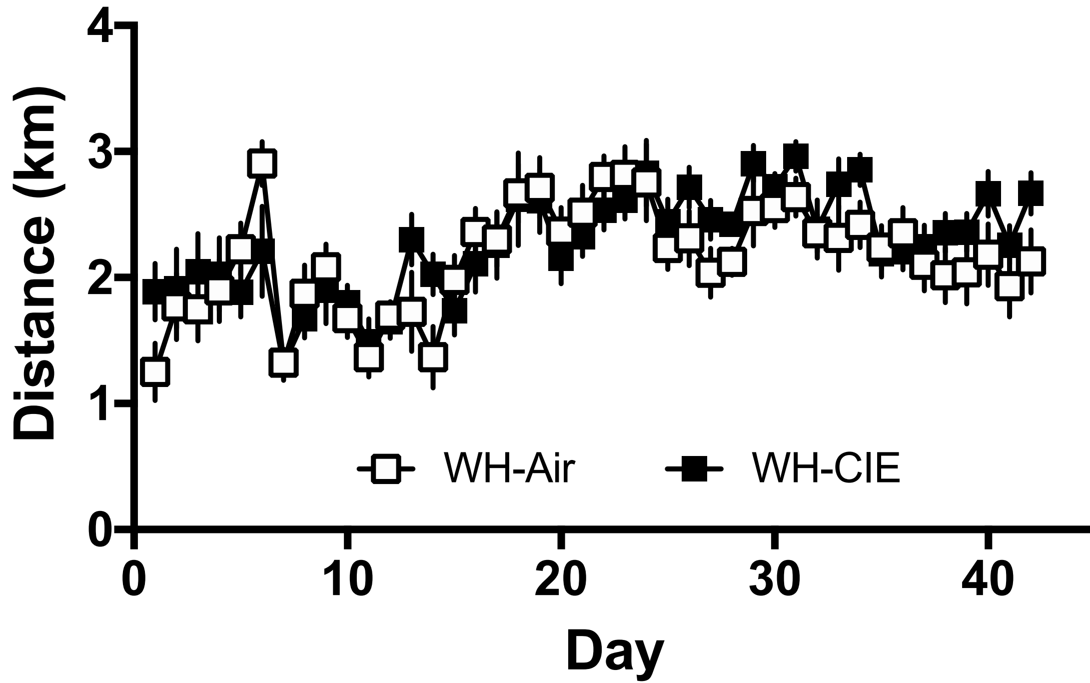


Figure 3.2 - Experiment 3: Daily Baseline Wheel Running.
Average daily running (km) at Baseline for mice that will be exposed to Air or CIE vapor. No differences between groups at Baseline over the last 7 days of running. Values are mean ± s.e.m ($p=0.23$; $N=9\text{-}10$/group)

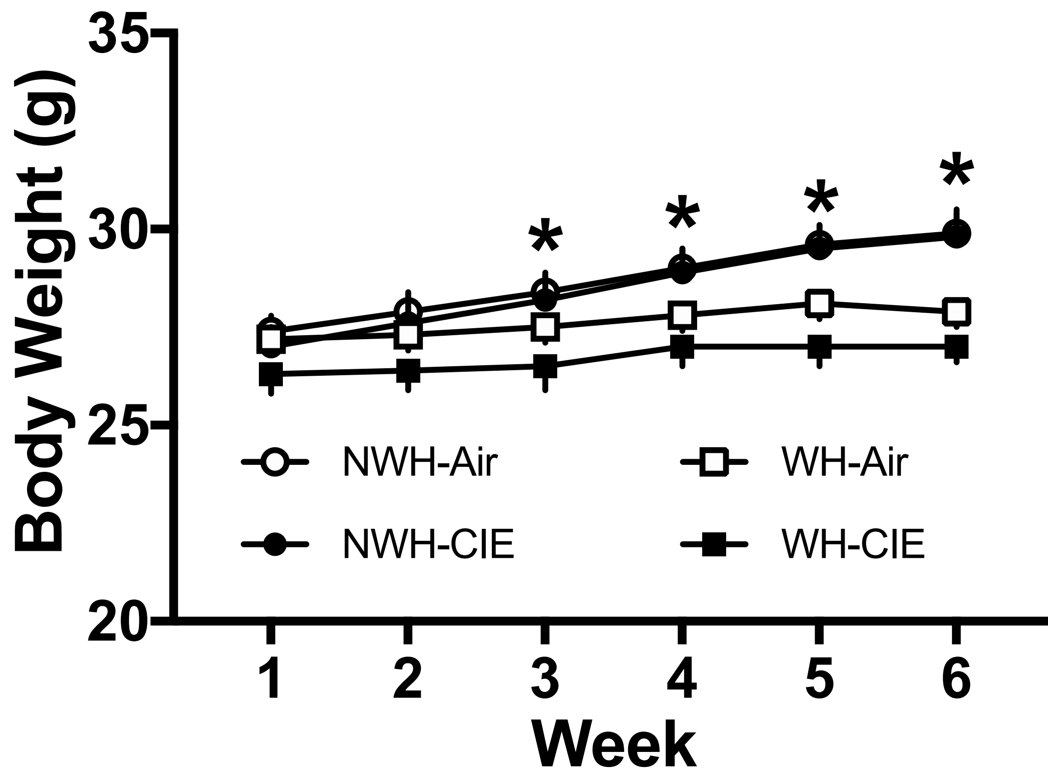


Figure 3.3 - Experiment 3: Average Weekly Body Weights
Average Group body weights (g) for exercising (WH) and sedentary mice (NWH) during Baseline. Values are mean ± s.e.m. (N= 9-11/group). Group x Week interaction ($p<0.005$), pair-wise comparisons (Exercise: No Wheel x Wheel, *$p<0.02$).

Wheel running distance (km) was determined over the last week of Baseline and 4 Test weeks for CIE and Air groups (Figure 3.4). Exposure to alcohol vapor on the whole resulted in reduced running compared to Air exposure. Repeated measure by Week of average distance ran during Baseline and Test 1-4 indicates a main effect of Group [$F(1, 17) = 4.052$, $p=0.06$] and a Group x Week interaction [$F(4,68) =7.21$, $p<0.0005$]. Pairwise comparisons reveal no Group (WH-Air vs WH-CIE) differences during the last 9 days of Baseline [$F(1, 17) = 0.415$, $p=0.528$], Test 1 [$F(1, 17) = 1.002$, $p=0.331$] or Test 2 [$F(1, 17) = 2.194$, $p=0.157$]. Significant Group differences were detected Test 3 [$F(1, 17) = 9.408$, $p<0.007$] and Test 4 [$F(1, 17) = 8.105$, $p<0.011$]. This data indicates that CIE exposure decreases wheel running activity, which worsens with repeated CIE exposures.

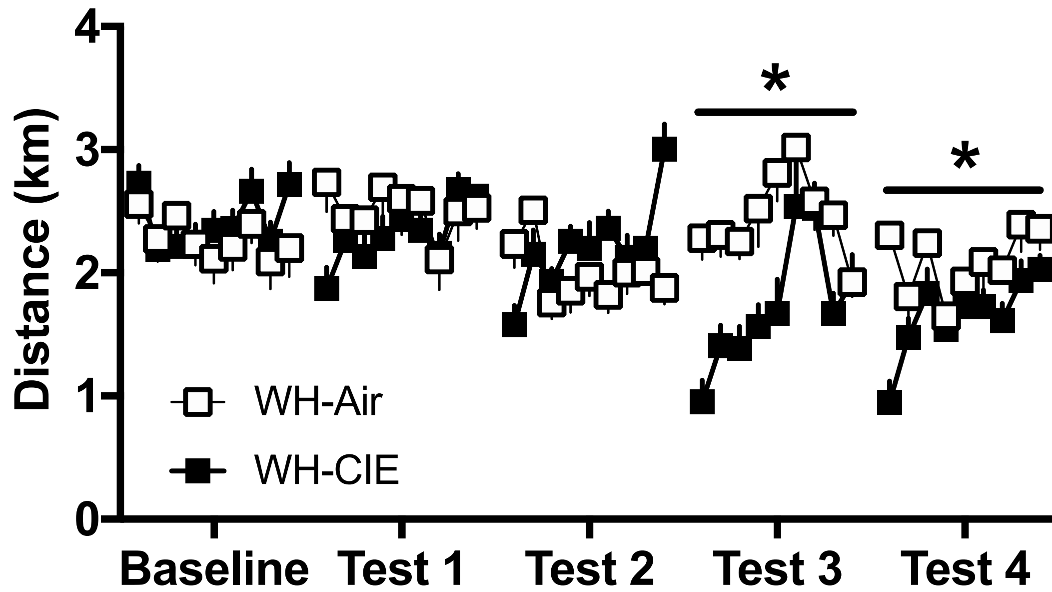
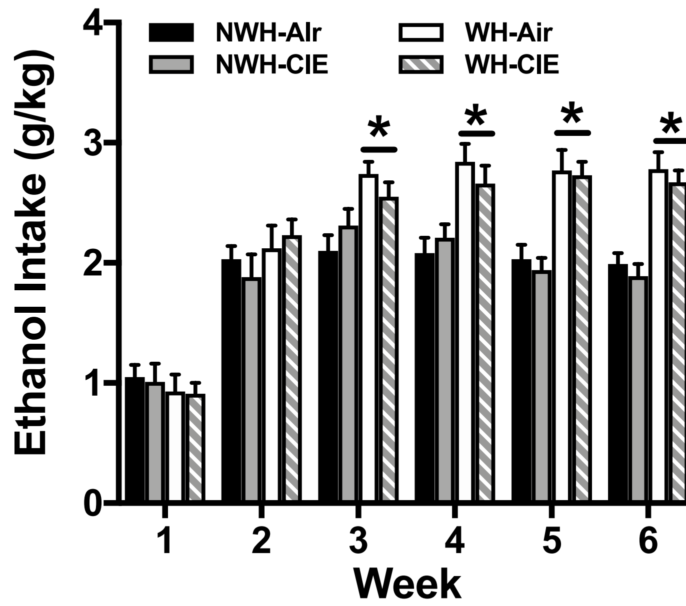


Figure 3.4 - Experiment 3: Wheel Running During Test Weeks.
Average daily running (km) of mice exposed to Air or CIE vapor. Values are mean ± s.e.m. Main effect of Group (*$p<0.05$; N=9-11/group).

As previously observed (Chapter 2), there was a small but significant increase in alcohol consumed in WH mice compared to NWH mice beginning at Week 3 during the Baseline period (Figure 3.5A). This was supported by ANOVA that revealed a main effect of Exercise [$F(1, 37) = 28.077$, $p<0.005$]. A significant Exercise x Week interaction [$F(5, 185) = 9.761$, $p<0.005$] followed by pairwise comparisons indicated WH mice consumed more alcohol than NWH mice during Weeks 3-6 (ps<0.005). Main effect of Group [$F(1,37)= 0.22$, $p=0.64$], and interactions of Group x Exercise x Week [$F(5,185)= 1.2$, $p=0.31$], Group x Exercise [$F(1,37)= 0.14$, $p=0.71$], and Group x Week [$F(5,185)= 0.13$, $p=0.99$] were not significant as at Baseline. Group was a pseudo-variable, indicating that Exercise mice had similar alcohol drinking history prior to being separated for CIE/Air exposure.

Due to lower body weights of exercising mice, volume (ml) of alcohol intake was also analyzed (Figure 3.5B). Similar to alcohol intake (g/kg), ANOVA revealed a significant main effect of Exercise [$F(1,35)= 7.32$, $p<0.02$], indicating greater alcohol intake in WH compared to NWH mice. ANOVA also indicated a main effect of Week [$F(5,175)= 98.68$, $p<0.0005$] and an Exercise x Week interaction [$F(5,175)= 6.29$, $p<0.005$]. Pairwise comparisons indicated that wheel access increased alcohol intake during Week 3 ($p<0.05$), Week 4 ($p<0.02$), Week 5 ($p<0.001$), and Week 6 ($p<0.0005$). As seen with g/kg intake, no significant main effect of Group [$F(1,35)= 0.08$, $p=0.8$] or significant interactions were detected between Group x Exercise x Week [$F(5,185)= 1.11$, $p=0.71$], Group x Exercise [$F(1,37)= 0.86$, $p=0.36$] and Group x Week [$F(5,185)= 0.26$, $p=0.94$]. Baseline Group was a pseudo-variable, indicating that Exercise mice had similar alcohol drinking history prior to CIE/Air exposure.

A



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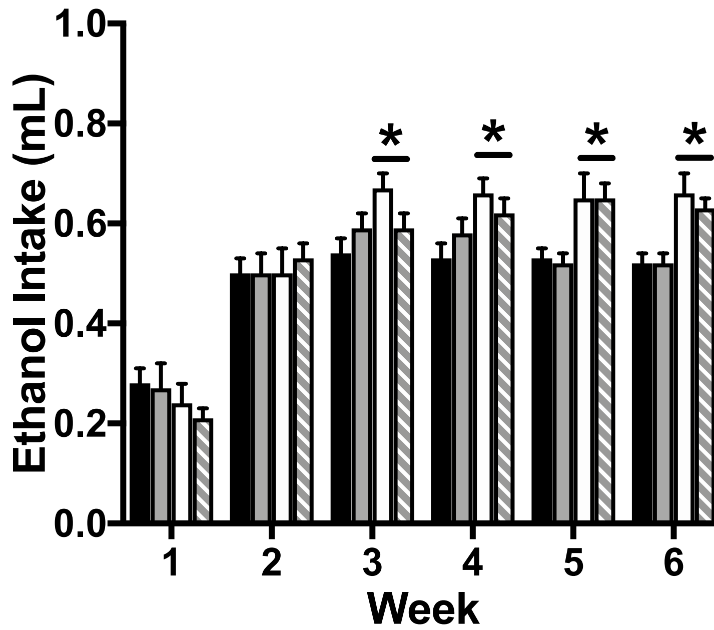

Figure 3.5 - Experiment 3: Baseline Alcohol Consumption.
Average weekly alcohol intake in **A)** g/kg, Exercise x Week interaction (*$p<0.005$) and **B)** mL, Exercise x Week I interaction (*$p<0.05$). Values are mean ± s.e.m. (N= 9-11/group).

Following 6 weeks of baseline, mice were exposed to 4 cycles of alcohol vapor (CIE) or air (Air), interleaved with weeks of Test drinking. Exercise (wheel-running) significantly attenuated CIE-induced escalated alcohol intake (g/kg) (Figure 3.6A). ANOVA indicated significant main effects of Group [$F(1, 33) = 62.38$, $p<0.0005$] (CIE>Air) and Exercise [$F(1, 33) = 7.95$, $p<0.008$] (NWH>WH), as well as a significant Exercise x Group interaction [$F(1,33) = 12.12$, $p<0.001$]. Pairwise comparisons with Bonferonni correction revealed significant differences between NWH-Air vs. NWH-CIE, WH-Air vs. WH-CIE, and NWH-CIE vs. WH-CIE groups ($ps<0.005$). ANOVA also revealed significant Exercise x Week [$F(3, 99) =2.59$, $p=0.057$] and Group x Week [$F(3, 99) =4.313$, $p<0.007$] interactions, but the Exercise x Group x Week interaction was not statistically significant [$F(3, 99) =1.07$, $p=0.37$].

Because alcohol intake between exercised (WH) and sedentary (NWH) mice was different at Baseline (Figure 3.5A & B), alcohol consumption was also expressed and analyzed as percent change from respective baseline levels for each group (Figure 3.6B). ANOVA indicated significant main effects of Group [$F(1, 33) = 45.67$, $p<0.0005$] and Exercise [$F(1, 33) = 85.74$, $p<0.0005$], and the Group x Exercise interaction [$F(1, 33) = 11.11$, $p<0.002$]. Pairwise comparisons reveal significant differences between NWH-CIE vs. NWH-Air, WH-CIE vs. NWH-CIE, WH-Air vs. NWH-Air ($ps<0.0005$), and WH-CIE vs. WH-Air ($p<0.019$) groups. While there was a Group x Week interaction [$F(3,99) =4.31$, $p =0.007$] there was not a significant Group x Exercise x Week interaction [$F(3,99) =1.3$, $p=0.28$]. Collectively, these data indicate that daily limited access to wheel-running significantly reduced the capacity from CIE exposure to escalate voluntary alcohol consumption.

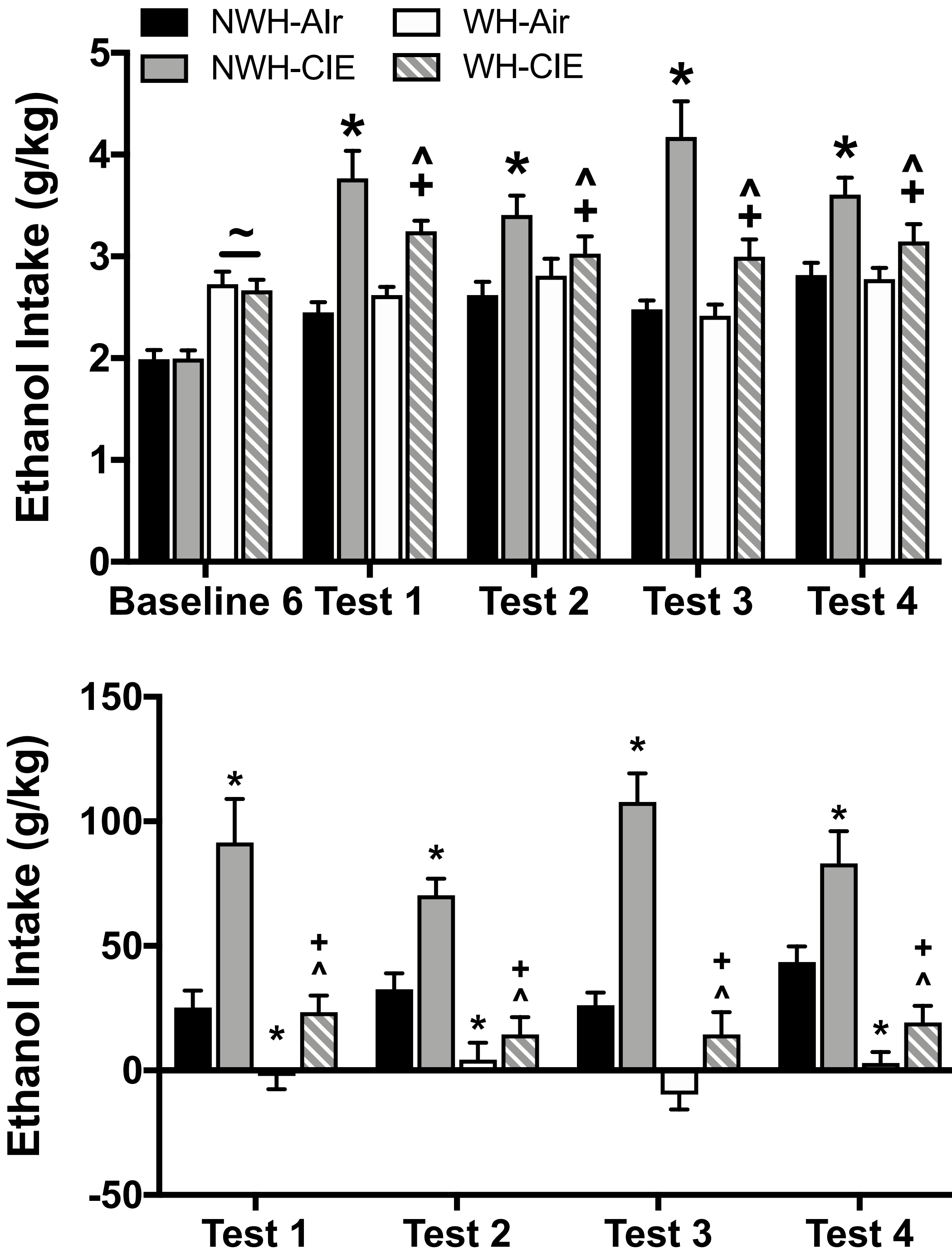

Figure 3.6 - Experiment 3: Test Alcohol Drinking.
Average weekly intake in **A)** g/kg, Group x Exercise interaction ($p<0.001$) and **B)** percent change from baseline, Group x Exercise interaction ($p<0.002$). Values are mean ± s.e.m. (N=9-11/group). Significantly differs from NWH-Air group (*p<0.005), from NWH-CIE (+p<0.005), from WH-Air (^p<0.005), main effect of Exercise (~p<0.005).

Exercise Blocks CIE-induced Reductions of *Bdnf* mRNA in mPFC

To determine the effects of exercise on *Bdnf* mRNA expression in mPFC, separate cohorts of mice were treated as described above, but they were sacrificed at the end of Baseline, immediately following the 4th CIE/Air exposure cycle, or the end of the 4th Test week (see Figure 3.1). *Bdnf* exon I, IV, and IX mRNA were analyzed separately at each time point by ANOVA.

Analysis of *Bdnf* mRNA expression in mPFC tissue 24-hr after the final 2-hr running session during Baseline conditions (Figure 3.7A) indicated that exercise produced significant increases in *Bdnf* exon I [$F(1,18) = 8.59$, $p<0.009$] and exon IV mRNA [$F(1, 18) = 4.89$, $p<0.05$], with a trend toward an increase in exon IX expression [$F(1, 18) = 3.75$, $p=0.07$] compared to sedentary (No Wheel) mice.

A cohort of mice exposed to 4 cycles of CIE or Air vapor, with or without exercise was sacrificed immediately out of chambers at the end of their last exposure cycle. *Bdnf* mRNA was analyzed by qPCR (Figure 3.7B) and demonstrated the ability of wheel running to increase *Bdnf* mRNA and prevent reductions induced by CIE exposure. Two-way ANOVA shows a main effect of Group for exon I [$F(1,33) =7.12$, $p<0.012$], exon IV [$F(1,35) =10.73$, $p<0.002$], and exon IX [$F(1,34) =18.98$, $p<0.0005$]. In addition there was a main effect of Exercise exon I [$F(1,33) =11.65$, $p<0.002$], exon IV [$F(1,35) =13.68$, $p<0.001$], and exon IX [$F(1,34) =17.36$, $p<0.0005$]. Although there were no significant Group x Exercise interactions for either exon I [$F(1,33) =1.33$, $p=0.26$], exon IV [$F(1,35) =0.18$, $p=0.69$], or exon IX [$F(1,35) =0.04$, $p=0.84$] this data does indicates exercise is capable of mitigating reductions in mPFC *Bdnf* mRNA caused by repeated cycles of chronic alcohol vapor.

Following a 4th Test week Exercise increased *Bdnf* exon I mRNA (Figure 3.7C) indicated by ANOVA. Analysis revealed a main effect of Exercise [F(1,33) = 17.67, p<0.005] and a trend toward a Group x Exercise interaction [F(1,33) = 3.74, p=0.062]. Based on the trend for the interaction and a visual inspection of the graph, post-hoc comparisons were performed. Post-hoc comparisons reveal a significant difference between CIE groups in the Exercise condition (NWH-CIE<WH-CIE, p<0.005) and between nonrunning mice in the Group condition (NWH-CIE<NWH-Air, p<0.04). The factor Group was not significant [F(1,33) =1.59, p=0.22]. Taken together these data indicate CIE exposure can produce prolonged reductions of exon I mRNA which are reversed by exercise.

Exon IV showed no Group x Exercise interaction [F(1, 35) =3.14, p=0.09], main effect of Group [F(1, 35) =0.054, p=0.82] or main effect of Exercise [F(1, 35) =1.34, p=0.26] on exon IV mRNA levels.

Exon IX mRNA displayed a recovery in expression following CIE exposure. Both NWH- and WH-CIE groups demonstrated significant increase in expression increase in expression of CIE exposed mice, indicated by a between-subjects main effect of CIE [F(1, 32) =6.59, p<0.02].

After a 4th Test week, qRT-PCR analysis evidenced wheel running increased *Bdnf* exon I indicated by a main effect of Exercise [F(1,33) = 17.67, p<0.005] (Figure 3.7C). While no main effect of Group emerged [F(1,33) =1.58, p=0.22] trend toward a Group x Exercise interaction was detected [F(1,33) = 3.74, p=0.062].

Based on the data and the trend toward an interaction, post-hoc comparisons were performed, revealing alcohol dependent sedentary mice had lower *Bdnf* exon I mRNA expression than alcohol dependent mice that exercised (NWH-CIE < WH-CIE; p<0.005)

and air exposed sedentary mice (NWH-CIE < NWH-Air; $p<0.04$). This data indicates wheel running can reverse CIE-induced reductions of exon I in the mPFC.

Analysis of *Bdnf* exon IV mRNA levels showed no effect of Group [$F(1, 35) = 0.054$, $p=0.818$], Exercise [$F(1, 35) = 1.336$, $p=0.256$] or Group x Exercise interaction [$F(1, 35) = 3.139$, $p=0.085$]. Exon IX mRNA displayed a recovery in expression following CIE exposure, regardless of access to a running wheel, indicated by a main effect of CIE [$F(1, 32) = 6.59$, $p<0.02$]. No effect of Exercise [$F(1,32) = 0.86$, $p=0.36$] or Group x Exercise interaction [$F(1,32) = 0.075$, $p=0.79$] were observed.

These results replicate previous findings validating the ability of wheel running to elevate *Bdnf* mRNA in the mPFC after several weeks of wheel access, while also demonstrating the ability of wheel running to protect against CIE-induced reductions in *Bdnf* mRNA in the mPFC.

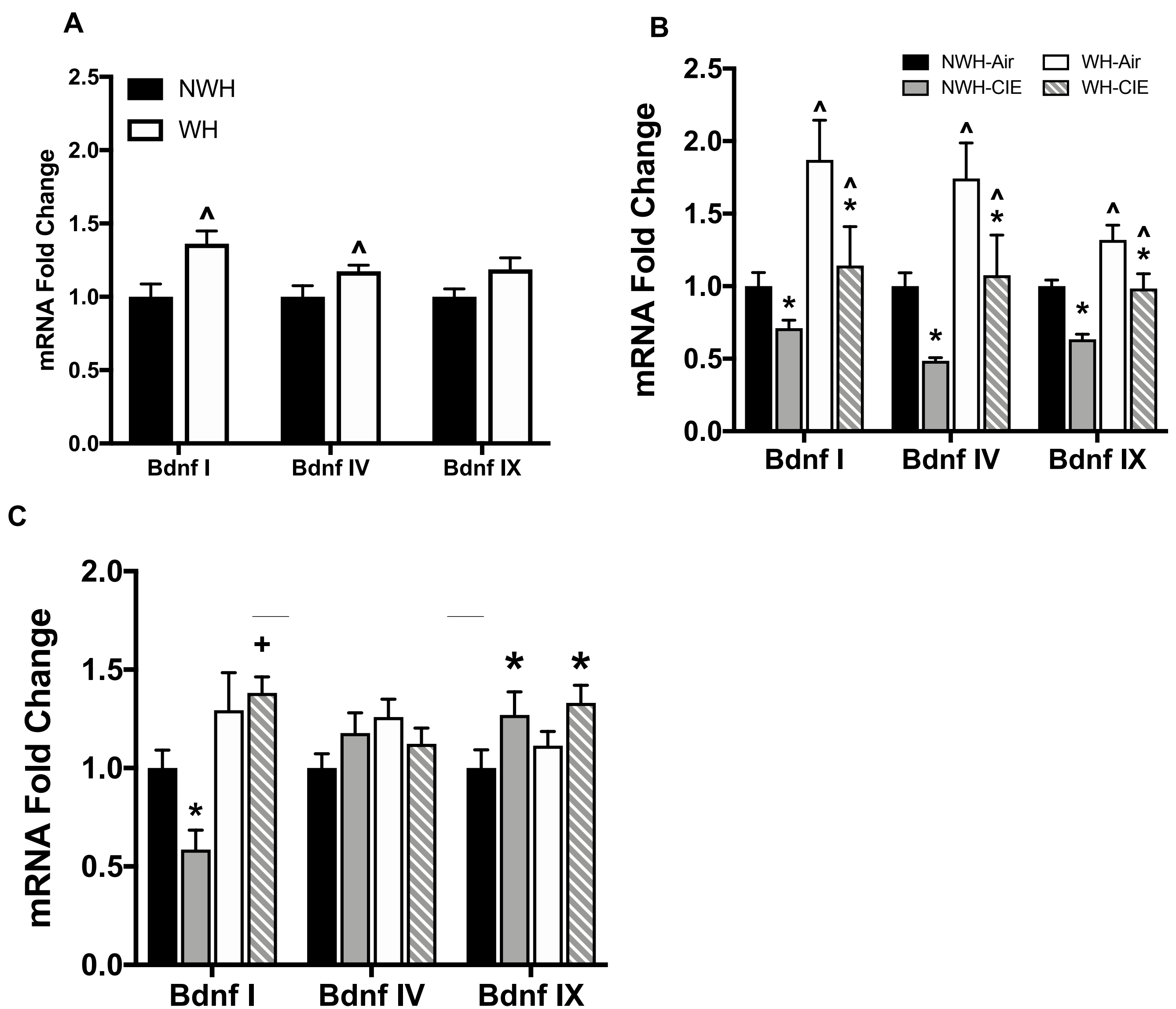

Figure 3.7 - Experiment 3: *Bdnf* mRNA Expression in the mPFC.
Bdnf mRNA fold change in exercising (WH) and sedentary (NWH) mice exposed to Air or CIE vapor at **(A)** the end of Baseline **(B)** immediately out of a 4th CIE/Air exposure cycle (0-hr) and **(C)** 24-hr after a 4th Test drinking week. Values are mean ± s.e.m. Expression fold change relative to NWH-Air group. Main effect of Exercise (^p<0.05); main effect of Group (*p<0.05), compared to NWH-CIE (+p<0.05), compared to NWH-Air (#p<0.05). N=7-10/group.

Discussion

Exercise is being used as an adjunct strategy in the treatment of drug and alcohol abuse and addiction (138, 139, 234-236). In this chapter we explored the ability of exercise, in the form of limited access voluntary wheel running, to attenuate the development of alcohol dependent-related escalated alcohol intake. We replicated finding from Chapter 2 showing 2-hr/day wheel access caused stable levels of running over several weeks reduced body weight, increased baseline levels of alcohol intake, and increased *Bdnf* mRNA in the mPFC of mice. We further show the ability of exercise (wheel running) to attenuate the escalation of alcohol intake and prevent reductions of *Bdnf* mRNA in the mPFC induced by an alcohol dependence model.

With only three FDA approved medications, alternative targets and therapies for the treatment of AUD are needed. One possible target is the BDNF-TrkB system as data shows an inverse relationship between BDNF-TrkB signaling and alcohol intake (299-303). CIE-exposed mice have decreased *Bdnf* mRNA and protein expression in the mPFC and increasing BDNF in this region blocked escalated alcohol drinking (174, 178, 326). Exercise has multiple health benefits including naturally increasing BDNF expression (137, 340, 349). Making the BDNF-TrkB system a reasonable target and exercise a potential therapeutic for alcohol dependence.

Mice exposed to repeated cycles of CIE vapor escalate their voluntary alcohol intake compared to their Baseline and compared to Air-exposed control mice (154, 157, 172, 174, 178, 326). Here, we demonstrated wheel running could blunt the escalation of alcohol intake, even after multiple CIE exposures. Replicating results from Chapter 2, running mice, even though they weighed less, consumed not only greater amounts of alcohol (ethanol; g/kg) but also drank a larger volume (mL). Due to these differences in alcohol

intake data was expressed as a percent change from Baseline intake. This allows for the assessment of how exercise affects the escalation of alcohol intake. Adjusting for Baseline differences, nonrunning mice exposed to chronic alcohol vapor robustly escalated their alcohol intake during each Test week. This effect was severely attenuated in mice with access to a running wheel. To our knowledge, this is the first-time exercise has been shown to reduced alcohol drinking in a rodent model of dependence.

Current data available describing the effects of exercise on alcohol drinking behavior in rodents is sparse, with most work focusing on nondependent drinking (136, 223, 235, 487). In this limited literature results have been mixed with increases, decreases, and no changes in alcohol consumption being detected. The different results are most likely due to variations in methodologies, species, and strains.

Our results indicate that exercise provides a protective mechanism to treat alcohol dependence related drinking by attenuating the escalation in alcohol intake. No data is currently available in regard to the effects of exercise on alcohol dependence related drinking however, data exists for other drugs of abuse. These studies provide evidence that wheel running will reduce self-administration of nicotine, methamphetamine, cocaine, and opioids (231, 236-243). Not only did aerobic exercise reduce drug intake but resistance training in rats decreased cocaine and heroin self-administration (242, 243). Human literature provides further support/confirmation of exercise's potential to treat AUD. Detoxified alcoholics who participated in low or moderate intensity exercise had a reduced urge to drink and when they did drink, they consumed less alcohol (228, 229). Similar to our results in mice, nondependent, adult humans who exercise consume greater levels alcohol than those that do not but also display a blunting in the development of alcohol abuse and dependence (240-243, 245-247). The increase in alcohol drinking at baseline

in exercising mice may create a ceiling effect, obscuring any escalations in alcohol intake. Additionally, for better comparisons between groups data should be adjusted to a percent change from baseline, to account for these baseline differences.

Evidence establishes an inverse relationship between *Bdnf* mRNA and BDNF protein levels in the mPFC and alcohol drinking behavior (174, 178, 325, 486). Our lab has shown a reduction in BDNF in the mPFC after repeated cycles of CIE exposure which can be blocked by replenishing or over-expression BDNF (mRNA and protein) in this region (174, 178, 486). Here, we replicated these finding demonstrating repeated cycles of CIE-exposure reduces *Bdnf* mRNA expression in the mPFC (174, 178, 486) and running at baseline (nondependent) increased expression in this same region (see Chapter 2). Excitingly, in addition to exercise's effect on the attenuation of escalated alcohol intake, we show mice that exercise can prevent reductions of *Bdnf* mRNA in the mPFC caused by heavy alcohol exposure. Thus, indicating a mechanism in which exercise functions in the treatment and prevention of AUD. Increased *Bdnf* mRNA, as a result of exercise, prior to heavy alcohol exposure, may function as a buffer to prevent or delay the development of AUD, thereby acting as a protective mechanism. Additionally, exercise after heavy alcohol exposure may serve to speed the rate of BDNF production to recover lost BDNF and begin to counteract the negative effects alcohol has on neuronal function and health.

One limitation to consider is BDNF protein was not analyzed in these samples due to lack of tissue being available. While we demonstrated in Chapter 2 that exercise increased both *Bdnf* mRNA and BDNF protein in the mPFC, and it is established that CIE exposure reduces both *Bdnf* mRNA and BDNF protein (174, 178, 486) we do not know if *Bdnf* mRNA and BDNF protein levels correlate in wheel running CIE exposure mice.

Evidence exists that BDNF protein can be trafficked to the mPFC from the HPC (492) and BDNF originating in the mPFC can be trafficked to the NAc (267, 268), thus *Bdnf* mRNA expression measured in the mPFC may not directly correlate with protein expression in this region depending on trafficking.

Occurring predominately in the early portion of the later Test weeks, repeated cycles of alcohol vapor exposure caused mice to run less than those exposed to air. Running activity progressively recovered throughout the week. Although we demonstrated mice which exercise attenuated alcohol intake after alcohol vapor exposure, it is unknown whether a history of wheel running is necessary or if acute exercise is sufficient to blunt intake during Test weeks. In Chapter 2 we replicated demonstrating 2-days of 2-hr running, after a period of inactivity, was able to rapidly elevate *Bdnf* mRNA and protein in these mice with a history of exercise (137). If the beneficial effect of exercise is through an acute increase of BDNF expression, proximal to CIE exposure, it may be critical for mice to have a "primed" BDNF system (137), allowing for rapid increases in BDNF expression. That is, does a history of running and BDNF elevation protect against alcohol dependence and how long does this effect last with repeated cycles of CIE? Or is it necessary for exercise to be introduced immediately after a heavy alcohol exposure in order to attenuate CIE-induced increases in alcohol intake?

The reduction in wheel activity is most likely due to early withdrawal effects of CIE vapor exposure. Acute withdrawal from heavy alcohol exposure produces negative affective states that include anxiety, depression, and anhedonia which will drive craving and relapse (11, 66-68). As with a human suffering from negative physical withdrawal effects, mice will be less likely and motivated to participate in physical activity. If a minimum amount of exercise needs to be performed after CIE exposure to increase *Bdnf*

mRNA expression, deficits in running activity early in the Test week may be detrimental to the beneficial effects of exercise.

Additionally, exercise prevents CIE from decreasing *Bdnf* mRNA in the mPFC and engenders an increase in *Bdnf* mRNA to prior to alcohol vapor exposure levels. Thus, providing a potential mechanism for exercise to exert its benefits in the treatment of AUD. In this Chapter we add to previously published findings showing CIE exposure, with interleaved alcohol drinking Test weeks, caused reductions of *Bdnf* mRNA in the mPFC of mice (178, 486). Here, it is revealed that wheel access prior to CIE-exposure prevented significant reductions in *Bdnf* mRNA in the mPFC and goes further to demonstrate wheel access during Test weeks will re-elevate *Bdnf* mRNA in this region and that these mice do not show as robust of an escalation of alcohol intake after CIE exposure. This information taken with published data revealing reduced *Bdnf* mRNA and protein, specifically in the mPFC, can lead to increased alcohol intake and that preventing these reductions can treat escalated alcohol intake (174) indicates a potential mechanism for exercise to attenuate CIE-induced escalated alcohol drinking. Here, we describe a role of exercise to prevent reductions in BDNF caused by alcohol vapor exposure and to attenuate escalated alcohol drinking, suggesting that wheel running can prevent increased drinking through elevations in BDNF.

In summary, mice that exercise display an attenuated response to chronic alcohol vapor exposure, mitigating the effects on reducing *Bdnf* mRNA in the mPFC and blunting the escalation of alcohol intake.

Chapter 4 - Role of BDNF-TrkB Signaling in the Effects of Exercise (Wheel-Running) on Alcohol Drinking in Dependent and Nondependent Mice

Introduction

Repeated bouts of heavy alcohol use and abstinence can lead to alcohol dependence, which is characterized by dysregulation of multiple neurotransmitter and neuropeptide systems in the brain (43, 44, 493, 494). Targeting these systems pharmacologically, to restore balance and function, is a means of treating excessive alcohol consumption. Unfortunately, there are only two FDA-approved medications (naltrexone and acamprosate) that target such adaptations (the third medication, disulfiram, influences alcohol metabolism) [51, 55, 77, 174, 180, 183]. As these treatments are not sufficiently effective (29-31), new targets for the treatment of alcohol dependence are being explored. One potential target is brain-derived neurotrophic factor (BDNF), as reduced BDNF expression in brain has been shown to be associated with alcohol dependence and increased alcohol drinking (318, 319).

As previously described, BDNF is a member of the neurotrophin family that binds predominantly to TrkB receptors [260]. A complex gene structure comprised of nine different exons, with exon IX coding for the BDNF protein and the other eight exons being differentially spliced to exon IX acting as promoter sequences (257). Through down-stream signaling, BDNF regulates growth, differentiation, and maintenance of neurons [246]. Roles in complex behaviors such as learning and memory [246, 247], neuropsychiatric disorders, and addiction [203-208]. Binding with its primary receptor target, tropomyosine-related kinase-B (TrkB) receptors, BDNF causes dimerization of the receptor and activation of one of three signaling cascades: PI3K, PLC-gamma, or the MAP kinase pathway (272). Global inhibition of brain TrkB activity is associated with elevated

alcohol consumption (178, 317, 320, 326, 486) and direct infusion of BDNF into this region blocks dependence-related escalated alcohol intake (174). Additionally, viral-mediated over-expression of BDNF in the mPFC prevented the emergence of excessive drinking in a model of alcohol dependence (174). Collectively, these data provide a strong basis for targeting the BDNF system in the treatment of alcohol dependence and excessive drinking.

It is well established that aerobic exercise increases *Bdnf* mRNA and BDNF protein expression in the brain (136-139, 221). Participation in exercise caused adolescents and young adults to use less drugs and alcohol and reduced the urge to drink and the amount consumed in detoxified alcoholics (231, 239-243, 247). In Chapter 3, we demonstrated that daily scheduled aerobic exercise (wheel-running) in the home-cage attenuated escalated drinking in mice following repeated cycles of chronic intermittent ethanol (CIE) exposure. Further, we showed exercise prevented CIE-induced reductions in *Bdnf* mRNA levels in the mPFC. This evidence demonstrates BDNF in the mPFC regulates alcohol drinking, suggests that increased BNDF-TrkB receptor signaling may be an important link between the effects of exercise and dependence-related excessive drinking.

In this chapter we aimed to examine this possibility by using a pharmacological antagonist of TrkB receptors. determine if voluntary wheel running attenuates CIE-induced escalated alcohol intake through BDNF-TrkB signaling. Specifically, we tested the hypothesis that TrkB antagonism with ANA-12 (495) would prevent or reduce the ability of exercise (wheel-running) to attenuate CIE-induced escalated alcohol drinking.

Methods

Subjects

Adult male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were individually housed under a 12-hr light/dark cycle (lights on at 8:00 AM). Mice were provided with food (Teklad rodent diet) and water ad libitum in a temperature and humidity controlled AAALAC-accredited facility at the Medical University of South Carolina. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental Design and Procedure

The study was performed in a balanced design of 2 (Exercise: Wheel, No-Wheel) x 2 (Group: CIE, Air) x 3 (Drug: Vehicle (1% DMSO in PBS), 0.5 mg/kg ANA-12, 1.0mg/kg ANA-12) (N= 9-11/group). During a 6-week Baseline period, mice received home-cage access to 1 bottle of alcohol (15% v/v ethanol) and 1 bottle of water, for 2-hr, starting 3-hr into the dark cycle. Starting 1-hr after the drinking session, some mice (WH groups) were given access to an activity wheel for 2-hr in their home cage. The remaining mice (NWH groups) were left undisturbed during this exercise period. The 2-hr alcohol drinking sessions occurred Monday – Friday each week and scheduled access to activity wheels was provided 7 days/week. Following the Baseline period, mice received CIE or Air exposure (16-hr/day for 4 days) in inhalation chambers, as described below. During CIE/Air inhalation exposure, alcohol drinking and exercise were not available to mice. Daily wheel-running (2-hr) resumed 24-hr after the final day of chamber exposure and alcohol drinking sessions (2-hr) commenced 72-hr after CIE/Air exposure for a 5-day test period (Test 1). Mice then received a 2nd CIE/Air exposure cycle before being were separated into three drug treatment groups (Vehicle, 0.5 mg/kg, or 1.0 mg/kg ANA-12).

During Test 2, ANA-12 (0.5 mg/kg or 1.0 mg/kg) or Vehicle was injected intraperitoneally (ip.) immediately following the 2-hr wheel-running sessions. Treatment started following the first day exercise commenced after the 2nd CIE/Air exposure cycle and continued for 6 days into Test 2 (Figure 4.1).

Chronic Intermittent Ethanol (CIE) Exposure

Mice were exposed to chronic intermittent ethanol vapor or air in inhalation chambers, as previously described (154, 157, 167). Prior to each day of ethanol vapor exposure, mice were administered 1.6 g/kg ethanol with 1 mmol/kg pyrazole in saline, to stabilize blood ethanol concentration (BEC). Air-exposed mice received only pyrazole in saline. Ethanol and pyrazole were administered intraperitoneally (ip.) in a volume of 0.02 mL/g body weight. Using an air stone, 95% ethanol was vaporized, mixed with fresh air, and delivered to the alcohol chambers. Air alone was delivered to the air chambers. The concentration of alcohol in the chambers were monitored daily, air and alcohol flow rates were adjusted to maintain BECs in the 200-250 mg/dl range throughout cycles of exposure (BECs were determined as previously described).

ANA-12 Preparation

The TrkB antagonist, ANA-12 (Selleckchem Cat no S7745), was resuspended in 1% (DMSO) in phosphate-buffered saline (PBS) (Sigma Aldrich) at 0.1 mg/mL for 1 mg/kg and 0.05 mg/mL for 0.5 mg/kg doses. ANA-12 (and vehicle) was administered intraperitoneally (ip.) in a volume of 0.01 mL/g body weight.

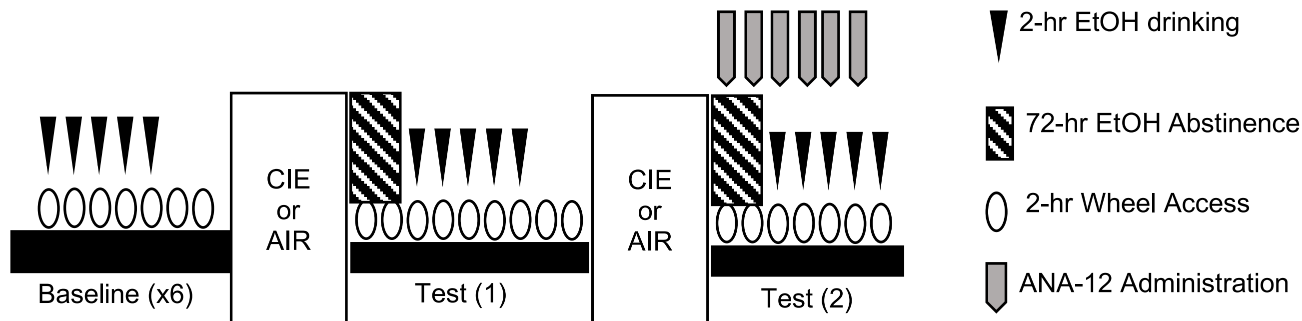

Figure 4.1 - Schematic of Experiment 4.
2 (Exercise: Wheel, No-Wheel) x 2 (Group: CIE, Air) x 3 (Drug: Vehicle (1% DMSO in PBS), 0.5 mg/kg ANA-12, 1.0mg/kg ANA-12) (N= 9-11/group). All mice had 6 weeks of Baseline Alcohol (15% v/v ethanol) drinking period. Exercise mice (WH-CIE, WH-Air) had 2-hr wheel access 1-hr after the drinking session. Following Baseline mice were exposed to alcohol vapor (CIE) or air (Air) 16-hr/day x 4 days. During Test 2 mice were treated ip. with ANA-12 (0, 0.5, or 1.0 mg/kg) immediately after wheel running.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 25 with significance set at $p < 0.05$. Average running distance (km) over the last 7 days of Baseline was analyzed by one-way ANOVA with Group as the main factor. Weekly body weight (grams) was analyzed by Repeated Measures ANOVA, with Exercise as the between-subjects factor and Week as a within-subjects factor. Likewise, average weekly alcohol intake (g/kg) during Baseline was analyzed by 2-way ANOVA (Exercise x Week). Average alcohol intake during Test 1 (expressed as g/kg and percent change from baseline) was analyzed by ANOVA, with Exercise and Group as between-subject factors. Similarly, alcohol intake during Test 2 was analyzed by ANOVA, with Drug as an additional factor. Pairwise comparisons with Bonferonni corrections were performed when appropriate. During the Test weeks the average distance (km) mice ran was analyzed by repeated measures ANOVA with CIE as the main factor. Weekly body weight (grams) was analyzed by Repeated Measures ANOVA, using CIE and Group as the between-subjects factor and Week as within-subjects factor. When applicable pairwise comparisons with Bonferonni corrections were performed. Two-way ANOVA was performed on Baseline and Test 1 alcohol intake (g/kg and percent change). Between-subject factors were Wheel Running and CIE/Air exposure with average weekly alcohol intake (g/kg) or percent change as the independent variable. In Test 2 ANOVA was performed on alcohol drinking (g/kg and percent change) with the Drug added as a between-subject factor. Pairwise comparisons with Bonferonni corrections were performed when applicable.

Results

During the 6-week Baseline period, wheel-running behavior stabilized for all mice and ANOVA revealed no significant difference in running distance between WH-CIE and WH-Air groups over the last 7 days of Baseline (prior to inhalation treatment) [$F(1,66)= 3.41$, $p= 0.07$] (Figure 4.2).

As previously reported (Chapter 2 Figure 2.3 & 2.6, Chapter 3 Figure 3.3), mice with access to activity wheels gained less weight than those not given an opportunity to exercise over the Baseline period (Figure 4.3). ANOVA indicated a significant main effect of Exercise [$F(1,113)= 5.57$, $p< 0.02$], and a significant Exercise x Week interaction [$F(6,68)= 23.10$, $p< 0.005$]. Pairwise comparisons showed WH mice weighed significantly less than NWH mice during Weeks 2-6 (ps< 0.05).

During Baseline, wheel-running mice displayed elevated alcohol consumption compared to mice that did not have access to activity wheels (Figure 4.4). This was supported by ANOVA, which indicated a significant main effect of Exercise [$F(1,111)= 27.17$, $p< 0.005$] and a significant Exercise x Week interaction [$F(5,565)= 23.58$, $p< 0.005$]. Pairwise comparisons indicated significant differences between Groups during Week 1 ($p< 0.02$) with the Exercise group consuming slightly less alcohol than sedentary mice and Week 3-6 (ps< 0.005) where exercise modestly increased alcohol intake. As demonstrated in Chapters 2 and 3, the modest increase in alcohol intake was also seen when milliliters of alcohol consumed was analyzed (data not shown).

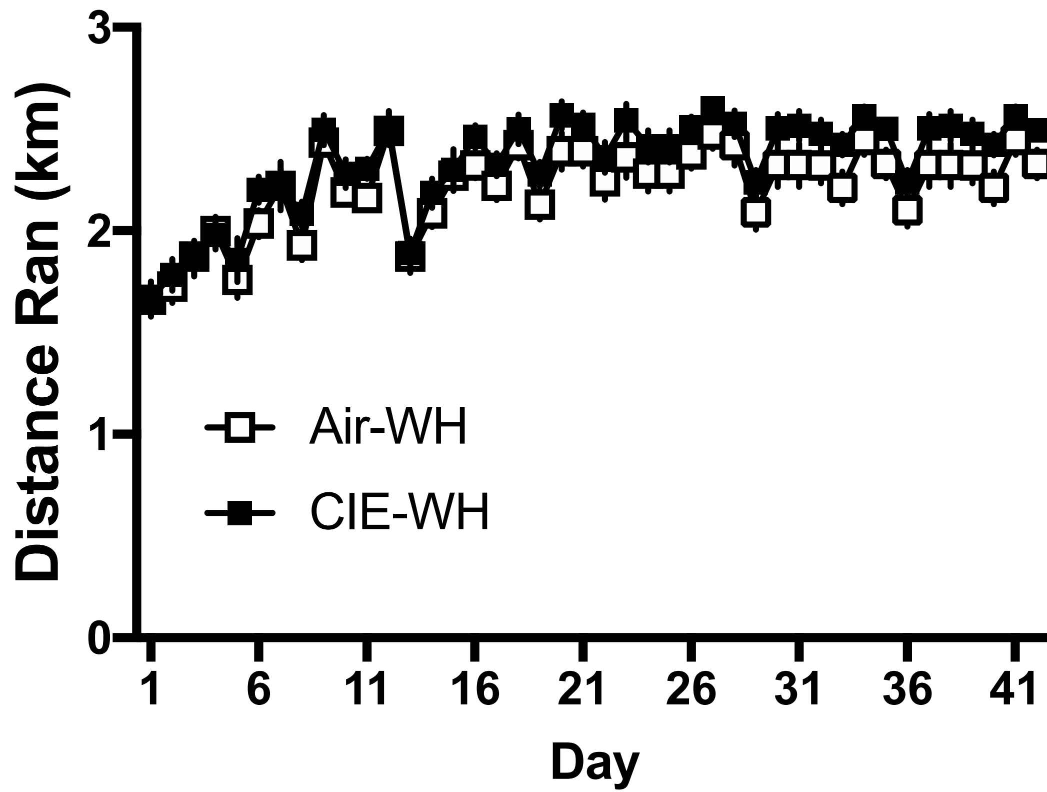

Figure 4.2 - Experiment 4: Daily Baseline Wheel Running.
Average daily running (km) at Baseline for mice to be exposed to Air or CIE vapor. No differences between groups at Baseline over the last 7 days of running. Values are mean ± s.e.m (N=27-29/group).

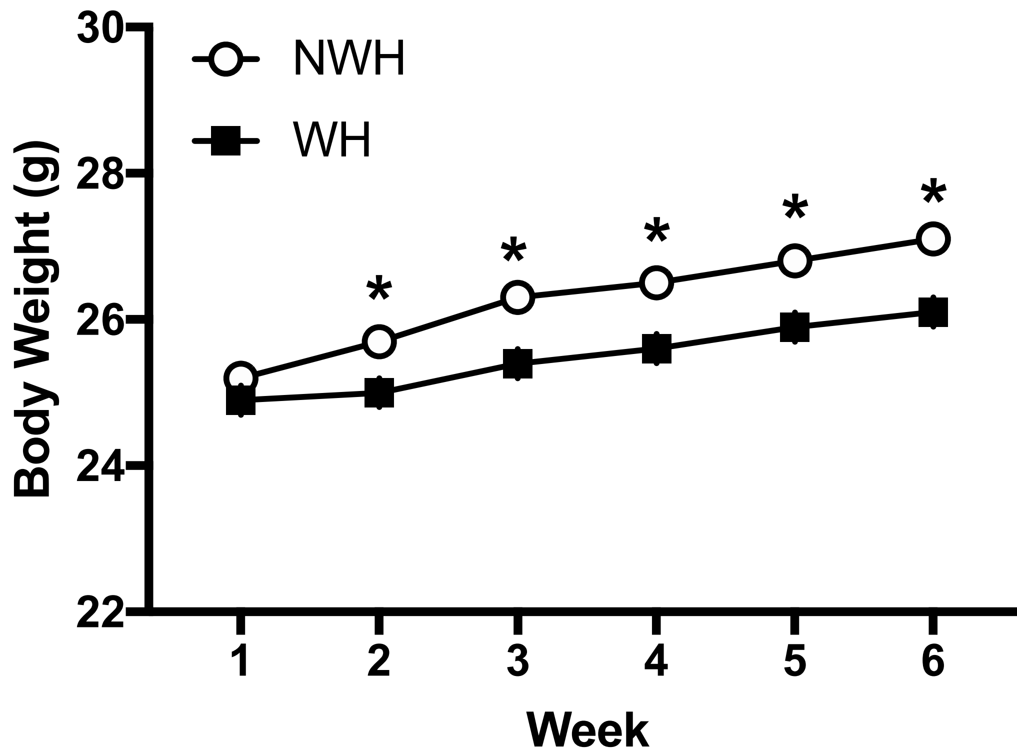

Figure 4.3 - Experiment 4: Weekly Baseline Body Weights.
Exercise (WH) resulted in lower body weight compared to sedentary mice (NWH). main effect of Exercise ($p<0.003$). Values are mean ± s.e.m. (N=55-56/group). Significantly differs from NWH (*$p<0.05$).

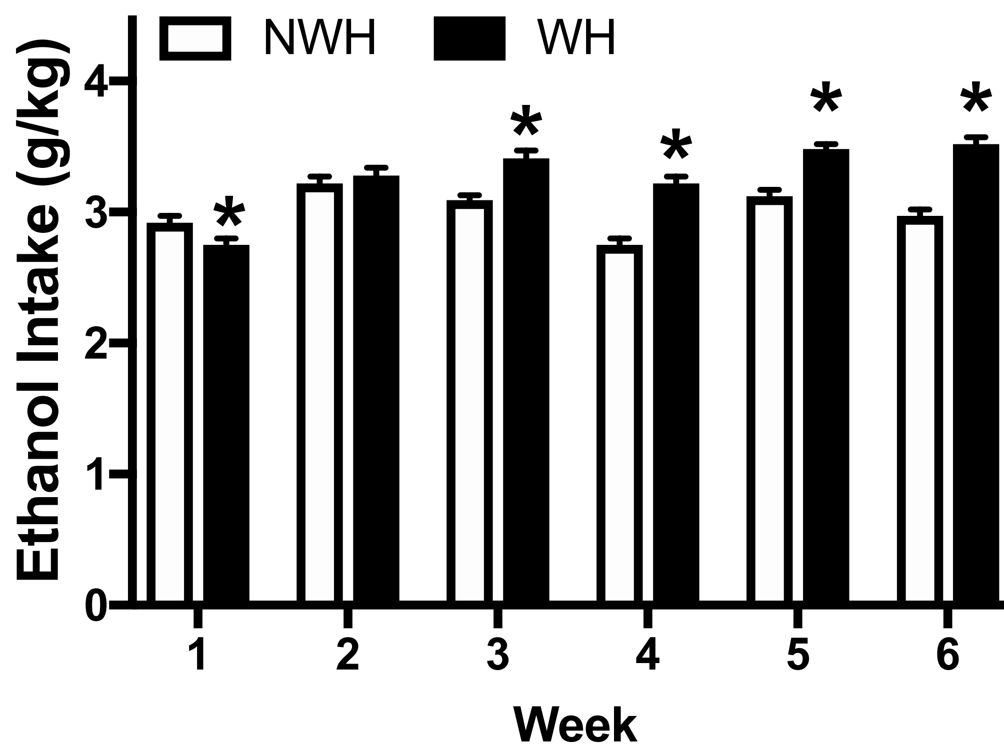

Figure 4.4 – Experiment 4: Baseline Alcohol Intake.
Baseline, weekly average alcohol intake (g/kg) in exercising (WH) and sedentary (NWH) mice. Exercise x Week Interaction ($p<0.005$) and main effect of Exercise ($p<0.01$). Values are mean ± s.e.m. (N =55-56/group). Differs from NWH (*$p<0.05$).

Exercise Attenuates Escalated Alcohol Intake

Analysis of alcohol intake (g/kg) during Test 1 indicated a significant Exercise x Group interaction [$F(1,111)= 7.65$, $p< 0.007$] (Figure 4.5A). Pairwise comparisons indicated all groups (WH-Air, NWH-CIE, WH-CIE) evidenced higher alcohol intake compared to the NWH-Air group ($ps< 0.05$). Main effects of Group [$F(1,111)= 33.24$, $p<0.0005$] and Exercise [$F(1,111)= 12.8$, $p<0.001$] we evidenced. Because exercise altered alcohol intake during Baseline (Figure 4.4), alcohol intake data were expressed as a percent change from baseline (Figure 4.5B), to determine how drinking behavior changed relative to each animal's individual baseline. Analysis of percent change data revealed significant main effects of Exercise [$F(1,111)= 12.80$, $p< 0.005$] and Group [$F(1,111)= 33.24$, $p< 0.005$]. Although Group x Exercise interaction was not significant [$F(1,111)= 2.46$, $p=0.12$], alcohol intake was greater in NWH-CIE mice compared to NWH-Air mice (NWH-CIE > NWH-Air; $p<0.0005$) and exercise was able to significantly attenuate escalated intake (WH-CIE < NWH-CIE; $p< 0.05$). As such, these results replicate findings observed in Chapter 3, demonstrating the ability of scheduled daily exercise to attenuate CIE-induced escalated alcohol drinking.

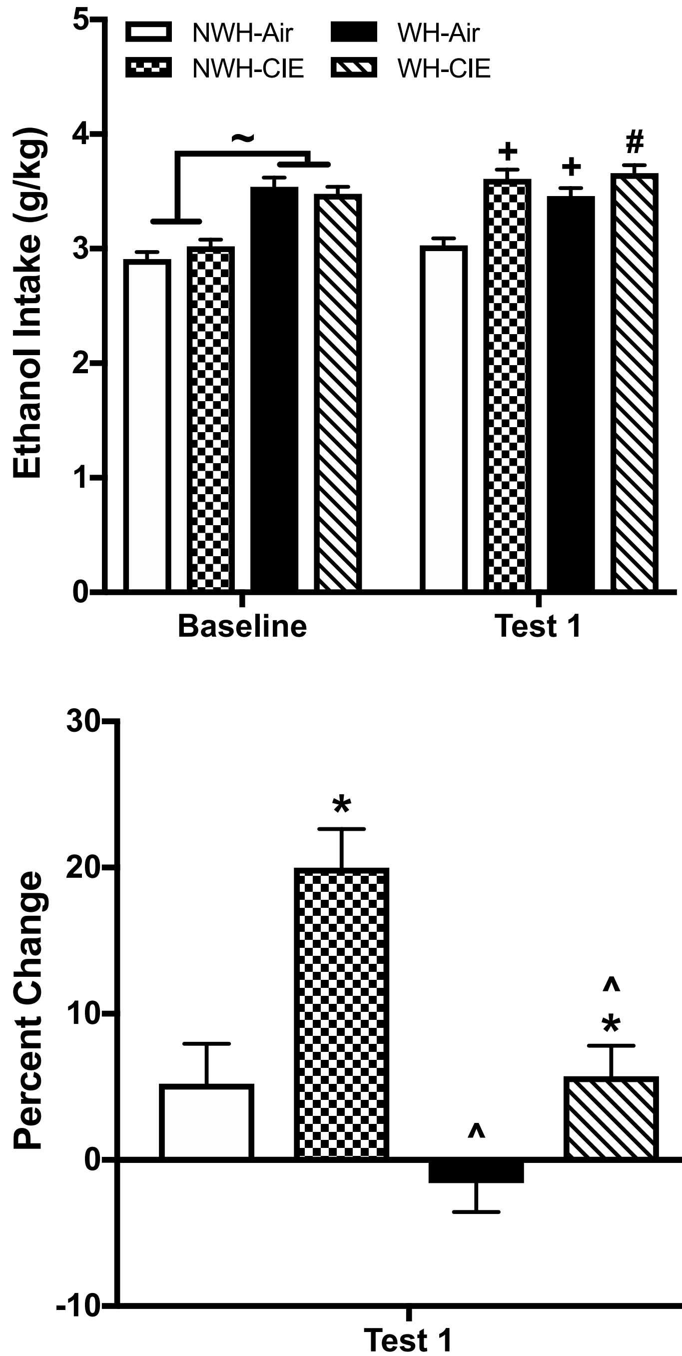

Figure 4.5 – Experiment 4: Test 1 Average Alcohol Intake
(**A**) g/kg intake at end of Baseline and Test 1. Baseline: main effect of Exercise (~$p<0.05$); Test 1 Group x Exercise interaction ($p<0.007$). Significant compared to NWH-Air (+$p<0.005$), compared to WH-Air (#$p<0.05$). (**B**) Percent change alcohol intake from Baseline. Main effect Group (*$p<0.005$) and main effect of Exercise (^$p<0.005$). Values are mean $\pm$ s.e.m. (N= 27-29/group).

TrkB Antagonism Blocks the Attenuation of Escalated Alcohol Intake by Exercise

Analysis of alcohol intake (g/kg) during Test 2 indicated a trend toward an effect of Exercise [$F(1,103)= 3.52$, $p=0.64$], a significant main effect of Group (CIE > Air) [$F(1,103)= 73.24$, $p< 0.0005$], and a Group x Exercise x Drug interaction [$F(2,103)= 3.161$, $p< 0.05$]. Pairwise comparisons of the interaction show the expected CIE-induced significant increase in alcohol drinking in mice with no access to activity wheels (NWH-CIE > NWH-Air), and this effect was not altered by ANA-12 treatment (all doses: $ps< 0.001$) (Figure 4.6A). Replicating our previous findings (Chapter 3), exercise attenuated CIE-induced escalated drinking in vehicle-treated mice (NWH-CIE > WH-CIE = WH-Air = NWH-Air) ($p< 0.05$). However, this attenuating effect of wheel-running was blocked by ANA-12 treatment. That is, both doses of ANA-12 significantly increased alcohol intake in WH-CIE mice compared to WH-Air mice (0.5 mg/kg: $p< 0.0005$; 1.0 mg/kg: $p< 0.0005$). Furthermore, ANA-12 increased alcohol intake in WH-CIE mice compared to vehicle-treated WH-CIE mice (Veh vs 0.5mg/kg: $p< 0.016$; Veh vs 1.0mg/kg: $p< 0.013$). No main effect of Dose [$F(2,103)= 0.53$, $p= 0.59$] and interactions of Group x Exercise [$F(1,103)= 0.5$, $p= 0.48$], Group x Dose [$F(2,103)= 1.12$, $p= 0.33$] or Exercise x Dose [$F(2,103)= 1.4$, $p= 0.25$] were detected.

Alcohol consumption during Test 2 expressed as a percent change from baseline is shown in Figure 4.6B. Analysis of these data revealed main effects of Group [$F(1,103)= 59.1$, $p< 0.0005$] and Exercise [$F(1,103)= 27.57$, $p< 0.0005$], but no effect of Dose [$2,103)= 0.8$, $p= 0.45$]. Interactions were nonsignificant for Group x Exercise [$F(1,103)= 0.054$, $p= 0.82$], Group x Dose [$F(2,103)= 0.4$, $p= 0.67$] and Exercise x Dose [$F(2,103)= 2.18$, $p= 0.12$]. However, a significant Exercise x Group x Drug interaction [$F(2,103) = 3.56$, $p< 0.05$] was detected. Post-hoc analyses indicated that a history of CIE exposure significantly increased alcohol drinking in mice that did not have access to activity wheels

in their home-cage (NWH-CIE groups) compared to NWH-Air mice, and this was true for all ANA-12 treatment conditions (Veh: $p < 0.0005$; 0.5 mg/kg ANA-12: $p < 0.001$; 1.0 mg/kg ANA-12: $p < 0.04$). In vehicle-treated mice, wheel-running prevented CIE-induced escalation of alcohol consumption (WH-CIE < NWH-CIE , $p < 0.005$). Of note, this attenuating effect of exercise was reversed by ANA-12 treatment. That is, despite having access to activity wheels, ANA-12 treated mice showed CIE-induced elevated alcohol intake (Veh: NWH-CIE > WH-CIE , $p < 0.05$; 0.5 mg/kg ANA-12: NWH-CIE = WH-CIE, $p = 0.09$; 1.0 mg/kg ANA12: NWH-CIE = WH-CIE, $p = 0.83$). Additionally, alcohol intake was significantly greater in WH-CIE groups that received 0.5 or 1.0 mg/kg ANA-12 compared to WH-CIE mice injected with vehicle (ps< 0.05).

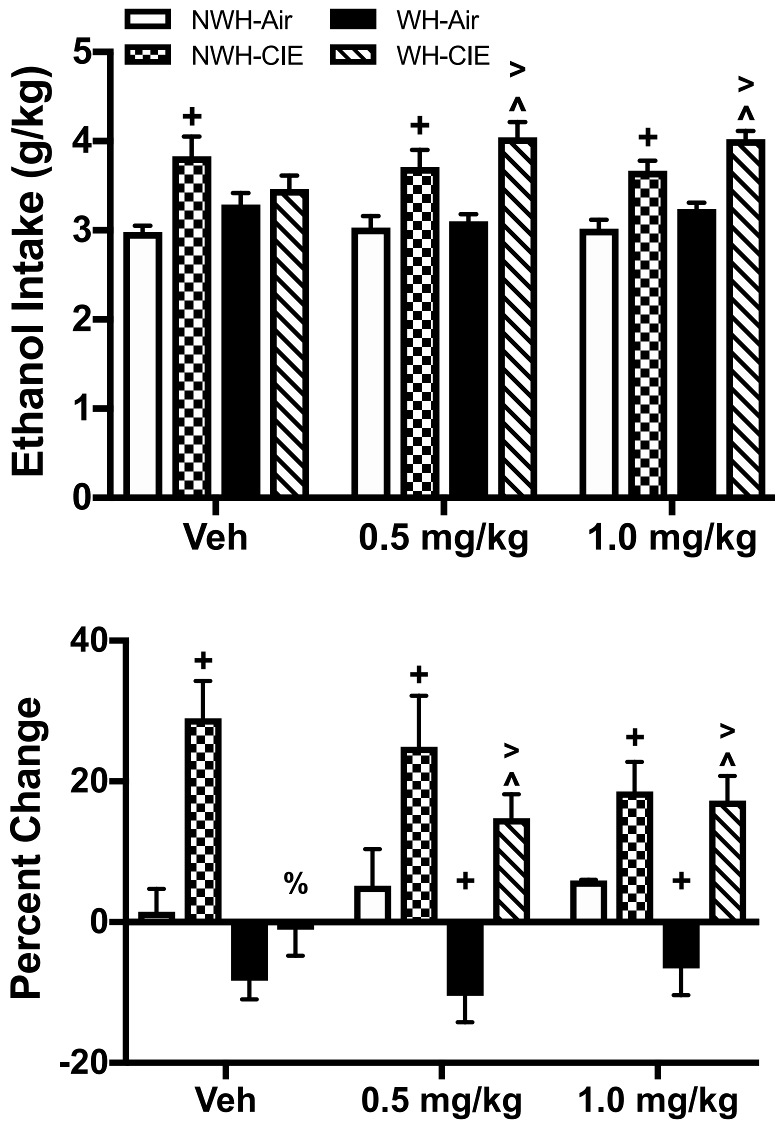

Figure 4.6 – Experiment 4: Test 2 Average Alcohol Intake
Test 2 average alcohol intake of CIE/Air exposed mice treated with vehicle or ANA-12 (0.5 or 1.0 mg/kg). (**A**) g/kg intake, Group x Exercise x Drug interaction ($p<0.05$). (**B**) Percent change alcohol intake from Baseline Group x Exercise x Drug interaction ($p<0.05$). Values are mean ± s.e.m. (N= 12-15/group). Significant compared to NWH-Air (+$p<0.005$), compared to WH-Air (^$p<0.005$), compared to NWH-CIE (%$p<0.05$), compared to Vehicle treated WH-CIE (>$p<0.05$).

Discussion

Exercise has shown efficacy in prolonging abstinence, reducing craving, and decreasing alcohol intake (138, 139, 231, 240-243, 267, 268). Using the CIE mouse model of alcohol dependence and relapse drinking, exercise was shown to attenuate escalated alcohol intake in dependent mice (Chapter 3). Further, daily scheduled limited access to activity wheels was shown to increase *Bdnf* mRNA (Chapters 2 and 3) and protein (Chapter 2) expression in the mPFC. Since CIE-induced elevated drinking is associated with reduced *Bdnf* mRNA and protein expression in the mPFC (174, 178, 325, 326), it was hypothesized that exercise may attenuate dependence-related excessive alcohol consumption by reversing reductions of BDNF activity in the mPFC following CIE exposure. To address this research question, a pharmacological antagonist of TrkB receptors, ANA-12, was administered in the model. Results from this study demonstrate that ANA-12 treatment blocked the ability of exercise (wheel-running) to reduce CIE-induced escalated alcohol drinking. As such, these results implicate a role for exercise-induced BDNF (possibly in the mPFC) in the reduction of alcohol intake in a model of alcohol dependence.

Mice with no opportunity to exercise showed the expected CIE-induced increased in alcohol drinking during Test 2, and this effect was not altered by ANA-12 treatment. Since CIE exposure reduces BDNF expression in brain, it may be that already depressed levels of BDNF masked any effects of ANA-12 in reducing BDNF-TrkB receptor signaling. In addition, ANA-12 treatment did not alter drinking in nondependent (NWH-Air) mice. It was previously shown that increasing BDNF levels in the mPFC (either by direct infusion or viral-mediated over-expression) did not alter drinking in nondependent mice but reduced intake in dependent animals (174). Thus, it may be that BNDF signaling in the

brain (mPFC) may be more effective in modulating alcohol consumption under conditions that engender excessive levels if intake. Support for this view comes from the fact that exercise does not appear to alter drinking in nondependent mice, and ANA-12 treatment did not affect alcohol drinking in nondependent exercising mice (WH-Air).

Confirming previous results obtained in Chapter 3, limited daily exercise (wheel-running) attenuated CIE-induced escalated drinking. Of significance, ANA-12 treatment blocked this effect. Both doses of ANA-12 tested were equally effective in reversing the ability of exercise to reduce CIE-induced excessive drinking This provides support for the hypothesis that exercise reduces CIE-induced excessive drinking by enhancing BDNF expression and signaling in brain. Studies have implicated mPFC BDNF activity in the regulation of alcohol dependent drinking mPFC (174, 178, 325).

While results from the present study demonstrate a potential role for BDNF-TrkB receptor signaling in the ability of exercise to modulate dependence-related drinking, one limitation to note is that the TrkB receptor antagonist was systemically administrated, thereby limiting the ability to establish if these effects are mediated in the mPFC. Future studies that directly manipulate BDNF-TrkB receptor signaling in the mPFC will help address this issue.

While the present study implicates a role for BDNF-TrkB receptor signaling in the effects of exercise on dependence-related alcohol drinking, the mechanism by which exercise modulates BDNF expression and activity in brain is not fully understood. Though BDNF can induce its own expression through TrkB receptor activation (272, 275), exercise can increase BDNF expression through two possible mechanisms, beta-hydroxybutyrate and irisin/FNDC5 (298, 299, 359, 367). Both of these mechanisms involve the metabolism of fat in the body to generate metabolites which function to stimulate the production of

BDNF in the brain. It has been suggested that exercise functions to prime the BDNF system (137), allowing for rapid increases in BDNF expression and TrkB signaling. This, in turn, may serve to mitigate deficits in BDNF activity that result from chronic alcohol (CIE) exposure and, thereby, temper excessive levels of drinking.

In summary, results from the present study demonstrate that exercise (wheel-running) is effective in attenuating escalated drinking associated with dependence. This effect was reversed by TrkB receptor antagonism. Taken together, these findings support a potential therapeutic effect of exercise in controlling alcohol consumption and implicate BDNF-TrkB receptor signaling as a target for the treatment of alcohol addiction.

Chapter 5 - General Discussion and Future Directions

Alcohol addiction is a health crisis affecting 16 million Americans every year, resulting in tens of thousands of deaths and injuries (1). The lack of fully efficacious pharmacological agents and treatments for alcohol use disorder (AUD) (30, 31) necessitates a strong need for additional pharmacological targets and therapeutic tools to improve treatment outcomes. Exercise is one such strategy showing promise in the treatment of neuropsychiatric conditions, including alcohol addiction (241-247).

An inverse correlation exists between alcohol consumption and brain-derived neurotrophic factor (BDNF) expression (mRNA and protein). Specifically, in the medial prefrontal cortex (mPFC), where reduction of BDNF expression and BDNF-TrkB signaling results in increased alcohol intake (174, 325). Elevating BDNF in the mPFC, by direct BDNF infusion or viral-overexpression, reversed or prevented escalated alcohol intake (174). Thus, demonstrating a role of prefrontal cortical BDNF in the treatment of alcohol use disorders.

Exercise naturally increases *Bdnf* mRNA and protein expression in the brain of rodents and in the blood of humans (136, 137, 221, 298, 341), making it a promising AUD treatment adjunct which can target the BDNF-TrkB system. Additionally, exercise can be easily implemented in a clinical or home setting and is of no monetary cost (walking, running, body weight exercise), making it accessible to all. And by understanding the mechanism by which exercise can exert its effects on attenuating dependence-related alcohol intake new pharmacological targets and substrates can be discovered and developed for the treatment of alcohol addiction.

In the previous chapters we tested the hypothesis that limited (2-hr) daily exercise (wheel running) could attenuate the escalation of alcohol drinking engendered by a model

of alcohol dependence. Data was provided demonstrating the ability of exercise to attenuate escalated alcohol intake in a mouse model of alcohol dependence (Chapter 3 & 4). Furthermore, this effect was shown to be mediated through increased BDNF (mRNA and protein) expression (Chapter 2 & 3) and BDNF-TrkB signaling (Chapter 4).

It was demonstrated (Chapter 2) that limited exercise for 4 or 6 weeks will increase *Bdnf* mRNA and protein expression in the mPFC, a region shown to regulate excessive alcohol intake through increased BDNF signaling (174, 325). Previously published finding showing a history of exercise caused a "BDNF priming" effect was replicated (137) in our model using 2-hr daily exercise, demonstrating exercise can rapidly elevate BDNF in the mPFC of previously exercising mice.

Chapter 3 explored whether limited exercise could attenuate escalated alcohol intake engendered by a model of alcohol dependence (CIE exposure) (154, 168, 171) and whether exercise could prevent CIE-induced reductions of *Bdnf* mRNA in the mPFC (174, 178, 326, 486). Results revealed exercise can prevent the development of alcohol dependence-related intake and this effect correlated with exercise preventing reductions of *Bdnf* mRNA in the mPFC. Suggesting a role of exercise induced BDNF expression in the mPFC in the attenuation of escalated alcohol intake.

To test the hypothesis that exercise attenuated escalated alcohol intake through mitigating chronic alcohol related reductions of BDNF in the brain (alcohol dependence decreases BDNF, exercise increases BDNF), TrkB receptors were antagonized by systemic administration of ANA-12 (Chapter 4). Blocking TrkB receptors prevented exercise from attenuating escalated alcohol intake, demonstrating a BDNF-TrkB mechanism responsible for the preventative effects of exercise.

The evidence presented showed the ability of exercise to prevent dependence related alcohol intake through a BDNF-TrkB mediated mechanism using systemic,

pharmacological antagonism of TrkB receptors. Although exercise attenuated escalated alcohol intake and prevented chronic alcohol exposure from reducing *Bdnf* mRNA in the mPFC, this data is highly correlational and does not specifically isolate the role of BDNF in the mPFC, limiting the interpretation of the results. Additional studies are needed to determine the specific role of exercise induced BDNF expression in the mPFC in alcohol dependence related drinking. Specifically blocking BDNF-TrkB signaling in the mPFC of exercising mice exposed to chronic alcohol. One such study required would use direct pharmacological antagonism of TrkB receptors or sequestration of BDNF protein using a scavenger protein (TrkB-Fc) in the mPFC of exercising mice exposed to chronic alcohol vapor. This would attempt to replicate results from Experiment 4 (Chapter 4) through specifically targeting the mPFC.

As described in Chapter 1, *Bdnf* mRNA is trafficked not only throughout the neuron (276-278, 280), but also to and from the mPFC (267, 268, 492). Therefore, another limitation in the work presented is that *Bdnf* mRNA measured in the mPFC may not correlate to the amount of functional BDNF protein actually found in the mPFC. While literature shows an inverse relationship between BDNF in the mPFC and alcohol drinking (174, 178, 325, 326, 486) we did not demonstrate that the *Bdnf* mRNA measured in the mPFC was in fact turned into BDNF protein that elicited the attenuation of escalated alcohol drinking. Studies described in the paragraph above would help to elucidate the role of exercise induced BDNF in the mPFC in this effect.

In addition to the mPFC, we found BDNF (mRNA and protein) to be upregulated in the dentate gyrus (DG) after exercise (2-hr/day x 4 weeks) (Chapter 2; Figure 2.4). Inhibition of the DG has been demonstrated to increase alcohol drinking behavior (496). Enhancing BDNF in the DG by exercise may suppress escalated alcohol intake by enhancing DG activity. The work presented in Chapter 3 and 4 focused on BDNF

expression in the mPFC and not the DG, therefore it can be speculated that the DG may be involved in the attenuating effects of exercise on alcohol dependence drinking. Studies replicating work that targeted BDNF in the mPFC (174) should be replicated targeting the DG to determine the role of BDNF in the DG on alcohol-dependence related drinking. Studies that pharmacologically target the BDNF-TrkB system (describe above for the mPFC) should be conducted in the DG to determine how the effects of exercise induced BDNF expression in the DG affects alcohol dependent drinking. This limitation can be expanded upon to include other brain regions not investigated that are known to be involved in addiction related behaviors.

Here the foundation was set, demonstrating a BDNF-TrkB mediated mechanism by which exercise attenuates dependence-related alcohol drinking. Currently pharmacological agonism of the TrkB receptors is severely limited, with only a couple of known molecules (314, 497). Determining a specific mechanism by which exercise increases BDNF expression will allow for the development of pharmacotherapies and treatments to target the mechanism to increase BDNF to treat AUD.

One overlapping mechanism is through the ketone body, beta-hydroxybutyrate. Exercise, fasting, and the high-fat ketogenic diet all result in the metabolism of fat and the production of beta-hydroxybutyrate (298, 354) which released into the blood stream and can cross the blood brain barrier (298, 354, 355). Beta-hydroxybutyrate is an HDAC inhibitor directly linked to increasing the expression of *Bdnf* mRNA exon I (298). Studies have administered HDAC inhibitors, e.g. sodium butyrate (beta-hydroxybutyrate analog) and SAHA, to rodents to reduce escalated alcohol intake (498, 499). This data along with work showing increasing BDNF in the mPFC can prevent the development of alcohol dependence and reduce alcohol dependent drinking (174), indicates a potential mechanism which can be targeted to increase BDNF and reduce drinking. Through the

high-fat ketogenic diet, beta-hydroxybutyrate levels are elevated in the blood and brain (298, 354). The ketogenic diet has been demonstrated to reduce alcohol withdrawal symptoms in rats (500) and the National Institute on Alcohol Abuse and Alcoholism (NIAAA) is currently funding a clinical trial examining the effects on withdrawal in humans (NCT03255031). Furthermore, at the recent 2019 Research Society on Alcoholism conference in Minneapolis, MN unpublished data was presented at Symposia Session #26 - Alcohol Metabolism and the Role of Ketone Bodies to Alleviate Symptoms of Alcohol Withdrawal, showing administration of exogenous ketone esters and the ketogenic diet could reduce craving and withdrawal symptoms. This information provides an additional natural, noninvasive mechanism by which BDNF levels can be elevated in the brain. Using fasting or ketogenic diet along with exercise, may work synergistically to reduce and prevent alcohol dependence drinking. Therefore, future studies could be performed to investigate the role of beta-hydroxybutyrate in the effects of exercise on BDNF levels and the attenuation of alcohol-dependence related drinking.

Additionally, exercise induces the browning of fat that produces irisin, a molecule that has been shown to increase the production of BDNF in the brain (329, 363, 367, 368). This is another mechanism which was not examined that may explain increases in BDNF expression detected and therefore makes another interesting mechanism to investigate for neuropsychiatric and substance use disorders.

Exercise increases the expression and release of multiple neurotransmitters (e.g. dopamine, norepinephrine, endogenous opioids) (415, 433, 461) in addition to enhancing BDNF production. It is well established that these other neurotransmitters are involved in alcohol use and addiction behaviors (Chapter 1, p. 36-45). The current studies only focused on BDNF-TrkB signaling and so may have overlooked the function of other exercise induced. Moreover, these signaling molecules have also been shown to positively

increase BDNF production (Chapter 1, p. 36-45). Future studies could be performed to examine how manipulation of these alternative signaling molecules, induced by exercise, could regulate alcohol intake. These studies could go further to determine if there is a relationship between neurotransmitter production and BDNF expression. Additionally, these other signaling molecules and pathways may provide better pharmacological targets which can still result in the enhancement of BDNF in the brain.

Another limitation to the studies presented is they only examined the effect of exercise as a treatment for alcohol dependence in mice that had a prior history of exercise. This limits the interpretation of the data for two reasons: 1) A history of exercise primes the BDNF system so that even after a period of inactivity, a single bout of exercise will rapidly elevate BDNF in rodents with a history of exercise (137) (Figure 4 A & B); 2) Human alcoholics have reduced BDNF in their blood (315, 316) and may require more bouts of exercise to sufficiently raise BDNF levels high enough, for a long enough duration to elicit a reduction in drinking.

However, exercise can still play an important, beneficial role in the maintenance of abstinence and in preventing the development of alcohol abuse and addiction. Future experiments should be pursued to evaluate the ability of exercise to reduce alcohol-dependence related drinking performed by alcohol dependent mice without prior exercise experience. This may prove to be more difficult to determine in mice using the methodology in the previous chapters, because as seen in Figure 13, as mice are exposed to repeated cycles of CIE, wheel running activity begins to decline. If mice are uncapable or unwilling to perform exercise, the benefits of exercise cannot be realized. To overcome this possible scenario, an alternative strategy of using forced treadmill exercise could be implemented.

Daily voluntary exercise increases BDNF expression in the brain of mice and can attenuate the escalation of alcohol intake caused by alcohol dependence (CIE vapor exposure). Data was provided demonstrating escalated alcohol-dependent intake is mediated through a BDNF-TrkB mediated signaling mechanism. Furthermore, we provided data to implicate the mPFC as a potential brain region through which exercised induced BDNF expression may exert its protective effects against the development of alcohol dependence. These data add to the growing body of literature revealing a therapeutic role of exercise in the treatment of alcohol dependence and provides data which indicates a role of BDNF in the mPFC in this effect.

References

1. (WHO) WHO. Global Status Report On Alcohol and Health. 2018 [Available from: https://apps.who.int/iris/bitstream/handle/10665/274603/9789241565639-eng.pdf?ua=1.
2. Koob GF, Volkow ND. Neurocircuitry of addiction. Neuropsychopharmacology. 2010;35(1):217-38.
3. Grant S, Watkins KE, Bogart A, Paddock SM, Hepner KA. Patient-Reported Offers of Alcohol Treatment for Primary Care Patients at High-Risk for an Alcohol Use Disorder. J Am Board Fam Med. 2016;29(6):682-7.
4. Hasin DS, Stinson FS, Ogburn E, Grant BF. Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Arch Gen Psychiatry. 2007;64(7):830-42.
5. Skinner HA, Allen BA. Alcohol dependence syndrome: measurement and validation. J Abnorm Psychol. 1982;91(3):199-209.
6. Koob GF, Le Moal M. Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology. 2001;24(2):97-129.
7. Nona CN, Hendershot CS, Le AD. Behavioural sensitization to alcohol: Bridging the gap between preclinical research and human models. Pharmacol Biochem Behav. 2018;173:15-26.
8. Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. JAMA. 2004;291(10):1238-45.
9. Rehm J, Lachenmeier DW, Room R. Why does society accept a higher risk for alcohol than for other voluntary or involuntary risks? BMC Med. 2014;12:189.
10. Rehm J, Parry CD. Alcohol consumption and infectious diseases in South Africa. Lancet. 2009;374(9707):2053.
11. Schuckit MA. Alcohol-use disorders. Lancet. 2009;373(9662):492-501.
12. Grant BF, Goldstein RB, Saha TD, Chou SP, Jung J, Zhang H, et al. Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry. 2015;72(8):757-66.
13. Kessler RC, McGonagle KA, Zhao S, Nelson CB, Hughes M, Eshleman S, et al. Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey. Arch Gen Psychiatry. 1994;51(1):8-19.
14. Robinson J, Sareen J, Cox BJ, Bolton JM. Role of self-medication in the development of comorbid anxiety and substance use disorders: a longitudinal investigation. Arch Gen Psychiatry. 2011;68(8):800-7.
15. Kessler RC, Crum RM, Warner LA, Nelson CB, Schulenberg J, Anthony JC. Lifetime co-occurrence of DSM-III-R alcohol abuse and dependence with other psychiatric disorders in the National Comorbidity Survey. Arch Gen Psychiatry. 1997;54(4):313-21.
16. DeWit DJ, Adlaf EM, Offord DR, Ogborne AC. Age at first alcohol use: a risk factor for the development of alcohol disorders. Am J Psychiatry. 2000;157(5):745-50.
17. Corte CM, Sommers MS. Alcohol and risky behaviors. Annu Rev Nurs Res. 2005;23:327-60.
18. Cook PA, Phillips-Howard PA, Morleo M, Harkins C, Briant L, Bellis MA. The Big Drink Debate: perceptions of the impact of price on alcohol consumption from a large scale cross-sectional convenience survey in north west England. BMC Public Health. 2011;11:664.
19. Edwards G, Anderson P, Babor TF, Casswell S, Ferrence R, Giesbrecht N, et al. Alcohol policy and the public good: a good public debate. Addiction. 1996;91(4):477-81.

20. Miller JW, Naimi TS, Brewer RD, Jones SE. Binge drinking and associated health risk behaviors among high school students. Pediatrics. 2007;119(1):76-85.
21. Anderson RI, Lopez MF, Griffin WC, Haun HL, Bloodgood DW, Pati D, et al. Dynorphin-kappa opioid receptor activity in the central amygdala modulates binge-like alcohol drinking in mice. Neuropsychopharmacology. 2018.
22. Spiga S, Talani G, Mulas G, Licheri V, Fois GR, Muggironi G, et al. Hampered long-term depression and thin spine loss in the nucleus accumbens of ethanol-dependent rats. Proc Natl Acad Sci U S A. 2014;111(35):E3745-54.
23. Kyzar EJ, Pandey SC. Molecular mechanisms of synaptic remodeling in alcoholism. Neurosci Lett. 2015;601:11-9.
24. Anton RF, Schacht JP, Book SW. Pharmacologic treatment of alcoholism. Handb Clin Neurol. 2014;125:527-42.
25. Humphreys K, Moos RH. Reduced substance-abuse-related health care costs among voluntary participants in Alcoholics Anonymous. Psychiatr Serv. 1996;47(7):709-13.
26. Moos RH, Moos BS. Participation in treatment and Alcoholics Anonymous: a 16-year follow-up of initially untreated individuals. J Clin Psychol. 2006;62(6):735-50.
27. Ouimette PC, Moos RH, Finney JW. Influence of outpatient treatment and 12-step group involvement on one-year substance abuse treatment outcomes. J Stud Alcohol. 1998;59(5):513-22.
28. McHugh RK, Hearon BA, Otto MW. Cognitive behavioral therapy for substance use disorders. Psychiatr Clin North Am. 2010;33(3):511-25.
29. Wackernah RC, Minnick MJ, Clapp P. Alcohol use disorder: pathophysiology, effects, and pharmacologic options for treatment. Subst Abuse Rehabil. 2014;5:1-12.
30. Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, et al. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet. 2013;382(9904):1575-86.
31. Jupp B, Lawrence AJ. New horizons for therapeutics in drug and alcohol abuse. Pharmacol Ther. 2010;125(1):138-68.
32. Chapman C, Slade T, Hunt C, Teesson M. Delay to first treatment contact for alcohol use disorder. Drug Alcohol Depend. 2015;147:116-21.
33. Schuler MS, Puttaiah S, Mojtabai R, Crum RM. Perceived Barriers to Treatment for Alcohol Problems: A Latent Class Analysis. Psychiatr Serv. 2015;66(11):1221-8.
34. Krampe H, Stawicki S, Hoehe MR, Ehrenreich H. Outpatient Long-term Intensive Therapy for Alcoholics (OLITA): a successful biopsychosocial approach to the treatment of alcoholism. Dialogues Clin Neurosci. 2007;9(4):399-412.
35. McKay JR, Franklin TR, Patapis N, Lynch KG. Conceptual, methodological, and analytical issues in the study of relapse. Clin Psychol Rev. 2006;26(2):109-27.
36. Moos RH, Moos BS. Rates and predictors of relapse after natural and treated remission from alcohol use disorders. Addiction. 2006;101(2):212-22.
37. Baler RD, Volkow ND. Drug addiction: the neurobiology of disrupted self-control. Trends Mol Med. 2006;12(12):559-66.
38. Le Moal M. Drug abuse: vulnerability and transition to addiction. Pharmacopsychiatry. 2009;42 Suppl 1:S42-55.
39. Volkow ND, Li TK. Drug addiction: the neurobiology of behaviour gone awry. Nat Rev Neurosci. 2004;5(12):963-70.
40. Nees F, Witt SH, Dinu-Biringer R, Lourdusamy A, Tzschoppe J, Vollstadt-Klein S, et al. BDNF Val66Met and reward-related brain function in adolescents: role for early alcohol consumption. Alcohol. 2015;49(2):103-10.

41. Rolland B, Naassila M. Binge Drinking: Current Diagnostic and Therapeutic Issues. CNS Drugs. 2017;31(3):181-6.
42. Courtney KE, Polich J. Binge drinking in young adults: Data, definitions, and determinants. Psychol Bull. 2009;135(1):142-56.
43. Dawson DA, Grant BF, Li TK. Quantifying the risks associated with exceeding recommended drinking limits. Alcohol Clin Exp Res. 2005;29(5):902-8.
44. Corso TD, Mostafa HM, Collins MA, Neafsey EJ. Brain neuronal degeneration caused by episodic alcohol intoxication in rats: effects of nimodipine, 6,7-dinitro-quinoxaline-2,3-dione, and MK-801. Alcohol Clin Exp Res. 1998;22(1):217-24.
45. Lee H, Heller AS, van Reekum CM, Nelson B, Davidson RJ. Amygdala-prefrontal coupling underlies individual differences in emotion regulation. Neuroimage. 2012;62(3):1575-81.
46. Peters S, Peper JS, Van Duijvenvoorde ACK, Braams BR, Crone EA. Amygdala-orbitofrontal connectivity predicts alcohol use two years later: a longitudinal neuroimaging study on alcohol use in adolescence. Dev Sci. 2017;20(4).
47. Crane NA, Gorka SM, Phan KL, Childs E. Amygdala-orbitofrontal functional connectivity mediates the relationship between sensation seeking and alcohol use among binge-drinking adults. Drug Alcohol Depend. 2018;192:208-14.
48. Koob GF, Roberts AJ, Schulteis G, Parsons LH, Heyser CJ, Hyytia P, et al. Neurocircuitry targets in ethanol reward and dependence. Alcohol Clin Exp Res. 1998;22(1):3-9.
49. Raeder H, Holter SM, Hartmann AM, Spanagel R, Moller HJ, Rujescu D. Expression of N-methyl-d-aspartate (NMDA) receptor subunits and splice variants in an animal model of long-term voluntary alcohol self-administration. Drug Alcohol Depend. 2008;96(1-2):16-21.
50. Boileau I, Assaad JM, Pihl RO, Benkelfat C, Leyton M, Diksic M, et al. Alcohol promotes dopamine release in the human nucleus accumbens. Synapse. 2003;49(4):226-31.
51. Roberto M, Schweitzer P, Madamba SG, Stouffer DG, Parsons LH, Siggins GR. Acute and chronic ethanol alter glutamatergic transmission in rat central amygdala: an in vitro and in vivo analysis. J Neurosci. 2004;24(7):1594-603.
52. Blednov YA, Harris RA. Metabotropic glutamate receptor 5 (mGluR5) regulation of ethanol sedation, dependence and consumption: relationship to acamprosate actions. Int J Neuropsychopharmacol. 2008;11(6):775-93.
53. Woodward JJ, Gonzales RA. Ethanol inhibition of N-methyl-D-aspartate-stimulated endogenous dopamine release from rat striatal slices: reversal by glycine. J Neurochem. 1990;54(2):712-5.
54. Lovinger DM, White G, Weight FF. Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science. 1989;243(4899):1721-4.
55. Anton RF, O'Malley SS, Ciraulo DA, Cisler RA, Couper D, Donovan DM, et al. Combined pharmacotherapies and behavioral interventions for alcohol dependence: the COMBINE study: a randomized controlled trial. JAMA. 2006;295(17):2003-17.
56. Koob GF. A role for GABA mechanisms in the motivational effects of alcohol. Biochem Pharmacol. 2004;68(8):1515-25.
57. Most D, Ferguson L, Harris RA. Molecular basis of alcoholism. Handb Clin Neurol. 2014;125:89-111.
58. Federici M, Nistico R, Giustizieri M, Bernardi G, Mercuri NB. Ethanol enhances GABAB-mediated inhibitory postsynaptic transmission on rat midbrain dopaminergic neurons by facilitating GIRK currents. Eur J Neurosci. 2009;29(7):1369-77.

59. Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, et al. Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. Nature. 1997;389(6649):385-9.
60. Addolorato G, Caputo F, Capristo E, Domenicali M, Bernardi M, Janiri L, et al. Baclofen efficacy in reducing alcohol craving and intake: a preliminary double-blind randomized controlled study. Alcohol Alcohol. 2002;37(5):504-8.
61. Maccioni P, Colombo G. Role of the GABA(B) receptor in alcohol-seeking and drinking behavior. Alcohol. 2009;43(7):555-8.
62. Vendruscolo LF, Barbier E, Schlosburg JE, Misra KK, Whitfield TW, Jr., Logrip ML, et al. Corticosteroid-dependent plasticity mediates compulsive alcohol drinking in rats. J Neurosci. 2012;32(22):7563-71.
63. Rasmussen DD, Boldt BM, Bryant CA, Mitton DR, Larsen SA, Wilkinson CW. Chronic daily ethanol and withdrawal: 1. Long-term changes in the hypothalamo-pituitary-adrenal axis. Alcohol Clin Exp Res. 2000;24(12):1836-49.
64. Zorrilla EP, Valdez GR, Weiss F. Changes in levels of regional CRF-like-immunoreactivity and plasma corticosterone during protracted drug withdrawal in dependent rats. Psychopharmacology (Berl). 2001;158(4):374-81.
65. Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F, Baler R. Addiction: decreased reward sensitivity and increased expectation sensitivity conspire to overwhelm the brain's control circuit. Bioessays. 2010;32(9):748-55.
66. Saitz R. Introduction to alcohol withdrawal. Alcohol Health Res World. 1998;22(1):5-12.
67. Becker HC. Animal models of alcohol withdrawal. Alcohol Res Health. 2000;24(2):105-13.
68. Heilig M, Egli M, Crabbe JC, Becker HC. Acute withdrawal, protracted abstinence and negative affect in alcoholism: are they linked? Addict Biol. 2010;15(2):169-84.
69. Heilig M, Koob GF. A key role for corticotropin-releasing factor in alcohol dependence. Trends Neurosci. 2007;30(8):399-406.
70. Melis M, Spiga S, Diana M. The dopamine hypothesis of drug addiction: hypodopaminergic state. Int Rev Neurobiol. 2005;63:101-54.
71. Volkow ND, Fowler JS, Wang GJ, Swanson JM, Telang F. Dopamine in drug abuse and addiction: results of imaging studies and treatment implications. Arch Neurol. 2007;64(11):1575-9.
72. Volkow ND, Wise RA, Baler R. The dopamine motive system: implications for drug and food addiction. Nat Rev Neurosci. 2017;18(12):741-52.
73. Coccaro EF, Fanning JR, Phan KL, Lee R. Serotonin and impulsive aggression. CNS Spectr. 2015;20(3):295-302.
74. Little HJ, Croft AP, O'Callaghan MJ, Brooks SP, Wang G, Shaw SG. Selective increases in regional brain glucocorticoid: a novel effect of chronic alcohol. Neuroscience. 2008;156(4):1017-27.
75. Koob GF. A role for brain stress systems in addiction. Neuron. 2008;59(1):11-34.
76. Jacquot C, Croft AP, Prendergast MA, Mulholland P, Shaw SG, Little HJ. Effects of the glucocorticoid antagonist, mifepristone, on the consequences of withdrawal from long term alcohol consumption. Alcohol Clin Exp Res. 2008;32(12):2107-16.
77. Pettinati HM, O'Brien CP, Rabinowitz AR, Wortman SP, Oslin DW, Kampman KM, et al. The status of naltrexone in the treatment of alcohol dependence: specific effects on heavy drinking. J Clin Psychopharmacol. 2006;26(6):610-25.

78. Walker BM, Zorrilla EP, Koob GF. Systemic kappa-opioid receptor antagonism by nor-binaltorphimine reduces dependence-induced excessive alcohol self-administration in rats. Addict Biol. 2011;16(1):116-9.
79. Bruchas MR, Land BB, Chavkin C. The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. Brain Res. 2010;1314:44-55.
80. Anderson RI, Becker HC. Role of the Dynorphin/Kappa Opioid Receptor System in the Motivational Effects of Ethanol. Alcohol Clin Exp Res. 2017;41(8):1402-18.
81. Kissler JL, Sirohi S, Reis DJ, Jansen HT, Quock RM, Smith DG, et al. The one-two punch of alcoholism: role of central amygdala dynorphins/kappa-opioid receptors. Biol Psychiatry. 2014;75(10):774-82.
82. Maisonneuve IM, Archer S, Glick SD. U50,488, a kappa opioid receptor agonist, attenuates cocaine-induced increases in extracellular dopamine in the nucleus accumbens of rats. Neurosci Lett. 1994;181(1-2):57-60.
83. Anderson RI, Lopez MF, Becker HC. Stress-Induced Enhancement of Ethanol Intake in C57BL/6J Mice with a History of Chronic Ethanol Exposure: Involvement of Kappa Opioid Receptors. Front Cell Neurosci. 2016;10:45.
84. Beardsley PM, Howard JL, Shelton KL, Carroll FI. Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats. Psychopharmacology (Berl). 2005;183(1):118-26.
85. Sinha R. The clinical neurobiology of drug craving. Curr Opin Neurobiol. 2013;23(4):649-54.
86. Sinha R. Modeling stress and drug craving in the laboratory: implications for addiction treatment development. Addict Biol. 2009;14(1):84-98.
87. Sinha R, Lacadie C, Skudlarski P, Fulbright RK, Rounsaville BJ, Kosten TR, et al. Neural activity associated with stress-induced cocaine craving: a functional magnetic resonance imaging study. Psychopharmacology (Berl). 2005;183(2):171-80.
88. Marinelli M, Piazza PV. Interaction between glucocorticoid hormones, stress and psychostimulant drugs. Eur J Neurosci. 2002;16(3):387-94.
89. Breese GR, Sinha R, Heilig M. Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. Pharmacol Ther. 2011;129(2):149-71.
90. Sinha R. New findings on biological factors predicting addiction relapse vulnerability. Curr Psychiatry Rep. 2011;13(5):398-405.
91. Sinha R. How does stress lead to risk of alcohol relapse? Alcohol Res. 2012;34(4):432-40.
92. Bottlender M, Soyka M. Impact of craving on alcohol relapse during, and 12 months following, outpatient treatment. Alcohol Alcohol. 2004;39(4):357-61.
93. Sinha R, Fox HC, Hong KI, Hansen J, Tuit K, Kreek MJ. Effects of adrenal sensitivity, stress- and cue-induced craving, and anxiety on subsequent alcohol relapse and treatment outcomes. Arch Gen Psychiatry. 2011;68(9):942-52.
94. Seo D, Lacadie CM, Tuit K, Hong KI, Constable RT, Sinha R. Disrupted ventromedial prefrontal function, alcohol craving, and subsequent relapse risk. JAMA Psychiatry. 2013;70(7):727-39.
95. Hampton AN, Bossaerts P, O'Doherty JP. The role of the ventromedial prefrontal cortex in abstract state-based inference during decision making in humans. J Neurosci. 2006;26(32):8360-7.
96. Urry HL, van Reekum CM, Johnstone T, Kalin NH, Thurow ME, Schaefer HS, et al. Amygdala and ventromedial prefrontal cortex are inversely coupled during regulation

of negative affect and predict the diurnal pattern of cortisol secretion among older adults. J Neurosci. 2006;26(16):4415-25.
97. Rando K, Hong KI, Bhagwagar Z, Li CS, Bergquist K, Guarnaccia J, et al. Association of frontal and posterior cortical gray matter volume with time to alcohol relapse: a prospective study. Am J Psychiatry. 2011;168(2):183-92.
98. Kalivas PW, Volkow ND. The neural basis of addiction: a pathology of motivation and choice. Am J Psychiatry. 2005;162(8):1403-13.
99. Di Chiara G. Alcohol and dopamine. Alcohol Health Res World. 1997;21(2):108-14.
100. Wrase J, Makris N, Braus DF, Mann K, Smolka MN, Kennedy DN, et al. Amygdala volume associated with alcohol abuse relapse and craving. Am J Psychiatry. 2008;165(9):1179-84.
101. Beck A, Wustenberg T, Genauck A, Wrase J, Schlagenhauf F, Smolka MN, et al. Effect of brain structure, brain function, and brain connectivity on relapse in alcohol-dependent patients. Arch Gen Psychiatry. 2012;69(8):842-52.
102. Li CS, Sinha R. Inhibitory control and emotional stress regulation: neuroimaging evidence for frontal-limbic dysfunction in psycho-stimulant addiction. Neurosci Biobehav Rev. 2008;32(3):581-97.
103. Koob GF, Le Moal M. Drug abuse: hedonic homeostatic dysregulation. Science. 1997;278(5335):52-8.
104. Jentsch JD, Taylor JR. Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for the control of behavior by reward-related stimuli. Psychopharmacology (Berl). 1999;146(4):373-90.
105. Abernathy K, Chandler LJ, Woodward JJ. Alcohol and the prefrontal cortex. Int Rev Neurobiol. 2010;91:289-320.
106. Goldstein RZ, Volkow ND. Drug addiction and its underlying neurobiological basis: neuroimaging evidence for the involvement of the frontal cortex. Am J Psychiatry. 2002;159(10):1642-52.
107. Goldstein RZ, Volkow ND. Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications. Nat Rev Neurosci. 2011;12(11):652-69.
108. Uylings HB, Groenewegen HJ, Kolb B. Do rats have a prefrontal cortex? Behav Brain Res. 2003;146(1-2):3-17.
109. Sierra-Mercado D, Padilla-Coreano N, Quirk GJ. Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. Neuropsychopharmacology. 2011;36(2):529-38.
110. Cassaday HJ, Nelson AJ, Pezze MA. From attention to memory along the dorsal-ventral axis of the medial prefrontal cortex: some methodological considerations. Front Syst Neurosci. 2014;8:160.
111. Moorman DE, James MH, McGlinchey EM, Aston-Jones G. Differential roles of medial prefrontal subregions in the regulation of drug seeking. Brain Res. 2015;1628(Pt A):130-46.
112. Peters J, Kalivas PW, Quirk GJ. Extinction circuits for fear and addiction overlap in prefrontal cortex. Learn Mem. 2009;16(5):279-88.
113. Everitt BJ, Robbins TW. Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci. 2005;8(11):1481-9.
114. Koob GF, Le Moal M. Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. Nat Neurosci. 2005;8(11):1442-4.
115. Koob GF, Volkow ND. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry. 2016;3(8):760-73.

116. Hoover WB, Vertes RP. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. Brain Struct Funct. 2007;212(2):149-79.
117. George O, Sanders C, Freiling J, Grigoryan E, Vu S, Allen CD, et al. Recruitment of medial prefrontal cortex neurons during alcohol withdrawal predicts cognitive impairment and excessive alcohol drinking. Proc Natl Acad Sci U S A. 2012;109(44):18156-61.
118. Buhler M, Mann K. Alcohol and the human brain: a systematic review of different neuroimaging methods. Alcohol Clin Exp Res. 2011;35(10):1771-93.
119. Myrick H, Anton RF, Li X, Henderson S, Drobes D, Voronin K, et al. Differential brain activity in alcoholics and social drinkers to alcohol cues: relationship to craving. Neuropsychopharmacology. 2004;29(2):393-402.
120. Grusser SM, Wrase J, Klein S, Hermann D, Smolka MN, Ruf M, et al. Cue-induced activation of the striatum and medial prefrontal cortex is associated with subsequent relapse in abstinent alcoholics. Psychopharmacology (Berl). 2004;175(3):296-302.
121. Rogers JL, Ghee S, See RE. The neural circuitry underlying reinstatement of heroin-seeking behavior in an animal model of relapse. Neuroscience. 2008;151(2):579-88.
122. Harper C, Matsumoto I. Ethanol and brain damage. Curr Opin Pharmacol. 2005;5(1):73-8.
123. Durazzo TC, Tosun D, Buckley S, Gazdzinski S, Mon A, Fryer SL, et al. Cortical thickness, surface area, and volume of the brain reward system in alcohol dependence: relationships to relapse and extended abstinence. Alcohol Clin Exp Res. 2011;35(6):1187-200.
124. Demirakca T, Ende G, Kammerer N, Welzel-Marquez H, Hermann D, Heinz A, et al. Effects of alcoholism and continued abstinence on brain volumes in both genders. Alcohol Clin Exp Res. 2011;35(9):1678-85.
125. Kril JJ, Halliday GM. Brain shrinkage in alcoholics: a decade on and what have we learned? Prog Neurobiol. 1999;58(4):381-7.
126. Stephens DN, Duka T. Review. Cognitive and emotional consequences of binge drinking: role of amygdala and prefrontal cortex. Philos Trans R Soc Lond B Biol Sci. 2008;363(1507):3169-79.
127. George O, Koob GF. Individual differences in prefrontal cortex function and the transition from drug use to drug dependence. Neurosci Biobehav Rev. 2010;35(2):232-47.
128. Bissonette GB, Martins GJ, Franz TM, Harper ES, Schoenbaum G, Powell EM. Double dissociation of the effects of medial and orbital prefrontal cortical lesions on attentional and affective shifts in mice. J Neurosci. 2008;28(44):11124-30.
129. Tsuchida A, Doll BB, Fellows LK. Beyond reversal: a critical role for human orbitofrontal cortex in flexible learning from probabilistic feedback. J Neurosci. 2010;30(50):16868-75.
130. Bijl S, de Bruin EA, Kenemans JL, Verbaten MN, Bocker KB. Effects of chronic alcohol consumption in a visual attention task and an auditory oddball task: an event-related potential study. Alcohol Clin Exp Res. 2005;29(11):2029-38.
131. Ratti MT, Bo P, Giardini A, Soragna D. Chronic alcoholism and the frontal lobe: which executive functions are imparied? Acta Neurol Scand. 2002;105(4):276-81.
132. Vyas S, Rodrigues AJ, Silva JM, Tronche F, Almeida OF, Sousa N, et al. Chronic Stress and Glucocorticoids: From Neuronal Plasticity to Neurodegeneration. Neural Plast. 2016;2016:6391686.

133. Meda SA, Hawkins KA, Dager AD, Tennen H, Khadka S, Austad CS, et al. Longitudinal Effects of Alcohol Consumption on the Hippocampus and Parahippocampus in College Students. Biol Psychiatry Cogn Neurosci Neuroimaging. 2018;3(7):610-7.
134. Agartz I, Momenan R, Rawlings RR, Kerich MJ, Hommer DW. Hippocampal volume in patients with alcohol dependence. Arch Gen Psychiatry. 1999;56(4):356-63.
135. Le Berre AP, Rauchs G, La Joie R, Mezenge F, Boudehent C, Vabret F, et al. Impaired decision-making and brain shrinkage in alcoholism. Eur Psychiatry. 2014;29(3):125-33.
136. Ehringer MA, Hoft NR, Zunhammer M. Reduced alcohol consumption in mice with access to a running wheel. Alcohol. 2009;43(6):443-52.
137. Berchtold NC, Chinn G, Chou M, Kesslak JP, Cotman CW. Exercise primes a molecular memory for brain-derived neurotrophic factor protein induction in the rat hippocampus. Neuroscience. 2005;133(3):853-61.
138. Gallego X, Cox RJ, Funk E, Foster RA, Ehringer MA. Voluntary exercise decreases ethanol preference and consumption in C57BL/6 adolescent mice: sex differences and hippocampal BDNF expression. Physiol Behav. 2015;138:28-36.
139. Darlington TM, McCarthy RD, Cox RJ, Ehringer MA. Mesolimbic transcriptional response to hedonic substitution of voluntary exercise and voluntary ethanol consumption. Behav Brain Res. 2014;259:313-20.
140. Sovik E, Barron AB. Invertebrate models in addiction research. Brain Behav Evol. 2013;82(3):153-65.
141. Lovinger DM, Crabbe JC. Laboratory models of alcoholism: treatment target identification and insight into mechanisms. Nat Neurosci. 2005;8(11):1471-80.
142. Banks ML, Czoty PW, Negus SS. Utility of Nonhuman Primates in Substance Use Disorders Research. ILAR J. 2017;58(2):202-15.
143. Anacker AM, Ryabinin AE. Biological contribution to social influences on alcohol drinking: evidence from animal models. Int J Environ Res Public Health. 2010;7(2):473-93.
144. Holter SM, Linthorst AC, Reul JM, Spanagel R. Withdrawal symptoms in a long-term model of voluntary alcohol drinking in Wistar rats. Pharmacol Biochem Behav. 2000;66(1):143-51.
145. Spanagel R. Recent animal models of alcoholism. Alcohol Res Health. 2000;24(2):124-31.
146. Becker HC, Ron D. Animal models of excessive alcohol consumption: recent advances and future challenges. Alcohol. 2014;48(3):205-8.
147. Lopez MF, Becker HC. Operant ethanol self-administration in ethanol dependent mice. Alcohol. 2014;48(3):295-9.
148. Crabbe JC, Phillips TJ, Belknap JK. The complexity of alcohol drinking: studies in rodent genetic models. Behav Genet. 2010;40(6):737-50.
149. Wiens F, Zitzmann A, Lachance MA, Yegles M, Pragst F, Wurst FM, et al. Chronic intake of fermented floral nectar by wild treeshrews. Proc Natl Acad Sci U S A. 2008;105(30):10426-31.
150. Mayfield RD, Harris RA, Schuckit MA. Genetic factors influencing alcohol dependence. Br J Pharmacol. 2008;154(2):275-87.
151. Lopez MF, Miles MF, Williams RW, Becker HC. Variable effects of chronic intermittent ethanol exposure on ethanol drinking in a genetically diverse mouse cohort. Alcohol. 2017;58:73-82.

152. Rinker JA, Fulmer DB, Trantham-Davidson H, Smith ML, Williams RW, Lopez MF, et al. Differential potassium channel gene regulation in BXD mice reveals novel targets for pharmacogenetic therapies to reduce heavy alcohol drinking. Alcohol. 2017;58:33-45.
153. van der Vaart AD, Wolstenholme JT, Smith ML, Harris GM, Lopez MF, Wolen AR, et al. The allostatic impact of chronic ethanol on gene expression: A genetic analysis of chronic intermittent ethanol treatment in the BXD cohort. Alcohol. 2017;58:93-106.
154. Becker HC, Lopez MF. Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. Alcohol Clin Exp Res. 2004;28(12):1829-38.
155. Gao J, Chen W, Dou L, Chen CC, Chang WH, Liu Y, et al. Elucidating double aggregation mechanisms in the morphology optimization of diketopyrrolopyrrole-based narrow bandgap polymer solar cells. Adv Mater. 2014;26(19):3142-7.
156. Griffin WC, 3rd. Alcohol dependence and free-choice drinking in mice. Alcohol. 2014;48(3):287-93.
157. Lopez MF, Becker HC. Effect of pattern and number of chronic ethanol exposures on subsequent voluntary ethanol intake in C57BL/6J mice. Psychopharmacology (Berl). 2005;181(4):688-96.
158. Hopf FW, Lesscher HM. Rodent models for compulsive alcohol intake. Alcohol. 2014;48(3):253-64.
159. June HL, Gilpin NW. Operant self-administration models for testing the neuropharmacological basis of ethanol consumption in rats. Curr Protoc Neurosci. 2010;Chapter 9:Unit 9 12 1-26.
160. Spanagel R. Animal models of addiction. Dialogues Clin Neurosci. 2017;19(3):247-58.
161. Bertola A, Mathews S, Ki SH, Wang H, Gao B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). Nat Protoc. 2013;8(3):627-37.
162. Guo F, Zheng K, Benede-Ubieto R, Cubero FJ, Nevzorova YA. The Lieber-DeCarli Diet-A Flagship Model for Experimental Alcoholic Liver Disease. Alcohol Clin Exp Res. 2018;42(10):1828-40.
163. Lieber CS, DeCarli LM, Sorrell MF. Experimental methods of ethanol administration. Hepatology. 1989;10(4):501-10.
164. Crabbe JC, Harris RA, Koob GF. Preclinical studies of alcohol binge drinking. Ann N Y Acad Sci. 2011;1216:24-40.
165. Crabbe JC, Harkness JH, Spence SE, Huang LC, Metten P. Intermittent availability of ethanol does not always lead to elevated drinking in mice. Alcohol Alcohol. 2012;47(5):509-17.
166. Crabbe JC, Metten P, Rhodes JS, Yu CH, Brown LL, Phillips TJ, et al. A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. Biol Psychiatry. 2009;65(8):662-70.
167. Griffin WC, 3rd, Lopez MF, Yanke AB, Middaugh LD, Becker HC. Repeated cycles of chronic intermittent ethanol exposure in mice increases voluntary ethanol drinking and ethanol concentrations in the nucleus accumbens. Psychopharmacology (Berl). 2009;201(4):569-80.
168. Becker HC. Animal models of excessive alcohol consumption in rodents. Curr Top Behav Neurosci. 2013;13:355-77.
169. Griffin WC, 3rd, Middaugh LD, Becker HC. Voluntary ethanol drinking in mice and ethanol concentrations in the nucleus accumbens. Brain Res. 2007;1138:208-13.

170. Melendez RI. Intermittent (every-other-day) drinking induces rapid escalation of ethanol intake and preference in adolescent and adult C57BL/6J mice. Alcohol Clin Exp Res. 2011;35(4):652-8.
171. Becker HC. Alcohol dependence, withdrawal, and relapse. Alcohol Res Health. 2008;31(4):348-61.
172. Griffin WC, 3rd, Lopez MF, Becker HC. Intensity and duration of chronic ethanol exposure is critical for subsequent escalation of voluntary ethanol drinking in mice. Alcohol Clin Exp Res. 2009;33(11):1893-900.
173. Becker HC, Hale RL. Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: an animal model of alcohol withdrawal "kindling". Alcohol Clin Exp Res. 1993;17(1):94-8.
174. Haun HL, Griffin WC, Lopez MF, Solomon MG, Mulholland PJ, Woodward JJ, et al. Increasing Brain-Derived Neurotrophic Factor (BDNF) in medial prefrontal cortex selectively reduces excessive drinking in ethanol dependent mice. Neuropharmacology. 2018;140:35-42.
175. DePoy L, Daut R, Brigman JL, MacPherson K, Crowley N, Gunduz-Cinar O, et al. Chronic alcohol produces neuroadaptations to prime dorsal striatal learning. Proc Natl Acad Sci U S A. 2013;110(36):14783-8.
176. O'Dell LE, Roberts AJ, Smith RT, Koob GF. Enhanced alcohol self-administration after intermittent versus continuous alcohol vapor exposure. Alcohol Clin Exp Res. 2004;28(11):1676-82.
177. Rimondini R, Thorsell A, Heilig M. Suppression of ethanol self-administration by the neuropeptide Y (NPY) Y2 receptor antagonist BIIE0246: evidence for sensitization in rats with a history of dependence. Neurosci Lett. 2005;375(2):129-33.
178. Melendez RI, McGinty JF, Kalivas PW, Becker HC. Brain region-specific gene expression changes after chronic intermittent ethanol exposure and early withdrawal in C57BL/6J mice. Addict Biol. 2012;17(2):351-64.
179. Carroll KM, Kiluk BD. Cognitive behavioral interventions for alcohol and drug use disorders: Through the stage model and back again. Psychol Addict Behav. 2017;31(8):847-61.
180. Schuckit MA. Comorbidity between substance use disorders and psychiatric conditions. Addiction. 2006;101 Suppl 1:76-88.
181. Gossop M, Stewart D, Marsden J. Attendance at Narcotics Anonymous and Alcoholics Anonymous meetings, frequency of attendance and substance use outcomes after residential treatment for drug dependence: a 5-year follow-up study. Addiction. 2008;103(1):119-25.
182. Gossop M, Harris J, Best D, Man LH, Manning V, Marshall J, et al. Is attendance at Alcoholics Anonymous meetings after inpatient treatment related to improved outcomes? A 6-month follow-up study. Alcohol Alcohol. 2003;38(5):421-6.
183. Kaskutas LA, Subbaraman MS, Witbrodt J, Zemore SE. Effectiveness of Making Alcoholics Anonymous Easier: a group format 12-step facilitation approach. J Subst Abuse Treat. 2009;37(3):228-39.
184. Swift RM. Medications and alcohol craving. Alcohol Res Health. 1999;23(3):207-13.
185. Kranzler HR, Soyka M. Diagnosis and Pharmacotherapy of Alcohol Use Disorder: A Review. JAMA. 2018;320(8):815-24.
186. Modesto-Lowe V, Fritz EM. The opioidergic-alcohol link : implications for treatment. CNS Drugs. 2005;19(8):693-707.

187. Heilig M, Goldman D, Berrettini W, O'Brien CP. Pharmacogenetic approaches to the treatment of alcohol addiction. Nat Rev Neurosci. 2011;12(11):670-84.
188. Gianoulakis C. Influence of the endogenous opioid system on high alcohol consumption and genetic predisposition to alcoholism. J Psychiatry Neurosci. 2001;26(4):304-18.
189. Benjamin D, Grant ER, Pohorecky LA. Naltrexone reverses ethanol-induced dopamine release in the nucleus accumbens in awake, freely moving rats. Brain Res. 1993;621(1):137-40.
190. Littleton JM. Acamprosate in alcohol dependence: implications of a unique mechanism of action. J Addict Med. 2007;1(3):115-25.
191. Naassila M, Pierrefiche O, Beauge FJ, Sebire N, Daoust M. Chronic ethanol exposure differentially regulates NOS1 mRNA levels depending on rat brain area. Neurosci Lett. 2003;338(3):221-4.
192. Gilpin NW, Koob GF. Neurobiology of alcohol dependence: focus on motivational mechanisms. Alcohol Res Health. 2008;31(3):185-95.
193. Kalk NJ, Lingford-Hughes AR. The clinical pharmacology of acamprosate. Br J Clin Pharmacol. 2014;77(2):315-23.
194. Spanagel R, Vengeliene V, Jandeleit B, Fischer WN, Grindstaff K, Zhang X, et al. Acamprosate produces its anti-relapse effects via calcium. Neuropsychopharmacology. 2014;39(4):783-91.
195. Fox HC, Anderson GM, Tuit K, Hansen J, Kimmerling A, Siedlarz KM, et al. Prazosin effects on stress- and cue-induced craving and stress response in alcohol-dependent individuals: preliminary findings. Alcohol Clin Exp Res. 2012;36(2):351-60.
196. Kenna GA, Haass-Koffler CL, Zywiak WH, Edwards SM, Brickley MB, Swift RM, et al. Role of the alpha1 blocker doxazosin in alcoholism: a proof-of-concept randomized controlled trial. Addict Biol. 2016;21(4):904-14.
197. Fredriksson I, Jayaram-Lindstrom N, Wirf M, Nylander E, Nystrom E, Jardemark K, et al. Evaluation of guanfacine as a potential medication for alcohol use disorder in long-term drinking rats: behavioral and electrophysiological findings. Neuropsychopharmacology. 2015;40(5):1130-40.
198. Palpacuer C, Laviolle B, Boussageon R, Reymann JM, Bellissant E, Naudet F. Risks and Benefits of Nalmefene in the Treatment of Adult Alcohol Dependence: A Systematic Literature Review and Meta-Analysis of Published and Unpublished Double-Blind Randomized Controlled Trials. PLoS Med. 2015;12(12):e1001924.
199. Guglielmo R, Martinotti G, Quatrale M, Ioime L, Kadilli I, Di Nicola M, et al. Topiramate in Alcohol Use Disorders: Review and Update. CNS Drugs. 2015;29(5):383-95.
200. Amiri A, Torabi Parizi G, Kousha M, Saadat F, Modabbernia MJ, Najafi K, et al. Changes in plasma Brain-derived neurotrophic factor (BDNF) levels induced by methylphenidate in children with Attention deficit-hyperactivity disorder (ADHD). Prog Neuropsychopharmacol Biol Psychiatry. 2013;47:20-4.
201. Oakes HV, DeVee CE, Farmer B, Allen SA, Hall AN, Ensley T, et al. Neurogenesis within the hippocampus after chronic methylphenidate exposure. J Neural Transm (Vienna). 2019;126(2):201-9.
202. Akay AP, Resmi H, Guney SA, Erkuran HO, Ozyurt G, Sargin E, et al. Serum brain-derived neurotrophic factor levels in treatment-naive boys with attention-deficit/hyperactivity disorder treated with methylphenidate: an 8-week, observational pretest-posttest study. Eur Child Adolesc Psychiatry. 2018;27(1):127-35.

203. Barrett SP, Pihl RO. Oral methylphenidate-alcohol co-abuse. J Clin Psychopharmacol. 2002;22(6):633-4.
204. Markowitz JS, Logan BK, Diamond F, Patrick KS. Detection of the novel metabolite ethylphenidate after methylphenidate overdose with alcohol coingestion. J Clin Psychopharmacol. 1999;19(4):362-6.
205. Caspersen CJ, Powell KE, Christenson GM. Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research. Public Health Rep. 1985;100(2):126-31.
206. Thivel D, Tremblay A, Genin PM, Panahi S, Riviere D, Duclos M. Physical Activity, Inactivity, and Sedentary Behaviors: Definitions and Implications in Occupational Health. Front Public Health. 2018;6:288.
207. Owen N, Leslie E, Salmon J, Fotheringham MJ. Environmental determinants of physical activity and sedentary behavior. Exerc Sport Sci Rev. 2000;28(4):153-8.
208. Warburton DE, Nicol CW, Bredin SS. Health benefits of physical activity: the evidence. CMAJ. 2006;174(6):801-9.
209. Bonaiuti D, Shea B, Iovine R, Negrini S, Robinson V, Kemper HC, et al. Exercise for preventing and treating osteoporosis in postmenopausal women. Cochrane Database Syst Rev. 2002(3):CD000333.
210. Bianchi VE. Weight loss is a critical factor to reduce inflammation. Clin Nutr ESPEN. 2018;28:21-35.
211. Petridou A, Siopi A, Mougios V. Exercise in the management of obesity. Metabolism. 2018.
212. Sellami M, Gasmi M, Denham J, Hayes LD, Stratton D, Padulo J, et al. Effects of Acute and Chronic Exercise on Immunological Parameters in the Elderly Aged: Can Physical Activity Counteract the Effects of Aging? Front Immunol. 2018;9:2187.
213. Phillips C, Baktir MA, Srivatsan M, Salehi A. Neuroprotective effects of physical activity on the brain: a closer look at trophic factor signaling. Front Cell Neurosci. 2014;8:170.
214. Swain RA, Harris AB, Wiener EC, Dutka MV, Morris HD, Theien BE, et al. Prolonged exercise induces angiogenesis and increases cerebral blood volume in primary motor cortex of the rat. Neuroscience. 2003;117(4):1037-46.
215. Nicastro TM, Greenwood BN. Central monoaminergic systems are a site of convergence of signals conveying the experience of exercise to brain circuits involved in cognition and emotional behavior. Curr Zool. 2016;62(3):293-306.
216. Colcombe SJ, Erickson KI, Raz N, Webb AG, Cohen NJ, McAuley E, et al. Aerobic fitness reduces brain tissue loss in aging humans. J Gerontol A Biol Sci Med Sci. 2003;58(2):176-80.
217. Paillard T, Rolland Y, de Souto Barreto P. Protective Effects of Physical Exercise in Alzheimer's Disease and Parkinson's Disease: A Narrative Review. J Clin Neurol. 2015;11(3):212-9.
218. Yuede CM, Timson BF, Hettinger JC, Yuede KM, Edwards HM, Lawson JE, et al. Interactions between stress and physical activity on Alzheimer's disease pathology. Neurobiol Stress. 2018;8:158-71.
219. Carek PJ, Laibstain SE, Carek SM. Exercise for the treatment of depression and anxiety. Int J Psychiatry Med. 2011;41(1):15-28.
220. Greenwood BN, Foley TE, Day HE, Campisi J, Hammack SH, Campeau S, et al. Freewheel running prevents learned helplessness/behavioral depression: role of dorsal raphe serotonergic neurons. J Neurosci. 2003;23(7):2889-98.

221. Smith MA, Lynch WJ. Exercise as a potential treatment for drug abuse: evidence from preclinical studies. Front Psychiatry. 2011;2:82.
222. Cooney GM, Dwan K, Greig CA, Lawlor DA, Rimer J, Waugh FR, et al. Exercise for depression. Cochrane Database Syst Rev. 2013(9):CD004366.
223. Darlington TM, McCarthy RD, Cox RJ, Miyamoto-Ditmon J, Gallego X, Ehringer MA. Voluntary wheel running reduces voluntary consumption of ethanol in mice: identification of candidate genes through striatal gene expression profiling. Genes Brain Behav. 2016;15(5):474-90.
224. Belke TW, Wagner JP. The reinforcing property and the rewarding aftereffect of wheel running in rats: a combination of two paradigms. Behav Processes. 2005;68(2):165-72.
225. Belke TW. Varying wheel-running reinforcer duration within a session: effect on the revolution-postreinforcement pause relation. J Exp Anal Behav. 2000;73(2):225-39.
226. Greenwood BN, Foley TE, Le TV, Strong PV, Loughridge AB, Day HE, et al. Long-term voluntary wheel running is rewarding and produces plasticity in the mesolimbic reward pathway. Behav Brain Res. 2011;217(2):354-62.
227. Herrera NA, Jesus I, Shinohara AL, Dionisio TJ, Santos CF, Amaral SL. Exercise training attenuates dexamethasone-induced hypertension by improving autonomic balance to the heart, sympathetic vascular modulation and skeletal muscle microcirculation. J Hypertens. 2016;34(10):1967-76.
228. Basso JC, Morrell JI. The medial prefrontal cortex and nucleus accumbens mediate the motivation for voluntary wheel running in the rat. Behav Neurosci. 2015;129(4):457-72.
229. Meijer JH, Robbers Y. Wheel running in the wild. Proc Biol Sci. 2014;281(1786).
230. Read JP, Brown RA, Marcus BH, Kahler CW, Ramsey SE, Dubreuil ME, et al. Exercise attitudes and behaviors among persons in treatment for alcohol use disorders. J Subst Abuse Treat. 2001;21(4):199-206.
231. Manthou E, Georgakouli K, Fatouros IG, Gianoulakis C, Theodorakis Y, Jamurtas AZ. Role of exercise in the treatment of alcohol use disorders. Biomed Rep. 2016;4(5):535-45.
232. Brene S, Bjornebekk A, Aberg E, Mathe AA, Olson L, Werme M. Running is rewarding and antidepressive. Physiol Behav. 2007;92(1-2):136-40.
233. Rasmussen EB, Hillman C. Naloxone and rimonabant reduce the reinforcing properties of exercise in rats. Exp Clin Psychopharmacol. 2011;19(6):389-400.
234. Lett BT, Grant VL, Byrne MJ, Koh MT. Pairings of a distinctive chamber with the aftereffect of wheel running produce conditioned place preference. Appetite. 2000;34(1):87-94.
235. Ozburn AR, Harris RA, Blednov YA. Wheel running, voluntary ethanol consumption, and hedonic substitution. Alcohol. 2008;42(5):417-24.
236. Werme M, Hermanson E, Carmine A, Buervenich S, Zetterstrom RH, Thoren P, et al. Decreased ethanol preference and wheel running in Nurr1-deficient mice. Eur J Neurosci. 2003;17(11):2418-24.
237. Martinsen M, Sundgot-Borgen J. Adolescent elite athletes' cigarette smoking, use of snus, and alcohol. Scand J Med Sci Sports. 2014;24(2):439-46.
238. Nelson MC, Gordon-Larsen P. Physical activity and sedentary behavior patterns are associated with selected adolescent health risk behaviors. Pediatrics. 2006;117(4):1281-90.

239. Terry-McElrath YM, O'Malley PM. Substance use and exercise participation among young adults: parallel trajectories in a national cohort-sequential study. Addiction. 2011;106(10):1855-65; discussion 66-7.
240. Ussher M, Sampuran AK, Doshi R, West R, Drummond DC. Acute effect of a brief bout of exercise on alcohol urges. Addiction. 2004;99(12):1542-7.
241. Brown RA, Abrantes AM, Read JP, Marcus BH, Jakicic J, Strong DR, et al. Aerobic exercise for alcohol recovery: rationale, program description, and preliminary findings. Behav Modif. 2009;33(2):220-49.
242. Brown RA, Abrantes AM, Minami H, Read JP, Marcus BH, Jakicic JM, et al. A preliminary, randomized trial of aerobic exercise for alcohol dependence. J Subst Abuse Treat. 2014;47(1):1-9.
243. Brown RA, Abrantes AM, Read JP, Marcus BH, Jakicic J, Strong DR, et al. A Pilot Study of Aerobic Exercise as an Adjunctive Treatment for Drug Dependence. Ment Health Phys Act. 2010;3(1):27-34.
244. Weinstock J, Barry D, Petry NM. Exercise-related activities are associated with positive outcome in contingency management treatment for substance use disorders. Addict Behav. 2008;33(8):1072-5.
245. French MT, Popovici I, Maclean JC. Do alcohol consumers exercise more? Findings from a national survey. Am J Health Promot. 2009;24(1):2-10.
246. Lisha NE, Martens M, Leventhal AM. Age and gender as moderators of the relationship between physical activity and alcohol use. Addict Behav. 2011;36(9):933-6.
247. Leichliter JS, Meilman PW, Presley CA, Cashin JR. Alcohol use and related consequences among students with varying levels of involvement in college athletics. J Am Coll Health. 1998;46(6):257-62.
248. Sanchez V, Lycas MD, Lynch WJ, Brunzell DH. Wheel running exercise attenuates vulnerability to self-administer nicotine in rats. Drug Alcohol Depend. 2015;156:193-8.
249. Miller ML, Vaillancourt BD, Wright MJ, Jr., Aarde SM, Vandewater SA, Creehan KM, et al. Reciprocal inhibitory effects of intravenous d-methamphetamine self-administration and wheel activity in rats. Drug Alcohol Depend. 2012;121(1-2):90-6.
250. Smith MA, Schmidt KT, Iordanou JC, Mustroph ML. Aerobic exercise decreases the positive-reinforcing effects of cocaine. Drug Alcohol Depend. 2008;98(1-2):129-35.
251. Smith MA, Pitts EG. Wheel running decreases the positive reinforcing effects of heroin. Pharmacol Rep. 2012;64(4):960-4.
252. Smith MA, Witte MA. The effects of exercise on cocaine self-administration, food-maintained responding, and locomotor activity in female rats: importance of the temporal relationship between physical activity and initial drug exposure. Exp Clin Psychopharmacol. 2012;20(6):437-46.
253. Lacy RT, Strickland JC, Brophy MK, Witte MA, Smith MA. Exercise decreases speedball self-administration. Life Sci. 2014;114(2):86-92.
254. Hosseini M, Alaei HA, Naderi A, Sharifi MR, Zahed R. Treadmill exercise reduces self-administration of morphine in male rats. Pathophysiology. 2009;16(1):3-7.
255. Strickland JC, Abel JM, Lacy RT, Beckmann JS, Witte MA, Lynch WJ, et al. The effects of resistance exercise on cocaine self-administration, muscle hypertrophy, and BDNF expression in the nucleus accumbens. Drug Alcohol Depend. 2016;163:186-94.
256. Smith MA, Fronk GE, Abel JM, Lacy RT, Bills SE, Lynch WJ. Resistance exercise decreases heroin self-administration and alters gene expression in the nucleus accumbens of heroin-exposed rats. Psychopharmacology (Berl). 2018;235(4):1245-55.
257. Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T. Mouse and rat BDNF gene structure and expression revisited. J Neurosci Res. 2007;85(3):525-35.

258. Bekinschtein P, Cammarota M, Medina JH. BDNF and memory processing. Neuropharmacology. 2014;76 Pt C:677-83.
259. Chen SD, Wu CL, Hwang WC, Yang DI. More Insight into BDNF against Neurodegeneration: Anti-Apoptosis, Anti-Oxidation, and Suppression of Autophagy. Int J Mol Sci. 2017;18(3).
260. Zuccato C, Cattaneo E. Huntington's disease. Handb Exp Pharmacol. 2014;220:357-409.
261. Maynard KR, Hill JL, Calcaterra NE, Palko ME, Kardian A, Paredes D, et al. Functional Role of BDNF Production from Unique Promoters in Aggression and Serotonin Signaling. Neuropsychopharmacology. 2016;41(8):1943-55.
262. Zheng F, Zhou X, Moon C, Wang H. Regulation of brain-derived neurotrophic factor expression in neurons. Int J Physiol Pathophysiol Pharmacol. 2012;4(4):188-200.
263. Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, et al. Anterograde transport of brain-derived neurotrophic factor and its role in the brain. Nature. 1997;389(6653):856-60.
264. Baquet ZC, Gorski JA, Jones KR. Early striatal dendrite deficits followed by neuron loss with advanced age in the absence of anterograde cortical brain-derived neurotrophic factor. J Neurosci. 2004;24(17):4250-8.
265. Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H, Cordelieres FP, et al. Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell. 2004;118(1):127-38.
266. Cattaneo E, Zuccato C, Tartari M. Normal huntingtin function: an alternative approach to Huntington's disease. Nat Rev Neurosci. 2005;6(12):919-30.
267. Park H, Popescu A, Poo MM. Essential role of presynaptic NMDA receptors in activity-dependent BDNF secretion and corticostriatal LTP. Neuron. 2014;84(5):1009-22.
268. Logrip ML, Janak PH, Ron D. Dynorphin is a downstream effector of striatal BDNF regulation of ethanol intake. FASEB J. 2008;22(7):2393-404.
269. Logrip ML, Koob GF, Zorrilla EP. Role of corticotropin-releasing factor in drug addiction: potential for pharmacological intervention. CNS Drugs. 2011;25(4):271-87.
270. McCarthy DM, Brown AN, Bhide PG. Regulation of BDNF expression by cocaine. Yale J Biol Med. 2012;85(4):437-46.
271. Kojima M, Takei N, Numakawa T, Ishikawa Y, Suzuki S, Matsumoto T, et al. Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activity-dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. J Neurosci Res. 2001;64(1):1-10.
272. Reichardt LF. Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci. 2006;361(1473):1545-64.
273. Tuvikene J, Pruunsild P, Orav E, Esvald EE, Timmusk T. AP-1 Transcription Factors Mediate BDNF-Positive Feedback Loop in Cortical Neurons. J Neurosci. 2016;36(4):1290-305.
274. Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, Konnerth A. Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. Nature. 2003;426(6962):74-8.
275. Sandhya VK, Raju R, Verma R, Advani J, Sharma R, Radhakrishnan A, et al. A network map of BDNF/TRKB and BDNF/p75NTR signaling system. J Cell Commun Signal. 2013;7(4):301-7.

276. Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, et al. Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron. 1993;10(3):475-89.
277. Chiaruttini C, Sonego M, Baj G, Simonato M, Tongiorgi E. BDNF mRNA splice variants display activity-dependent targeting to distinct hippocampal laminae. Mol Cell Neurosci. 2008;37(1):11-9.
278. Baj G, Leone E, Chao MV, Tongiorgi E. Spatial segregation of BDNF transcripts enables BDNF to differentially shape distinct dendritic compartments. Proc Natl Acad Sci U S A. 2011;108(40):16813-8.
279. Pruunsild P, Kazantseva A, Aid T, Palm K, Timmusk T. Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. Genomics. 2007;90(3):397-406.
280. Chiaruttini C, Vicario A, Li Z, Baj G, Braiuca P, Wu Y, et al. Dendritic trafficking of BDNF mRNA is mediated by translin and blocked by the G196A (Val66Met) mutation. Proc Natl Acad Sci U S A. 2009;106(38):16481-6.
281. Sakata K, Woo NH, Martinowich K, Greene JS, Schloesser RJ, Shen L, et al. Critical role of promoter IV-driven BDNF transcription in GABAergic transmission and synaptic plasticity in the prefrontal cortex. Proc Natl Acad Sci U S A. 2009;106(14):5942-7.
282. Dalley JW, Roiser JP. Dopamine, serotonin and impulsivity. Neuroscience. 2012;215:42-58.
283. Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell. 2003;112(2):257-69.
284. Colle R, Trabado S, David DJ, Brailly-Tabard S, Hardy P, Falissard B, et al. Plasma BDNF Level in Major Depression: Biomarker of the Val66Met BDNF Polymorphism and of the Clinical Course in Met Carrier Patients. Neuropsychobiology. 2017;75(1):39-45.
285. Li M, Chang H, Xiao X. BDNF Val66Met polymorphism and bipolar disorder in European populations: A risk association in case-control, family-based and GWAS studies. Neurosci Biobehav Rev. 2016;68:218-33.
286. Fan J, Sklar P. Genetics of bipolar disorder: focus on BDNF Val66Met polymorphism. Novartis Found Symp. 2008;289:60-72; discussion -3, 87-93.
287. Altmann V, Schumacher-Schuh AF, Rieck M, Callegari-Jacques SM, Rieder CR, Hutz MH. Val66Met BDNF polymorphism is associated with Parkinson's disease cognitive impairment. Neurosci Lett. 2016;615:88-91.
288. Boots EA, Schultz SA, Clark LR, Racine AM, Darst BF, Koscik RL, et al. BDNF Val66Met predicts cognitive decline in the Wisconsin Registry for Alzheimer's Prevention. Neurology. 2017;88(22):2098-106.
289. Kennedy KM, Reese ED, Horn MM, Sizemore AN, Unni AK, Meerbrey ME, et al. BDNF val66met polymorphism affects aging of multiple types of memory. Brain Res. 2015;1612:104-17.
290. Matsushita S, Kimura M, Miyakawa T, Yoshino A, Murayama M, Masaki T, et al. Association study of brain-derived neurotrophic factor gene polymorphism and alcoholism. Alcohol Clin Exp Res. 2004;28(11):1609-12.
291. Klimkiewicz A, Mach A, Jakubczyk A, Klimkiewicz J, Wnorowska A, Kopera M, et al. COMT and BDNF Gene Variants Help to Predict Alcohol Consumption in Alcohol-dependent Patients. J Addict Med. 2017;11(2):114-8.
292. Benzerouk F, Gierski F, Gorwood P, Ramoz N, Stefaniak N, Hubsch B, et al. Brain-derived neurotrophic factor (BDNF) Val66Met polymorphism and its implication in

executive functions in adult offspring of alcohol-dependent probands. Alcohol. 2013;47(4):271-4.
293. Wojnar M, Brower KJ, Strobbe S, Ilgen M, Matsumoto H, Nowosad I, et al. Association between Val66Met brain-derived neurotrophic factor (BDNF) gene polymorphism and post-treatment relapse in alcohol dependence. Alcohol Clin Exp Res. 2009;33(4):693-702.
294. Heilig M, Barbier E, Johnstone AL, Tapocik J, Meinhardt MW, Pfarr S, et al. Reprogramming of mPFC transcriptome and function in alcohol dependence. Genes Brain Behav. 2017;16(1):86-100.
295. Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, et al. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. Science. 2003;302(5646):890-3.
296. Sakharkar AJ, Vetreno RP, Zhang H, Kokare DM, Crews FT, Pandey SC. A role for histone acetylation mechanisms in adolescent alcohol exposure-induced deficits in hippocampal brain-derived neurotrophic factor expression and neurogenesis markers in adulthood. Brain Struct Funct. 2016;221(9):4691-703.
297. Pandey SC, Sakharkar AJ, Tang L, Zhang H. Potential role of adolescent alcohol exposure-induced amygdaloid histone modifications in anxiety and alcohol intake during adulthood. Neurobiol Dis. 2015;82:607-19.
298. Sleiman SF, Henry J, Al-Haddad R, El Hayek L, Abou Haidar E, Stringer T, et al. Exercise promotes the expression of brain derived neurotrophic factor (BDNF) through the action of the ketone body beta-hydroxybutyrate. Elife. 2016;5.
299. Intlekofer KA, Berchtold NC, Malvaez M, Carlos AJ, McQuown SC, Cunningham MJ, et al. Exercise and sodium butyrate transform a subthreshold learning event into long-term memory via a brain-derived neurotrophic factor-dependent mechanism. Neuropsychopharmacology. 2013;38(10):2027-34.
300. Lin Y, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, et al. Activity-dependent regulation of inhibitory synapse development by Npas4. Nature. 2008;455(7217):1198-204.
301. Kairisalo M, Korhonen L, Sepp M, Pruunsild P, Kukkonen JP, Kivinen J, et al. NF-kappaB-dependent regulation of brain-derived neurotrophic factor in hippocampal neurons by X-linked inhibitor of apoptosis protein. Eur J Neurosci. 2009;30(6):958-66.
302. Hara D, Fukuchi M, Miyashita T, Tabuchi A, Takasaki I, Naruse Y, et al. Remote control of activity-dependent BDNF gene promoter-I transcription mediated by REST/NRSF. Biochem Biophys Res Commun. 2009;384(4):506-11.
303. Pruunsild P, Sepp M, Orav E, Koppel I, Timmusk T. Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. J Neurosci. 2011;31(9):3295-308.
304. Flavell SW, Kim TK, Gray JM, Harmin DA, Hemberg M, Hong EJ, et al. Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. Neuron. 2008;60(6):1022-38.
305. Lyons MR, Schwarz CM, West AE. Members of the myocyte enhancer factor 2 transcription factor family differentially regulate Bdnf transcription in response to neuronal depolarization. J Neurosci. 2012;32(37):12780-5.
306. Hansson AC, Sommer WH, Metsis M, Stromberg I, Agnati LF, Fuxe K. Corticosterone actions on the hippocampal brain-derived neurotrophic factor expression are mediated by exon IV promoter. J Neuroendocrinol. 2006;18(2):104-14.

307. Takeuchi Y, Yamamoto H, Miyakawa T, Miyamoto E. Increase of brain-derived neurotrophic factor gene expression in NG108-15 cells by the nuclear isoforms of Ca2+/ calmodulin-dependent protein kinase II. J Neurochem. 2000;74(5):1913-22.
308. Roth TL, Lubin FD, Funk AJ, Sweatt JD. Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry. 2009;65(9):760-9.
309. Moonat S, Pandey SC. Stress, epigenetics, and alcoholism. Alcohol Res. 2012;34(4):495-505.
310. Ballestar E, Wolffe AP. Methyl-CpG-binding proteins. Targeting specific gene repression. Eur J Biochem. 2001;268(1):1-6.
311. Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, et al. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science. 2006;314(5796):140-3.
312. Sheikh HI, Hayden EP, Kryski KR, Smith HJ, Singh SM. Genotyping the BDNF rs6265 (val66met) polymorphism by one-step amplified refractory mutation system PCR. Psychiatr Genet. 2010;20(3):109-12.
313. Colzato LS, Van der Does AJ, Kouwenhoven C, Elzinga BM, Hommel B. BDNF Val66Met polymorphism is associated with higher anticipatory cortisol stress response, anxiety, and alcohol consumption in healthy adults. Psychoneuroendocrinology. 2011;36(10):1562-9.
314. Warnault V, Darcq E, Morisot N, Phamluong K, Wilbrecht L, Massa SM, et al. The BDNF Valine 68 to Methionine Polymorphism Increases Compulsive Alcohol Drinking in Mice That Is Reversed by Tropomyosin Receptor Kinase B Activation. Biol Psychiatry. 2016;79(6):463-73.
315. Joe KH, Kim YK, Kim TS, Roh SW, Choi SW, Kim YB, et al. Decreased plasma brain-derived neurotrophic factor levels in patients with alcohol dependence. Alcohol Clin Exp Res. 2007;31(11):1833-8.
316. Zanardini R, Fontana A, Pagano R, Mazzaro E, Bergamasco F, Romagnosi G, et al. Alterations of brain-derived neurotrophic factor serum levels in patients with alcohol dependence. Alcohol Clin Exp Res. 2011;35(8):1529-33.
317. McGough NN, He DY, Logrip ML, Jeanblanc J, Phamluong K, Luong K, et al. RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. J Neurosci. 2004;24(46):10542-52.
318. Hensler JG, Ladenheim EE, Lyons WE. Ethanol consumption and serotonin-1A (5-HT1A) receptor function in heterozygous BDNF (+/-) mice. J Neurochem. 2003;85(5):1139-47.
319. Pandey SC, Roy A, Zhang H, Xu T. Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. J Neurosci. 2004;24(21):5022-30.
320. Jeanblanc J, He DY, McGough NN, Logrip ML, Phamluong K, Janak PH, et al. The dopamine D3 receptor is part of a homeostatic pathway regulating ethanol consumption. J Neurosci. 2006;26(5):1457-64.
321. Prakash A, Zhang H, Pandey SC. Innate differences in the expression of brain-derived neurotrophic factor in the regions within the extended amygdala between alcohol preferring and nonpreferring rats. Alcohol Clin Exp Res. 2008;32(6):909-20.
322. Moonat S, Sakharkar AJ, Zhang H, Tang L, Pandey SC. Aberrant histone deacetylase2-mediated histone modifications and synaptic plasticity in the amygdala predisposes to anxiety and alcoholism. Biol Psychiatry. 2013;73(8):763-73.

323. Pandey SC, Zhang H, Roy A, Misra K. Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors. J Neurosci. 2006;26(32):8320-31.
324. Somkuwar SS, Fannon MJ, Staples MC, Zamora-Martinez ER, Navarro AI, Kim A, et al. Alcohol dependence-induced regulation of the proliferation and survival of adult brain progenitors is associated with altered BDNF-TrkB signaling. Brain Struct Funct. 2016;221(9):4319-35.
325. Tapocik JD, Barbier E, Flanigan M, Solomon M, Pincus A, Pilling A, et al. microRNA-206 in rat medial prefrontal cortex regulates BDNF expression and alcohol drinking. J Neurosci. 2014;34(13):4581-8.
326. Tapocik JD, Solomon M, Flanigan M, Meinhardt M, Barbier E, Schank JR, et al. Coordinated dysregulation of mRNAs and microRNAs in the rat medial prefrontal cortex following a history of alcohol dependence. Pharmacogenomics J. 2013;13(3):286-96.
327. Orru A, Caffino L, Moro F, Cassina C, Giannotti G, Di Clemente A, et al. Contingent and non-contingent recreational-like exposure to ethanol alters BDNF expression and signaling in the cortico-accumbal network differently. Psychopharmacology (Berl). 2016;233(17):3149-60.
328. Logrip ML, Janak PH, Ron D. Escalating ethanol intake is associated with altered corticostriatal BDNF expression. J Neurochem. 2009;109(5):1459-68.
329. Wrann CD, White JP, Salogiannnis J, Laznik-Bogoslavski D, Wu J, Ma D, et al. Exercise induces hippocampal BDNF through a PGC-1alpha/FNDC5 pathway. Cell Metab. 2013;18(5):649-59.
330. Balleine BW, O'Doherty JP. Human and rodent homologies in action control: corticostriatal determinants of goal-directed and habitual action. Neuropsychopharmacology. 2010;35(1):48-69.
331. Jeanblanc J, He DY, Carnicella S, Kharazia V, Janak PH, Ron D. Endogenous BDNF in the dorsolateral striatum gates alcohol drinking. J Neurosci. 2009;29(43):13494-502.
332. Leggio GM, Camillieri G, Platania CB, Castorina A, Marrazzo G, Torrisi SA, et al. Dopamine D3 receptor is necessary for ethanol consumption: an approach with buspirone. Neuropsychopharmacology. 2014;39(8):2017-28.
333. Guillin O, Diaz J, Carroll P, Griffon N, Schwartz JC, Sokoloff P. BDNF controls dopamine D3 receptor expression and triggers behavioural sensitization. Nature. 2001;411(6833):86-9.
334. Balleine BW, Delgado MR, Hikosaka O. The role of the dorsal striatum in reward and decision-making. J Neurosci. 2007;27(31):8161-5.
335. Giuliano C, Belin D, Everitt BJ. Compulsive Alcohol Seeking Results from a Failure to Disengage Dorsolateral Striatal Control over Behavior. J Neurosci. 2019;39(9):1744-54.
336. Bahi A, Dreyer JL. Striatal modulation of BDNF expression using microRNA124a-expressing lentiviral vectors impairs ethanol-induced conditioned-place preference and voluntary alcohol consumption. Eur J Neurosci. 2013;38(2):2328-37.
337. Chen G, Cuzon Carlson VC, Wang J, Beck A, Heinz A, Ron D, et al. Striatal involvement in human alcoholism and alcohol consumption, and withdrawal in animal models. Alcohol Clin Exp Res. 2011;35(10):1739-48.
338. Peterson AB, Abel JM, Lynch WJ. Dose-dependent effects of wheel running on cocaine-seeking and prefrontal cortex Bdnf exon IV expression in rats. Psychopharmacology (Berl). 2014;231(7):1305-14.

339. Huang AM, Jen CJ, Chen HF, Yu L, Kuo YM, Chen HI. Compulsive exercise acutely upregulates rat hippocampal brain-derived neurotrophic factor. J Neural Transm (Vienna). 2006;113(7):803-11.
340. Chen MJ, Russo-Neustadt AA. Running exercise-induced up-regulation of hippocampal brain-derived neurotrophic factor is CREB-dependent. Hippocampus. 2009;19(10):962-72.
341. Oliff HS, Berchtold NC, Isackson P, Cotman CW. Exercise-induced regulation of brain-derived neurotrophic factor (BDNF) transcripts in the rat hippocampus. Brain Res Mol Brain Res. 1998;61(1-2):147-53.
342. Soya H, Nakamura T, Deocaris CC, Kimpara A, Iimura M, Fujikawa T, et al. BDNF induction with mild exercise in the rat hippocampus. Biochem Biophys Res Commun. 2007;358(4):961-7.
343. Ke Z, Yip SP, Li L, Zheng XX, Tong KY. The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model. PLoS One. 2011;6(2):e16643.
344. Ding Y, Li J, Luan X, Ding YH, Lai Q, Rafols JA, et al. Exercise pre-conditioning reduces brain damage in ischemic rats that may be associated with regional angiogenesis and cellular overexpression of neurotrophin. Neuroscience. 2004;124(3):583-91.
345. Church DD, Hoffman JR, Mangine GT, Jajtner AR, Townsend JR, Beyer KS, et al. Comparison of high-intensity vs. high-volume resistance training on the BDNF response to exercise. J Appl Physiol (1985). 2016;121(1):123-8.
346. Zoladz JA, Pilc A, Majerczak J, Grandys M, Zapart-Bukowska J, Duda K. Endurance training increases plasma brain-derived neurotrophic factor concentration in young healthy men. J Physiol Pharmacol. 2008;59 Suppl 7:119-32.
347. Krabbe KS, Nielsen AR, Krogh-Madsen R, Plomgaard P, Rasmussen P, Erikstrup C, et al. Brain-derived neurotrophic factor (BDNF) and type 2 diabetes. Diabetologia. 2007;50(2):431-8.
348. Rasmussen P, Brassard P, Adser H, Pedersen MV, Leick L, Hart E, et al. Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. Exp Physiol. 2009;94(10):1062-9.
349. Seifert T, Brassard P, Wissenberg M, Rasmussen P, Nordby P, Stallknecht B, et al. Endurance training enhances BDNF release from the human brain. Am J Physiol Regul Integr Comp Physiol. 2010;298(2):R372-7.
350. Gold SM, Schulz KH, Hartmann S, Mladek M, Lang UE, Hellweg R, et al. Basal serum levels and reactivity of nerve growth factor and brain-derived neurotrophic factor to standardized acute exercise in multiple sclerosis and controls. J Neuroimmunol. 2003;138(1-2):99-105.
351. Ferris LT, Williams JS, Shen CL. The effect of acute exercise on serum brain-derived neurotrophic factor levels and cognitive function. Med Sci Sports Exerc. 2007;39(4):728-34.
352. Rojas Vega S, Struder HK, Vera Wahrmann B, Schmidt A, Bloch W, Hollmann W. Acute BDNF and cortisol response to low intensity exercise and following ramp incremental exercise to exhaustion in humans. Brain Res. 2006;1121(1):59-65.
353. Schmolesky MT, Webb DL, Hansen RA. The effects of aerobic exercise intensity and duration on levels of brain-derived neurotrophic factor in healthy men. J Sports Sci Med. 2013;12(3):502-11.
354. Newman JC, Verdin E. beta-hydroxybutyrate: much more than a metabolite. Diabetes Res Clin Pract. 2014;106(2):173-81.

355. Pellerin L, Bergersen LH, Halestrap AP, Pierre K. Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain. J Neurosci Res. 2005;79(1-2):55-64.
356. Henderson ST. Ketone bodies as a therapeutic for Alzheimer's disease. Neurotherapeutics. 2008;5(3):470-80.
357. Koppel I, Timmusk T. Differential regulation of Bdnf expression in cortical neurons by class-selective histone deacetylase inhibitors. Neuropharmacology. 2013;75:106-15.
358. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, et al. Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science. 2013;339(6116):211-4.
359. Marosi K, Kim SW, Moehl K, Scheibye-Knudsen M, Cheng A, Cutler R, et al. 3-Hydroxybutyrate regulates energy metabolism and induces BDNF expression in cerebral cortical neurons. J Neurochem. 2016;139(5):769-81.
360. Xie Z, Zhang D, Chung D, Tang Z, Huang H, Dai L, et al. Metabolic Regulation of Gene Expression by Histone Lysine beta-Hydroxybutyrylation. Mol Cell. 2016;62(2):194-206.
361. Lin J, Puigserver P, Donovan J, Tarr P, Spiegelman BM. Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. J Biol Chem. 2002;277(3):1645-8.
362. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Invest. 2006;116(3):615-22.
363. Belviranli M, Okudan N. Exercise Training Protects Against Aging-Induced Cognitive Dysfunction via Activation of the Hippocampal PGC-1alpha/FNDC5/BDNF Pathway. Neuromolecular Med. 2018;20(3):386-400.
364. Steiner JL, Murphy EA, McClellan JL, Carmichael MD, Davis JM. Exercise training increases mitochondrial biogenesis in the brain. J Appl Physiol (1985). 2011;111(4):1066-71.
365. Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, et al. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J. 2002;16(14):1879-86.
366. Norheim F, Langleite TM, Hjorth M, Holen T, Kielland A, Stadheim HK, et al. The effects of acute and chronic exercise on PGC-1alpha, irisin and browning of subcutaneous adipose tissue in humans. FEBS J. 2014;281(3):739-49.
367. Nygaard H, Slettalokken G, Vegge G, Hollan I, Whist JE, Strand T, et al. Irisin in blood increases transiently after single sessions of intense endurance exercise and heavy strength training. PLoS One. 2015;10(3):e0121367.
368. Lecker SH, Zavin A, Cao P, Arena R, Allsup K, Daniels KM, et al. Expression of the irisin precursor FNDC5 in skeletal muscle correlates with aerobic exercise performance in patients with heart failure. Circ Heart Fail. 2012;5(6):812-8.
369. McCutcheon JE, Beeler JA, Roitman MF. Sucrose-predictive cues evoke greater phasic dopamine release than saccharin-predictive cues. Synapse. 2012;66(4):346-51.
370. Greenwood BN. The role of dopamine in overcoming aversion with exercise. Brain Res. 2018.
371. Roitman MF, Wheeler RA, Wightman RM, Carelli RM. Real-time chemical responses in the nucleus accumbens differentiate rewarding and aversive stimuli. Nat Neurosci. 2008;11(12):1376-7.

372. Larsson A, Svensson L, Soderpalm B, Engel JA. Role of different nicotinic acetylcholine receptors in mediating behavioral and neurochemical effects of ethanol in mice. Alcohol. 2002;28(3):157-67.
373. Larsson A, Engel JA. Neurochemical and behavioral studies on ethanol and nicotine interactions. Neurosci Biobehav Rev. 2004;27(8):713-20.
374. Engel JA, Jerlhag E. Alcohol: mechanisms along the mesolimbic dopamine system. Prog Brain Res. 2014;211:201-33.
375. Nestler EJ, Carlezon WA, Jr. The mesolimbic dopamine reward circuit in depression. Biol Psychiatry. 2006;59(12):1151-9.
376. Lawford BR, Young R, Noble EP, Kann B, Ritchie T. The D2 dopamine receptor (DRD2) gene is associated with co-morbid depression, anxiety and social dysfunction in untreated veterans with post-traumatic stress disorder. Eur Psychiatry. 2006;21(3):180-5.
377. Zhu X, Peng S, Zhang S, Zhang X. Stress-induced depressive behaviors are correlated with Par-4 and DRD2 expression in rat striatum. Behav Brain Res. 2011;223(2):329-35.
378. Clark PJ, Ghasem PR, Mika A, Day HE, Herrera JJ, Greenwood BN, et al. Wheel running alters patterns of uncontrollable stress-induced cfos mRNA expression in rat dorsal striatum direct and indirect pathways: A possible role for plasticity in adenosine receptors. Behav Brain Res. 2014;272:252-63.
379. Foley TE, Fleshner M. Neuroplasticity of dopamine circuits after exercise: implications for central fatigue. Neuromolecular Med. 2008;10(2):67-80.
380. Williams SN, Undieh AS. Dopamine D1-like receptor activation induces brain-derived neurotrophic factor protein expression. Neuroreport. 2009;20(6):606-10.
381. Kuppers E, Ivanova T, Karolczak M, Beyer C. Estrogen: a multifunctional messenger to nigrostriatal dopaminergic neurons. J Neurocytol. 2000;29(5-6):375-85.
382. Kuppers E, Beyer C. Dopamine regulates brain-derived neurotrophic factor (BDNF) expression in cultured embryonic mouse striatal cells. Neuroreport. 2001;12(6):1175-9.
383. Iwakura Y, Nawa H, Sora I, Chao MV. Dopamine D1 receptor-induced signaling through TrkB receptors in striatal neurons. J Biol Chem. 2008;283(23):15799-806.
384. Adachi N, Yoshimura A, Chiba S, Ogawa S, Kunugi H. Rotigotine, a dopamine receptor agonist, increased BDNF protein levels in the rat cortex and hippocampus. Neurosci Lett. 2018;662:44-50.
385. Juric DM, Miklic S, Carman-Krzan M. Monoaminergic neuronal activity up-regulates BDNF synthesis in cultured neonatal rat astrocytes. Brain Res. 2006;1108(1):54-62.
386. Miklic S, Juric DM, Carman-Krzan M. Differences in the regulation of BDNF and NGF synthesis in cultured neonatal rat astrocytes. Int J Dev Neurosci. 2004;22(3):119-30.
387. Schmidt U, Pilgrim C, Beyer C. Differentiative effects of dopamine on striatal neurons involve stimulation of the cAMP/PKA pathway. Mol Cell Neurosci. 1998;11(1-2):9-18.
388. Do T, Kerr B, Kuzhikandathil EV. Brain-derived neurotrophic factor regulates the expression of D1 dopamine receptors. J Neurochem. 2007;100(2):416-28.
389. Brito V, Beyer C, Kuppers E. BDNF-dependent stimulation of dopamine D5 receptor expression in developing striatal astrocytes involves PI3-kinase signaling. Glia. 2004;46(3):284-95.
390. Nicholls DG, Coffey ET. Glutamate exocytosis from isolated nerve terminals. Adv Second Messenger Phosphoprotein Res. 1994;29:189-203.

391. Willard SS, Koochekpour S. Glutamate, glutamate receptors, and downstream signaling pathways. Int J Biol Sci. 2013;9(9):948-59.
392. Banerjee N. Neurotransmitters in alcoholism: A review of neurobiological and genetic studies. Indian J Hum Genet. 2014;20(1):20-31.
393. Sari Y, Sakai M, Weedman JM, Rebec GV, Bell RL. Ceftriaxone, a beta-lactam antibiotic, reduces ethanol consumption in alcohol-preferring rats. Alcohol Alcohol. 2011;46(3):239-46.
394. Selim M, Bradberry CW. Effect of ethanol on extracellular 5-HT and glutamate in the nucleus accumbens and prefrontal cortex: comparison between the Lewis and Fischer 344 rat strains. Brain Res. 1996;716(1-2):157-64.
395. Fadda F, Rossetti ZL. Chronic ethanol consumption: from neuroadaptation to neurodegeneration. Prog Neurobiol. 1998;56(4):385-431.
396. Gonzales RA, Jaworski JN. Alcohol and glutamate. Alcohol Health Res World. 1997;21(2):120-7.
397. Maddock RJ, Casazza GA, Fernandez DH, Maddock MI. Acute Modulation of Cortical Glutamate and GABA Content by Physical Activity. J Neurosci. 2016;36(8):2449-57.
398. Venezia AC, Quinlan E, Roth SM. A single bout of exercise increases hippocampal Bdnf: influence of chronic exercise and noradrenaline. Genes Brain Behav. 2017;16(8):800-11.
399. Dietrich MO, Mantese CE, Porciuncula LO, Ghisleni G, Vinade L, Souza DO, et al. Exercise affects glutamate receptors in postsynaptic densities from cortical mice brain. Brain Res. 2005;1065(1-2):20-5.
400. Zhang F, Jia J, Wu Y, Hu Y, Wang Y. The effect of treadmill training pre-exercise on glutamate receptor expression in rats after cerebral ischemia. Int J Mol Sci. 2010;11(7):2658-69.
401. Herbst EA, Holloway GP. Exercise increases mitochondrial glutamate oxidation in the mouse cerebral cortex. Appl Physiol Nutr Metab. 2016;41(7):799-801.
402. Mackowiak M, O'Neill MJ, Hicks CA, Bleakman D, Skolnick P. An AMPA receptor potentiator modulates hippocampal expression of BDNF: an in vivo study. Neuropharmacology. 2002;43(1):1-10.
403. Legutko B, Li X, Skolnick P. Regulation of BDNF expression in primary neuron culture by LY392098, a novel AMPA receptor potentiator. Neuropharmacology. 2001;40(8):1019-27.
404. Vaynman S, Ying Z, Gomez-Pinilla F. Interplay between brain-derived neurotrophic factor and signal transduction modulators in the regulation of the effects of exercise on synaptic-plasticity. Neuroscience. 2003;122(3):647-57.
405. Alt A, Nisenbaum ES, Bleakman D, Witkin JM. A role for AMPA receptors in mood disorders. Biochem Pharmacol. 2006;71(9):1273-88.
406. Berridge CW, Spencer RC. Differential cognitive actions of norepinephrine a2 and a1 receptor signaling in the prefrontal cortex. Brain Res. 2016;1641(Pt B):189-96.
407. Becker HC. Effects of alcohol dependence and withdrawal on stress responsiveness and alcohol consumption. Alcohol Res. 2012;34(4):448-58.
408. Kent JM, Mathew SJ, Gorman JM. Molecular targets in the treatment of anxiety. Biol Psychiatry. 2002;52(10):1008-30.
409. Amit Z, Levitan DE, Brown ZE, Sutherland EA. Catecholaminergic involvement in alcohol's rewarding properties: implications for a treatment model for alcoholics. Adv Exp Med Biol. 1977;85A:485-94.

410. Umhau JC, Schwandt ML, Usala J, Geyer C, Singley E, George DT, et al. Pharmacologically induced alcohol craving in treatment seeking alcoholics correlates with alcoholism severity, but is insensitive to acamprosate. Neuropsychopharmacology. 2011;36(6):1178-86.
411. Simms JA, Haass-Koffler CL, Bito-Onon J, Li R, Bartlett SE. Mifepristone in the central nucleus of the amygdala reduces yohimbine stress-induced reinstatement of ethanol-seeking. Neuropsychopharmacology. 2012;37(4):906-18.
412. Ivy AS, Rodriguez FG, Garcia C, Chen MJ, Russo-Neustadt AA. Noradrenergic and serotonergic blockade inhibits BDNF mRNA activation following exercise and antidepressant. Pharmacol Biochem Behav. 2003;75(1):81-8.
413. Garcia C, Chen MJ, Garza AA, Cotman CW, Russo-Neustadt A. The influence of specific noradrenergic and serotonergic lesions on the expression of hippocampal brain-derived neurotrophic factor transcripts following voluntary physical activity. Neuroscience. 2003;119(3):721-32.
414. Dishman RK, Renner KJ, White-Welkley JE, Burke KA, Bunnell BN. Treadmill exercise training augments brain norepinephrine response to familiar and novel stress. Brain Res Bull. 2000;52(5):337-42.
415. Dunn AL, Reigle TG, Youngstedt SD, Armstrong RB, Dishman RK. Brain norepinephrine and metabolites after treadmill training and wheel running in rats. Med Sci Sports Exerc. 1996;28(2):204-9.
416. Zouhal H, Jacob C, Delamarche P, Gratas-Delamarche A. Catecholamines and the effects of exercise, training and gender. Sports Med. 2008;38(5):401-23.
417. Helmich I, Latini A, Sigwalt A, Carta MG, Machado S, Velasques B, et al. Neurobiological alterations induced by exercise and their impact on depressive disorders [corrected]. Clin Pract Epidemiol Ment Health. 2010;6:115-25.
418. Kraemer WJ, Fleck SJ, Maresh CM, Ratamess NA, Gordon SE, Goetz KL, et al. Acute hormonal responses to a single bout of heavy resistance exercise in trained power lifters and untrained men. Can J Appl Physiol. 1999;24(6):524-37.
419. Follesa P, Biggio F, Gorini G, Caria S, Talani G, Dazzi L, et al. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. Brain Res. 2007;1179:28-34.
420. Zafra F, Lindholm D, Castren E, Hartikka J, Thoenen H. Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. J Neurosci. 1992;12(12):4793-9.
421. Chen MJ, Russo-Neustadt AA. Nitric oxide signaling participates in norepinephrine-induced activity of neuronal intracellular survival pathways. Life Sci. 2007;81(16):1280-90.
422. Russo-Neustadt AA, Alejandre H, Garcia C, Ivy AS, Chen MJ. Hippocampal brain-derived neurotrophic factor expression following treatment with reboxetine, citalopram, and physical exercise. Neuropsychopharmacology. 2004;29(12):2189-99.
423. Rantamaki T, Hendolin P, Kankaanpaa A, Mijatovic J, Piepponen P, Domenici E, et al. Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor TrkB and induce phospholipase-Cgamma signaling pathways in mouse brain. Neuropsychopharmacology. 2007;32(10):2152-62.
424. Chen MJ, Nguyen TV, Pike CJ, Russo-Neustadt AA. Norepinephrine induces BDNF and activates the PI-3K and MAPK cascades in embryonic hippocampal neurons. Cell Signal. 2007;19(1):114-28.
425. Sari Y, Johnson VR, Weedman JM. Role of the serotonergic system in alcohol dependence: from animal models to clinics. Prog Mol Biol Transl Sci. 2011;98:401-43.

426. Hammoumi S, Payen A, Favre JD, Balmes JL, Benard JY, Husson M, et al. Does the short variant of the serotonin transporter linked polymorphic region constitute a marker of alcohol dependence? Alcohol. 1999;17(2):107-12.
427. LeMarquand D, Pihl RO, Benkelfat C. Serotonin and alcohol intake, abuse, and dependence: findings of animal studies. Biol Psychiatry. 1994;36(6):395-421.
428. Wong DT, Threlkeld PG, Lumeng L, Li TK. Higher density of serotonin-1A receptors in the hippocampus and cerebral cortex of alcohol-preferring P rats. Life Sci. 1990;46(3):231-5.
429. Maurel S, De Vry J, Schreiber R. 5-HT receptor ligands differentially affect operant oral self-administration of ethanol in the rat. Eur J Pharmacol. 1999;370(3):217-23.
430. Ciccocioppo R, Economidou D, Cippitelli A, Cucculelli M, Ubaldi M, Soverchia L, et al. Genetically selected Marchigian Sardinian alcohol-preferring (msP) rats: an animal model to study the neurobiology of alcoholism. Addict Biol. 2006;11(3-4):339-55.
431. Kranzler HR, Feinn R, Armeli S, Tennen H. Comparison of alcoholism subtypes as moderators of the response to sertraline treatment. Alcohol Clin Exp Res. 2012;36(3):509-16.
432. Patrick RP, Ames BN. Vitamin D and the omega-3 fatty acids control serotonin synthesis and action, part 2: relevance for ADHD, bipolar disorder, schizophrenia, and impulsive behavior. FASEB J. 2015;29(6):2207-22.
433. Cordeiro LMS, Rabelo PCR, Moraes MM, Teixeira-Coelho F, Coimbra CC, Wanner SP, et al. Physical exercise-induced fatigue: the role of serotonergic and dopaminergic systems. Braz J Med Biol Res. 2017;50(12):e6432.
434. Mizutani K, Sonoda S, Karasawa N, Yamada K, Shimpo K, Chihara T, et al. Effects of exercise after focal cerebral cortex infarction on basal ganglion. Neurol Sci. 2013;34(6):861-7.
435. Ohmatsu S, Nakano H, Tominaga T, Terakawa Y, Murata T, Morioka S. Activation of the serotonergic system by pedaling exercise changes anterior cingulate cortex activity and improves negative emotion. Behav Brain Res. 2014;270:112-7.
436. Zimmer P, Stritt C, Bloch W, Schmidt FP, Hubner ST, Binnebossel S, et al. The effects of different aerobic exercise intensities on serum serotonin concentrations and their association with Stroop task performance: a randomized controlled trial. Eur J Appl Physiol. 2016;116(10):2025-34.
437. Nakatani Y, Sato-Suzuki I, Tsujino N, Nakasato A, Seki Y, Fumoto M, et al. Augmented brain 5-HT crosses the blood-brain barrier through the 5-HT transporter in rat. Eur J Neurosci. 2008;27(9):2466-72.
438. Young LW, Darios ES, Watts SW. An immunohistochemical analysis of SERT in the blood-brain barrier of the male rat brain. Histochem Cell Biol. 2015;144(4):321-9.
439. Nibuya M, Morinobu S, Duman RS. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. J Neurosci. 1995;15(11):7539-47.
440. Russo-Neustadt A, Beard RC, Cotman CW. Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. Neuropsychopharmacology. 1999;21(5):679-82.
441. Duman RS. Role of neurotrophic factors in the etiology and treatment of mood disorders. Neuromolecular Med. 2004;5(1):11-25.
442. Saarelainen T, Hendolin P, Lucas G, Koponen E, Sairanen M, MacDonald E, et al. Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. J Neurosci. 2003;23(1):349-57.

443. Musazzi L, Rimland JM, Ieraci A, Racagni G, Domenici E, Popoli M. Pharmacological characterization of BDNF promoters I, II and IV reveals that serotonin and norepinephrine input is sufficient for transcription activation. Int J Neuropsychopharmacol. 2014;17(5):779-91.
444. Jiang DG, Jin SL, Li GY, Li QQ, Li ZR, Ma HX, et al. Serotonin regulates brain-derived neurotrophic factor expression in select brain regions during acute psychological stress. Neural Regen Res. 2016;11(9):1471-9.
445. Altar CA, Whitehead RE, Chen R, Wortwein G, Madsen TM. Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain. Biol Psychiatry. 2003;54(7):703-9.
446. Kronenberg G, Mosienko V, Gertz K, Alenina N, Hellweg R, Klempin F. Increased brain-derived neurotrophic factor (BDNF) protein concentrations in mice lacking brain serotonin. Eur Arch Psychiatry Clin Neurosci. 2016;266(3):281-4.
447. Chemel BR, Roth BL, Armbruster B, Watts VJ, Nichols DE. WAY-100635 is a potent dopamine D4 receptor agonist. Psychopharmacology (Berl). 2006;188(2):244-51.
448. Marona-Lewicka D, Nichols DE. WAY 100635 produces discriminative stimulus effects in rats mediated by dopamine D(4) receptor activation. Behav Pharmacol. 2009;20(1):114-8.
449. Lam MP, Marinelli PW, Bai L, Gianoulakis C. Effects of acute ethanol on opioid peptide release in the central amygdala: an in vivo microdialysis study. Psychopharmacology (Berl). 2008;201(2):261-71.
450. Olive MF, Koenig HN, Nannini MA, Hodge CW. Stimulation of endorphin neurotransmission in the nucleus accumbens by ethanol, cocaine, and amphetamine. J Neurosci. 2001;21(23):RC184.
451. de Waele JP, Gianoulakis C. Effects of single and repeated exposures to ethanol on hypothalamic beta-endorphin and CRH release by the C57BL/6 and DBA/2 strains of mice. Neuroendocrinology. 1993;57(4):700-9.
452. Roberts AJ, McDonald JS, Heyser CJ, Kieffer BL, Matthes HW, Koob GF, et al. mu-Opioid receptor knockout mice do not self-administer alcohol. J Pharmacol Exp Ther. 2000;293(3):1002-8.
453. Kovacs KM, Szakall I, O'Brien D, Wang R, Vinod KY, Saito M, et al. Decreased oral self-administration of alcohol in kappa-opioid receptor knock-out mice. Alcohol Clin Exp Res. 2005;29(5):730-8.
454. Bienkowski P, Kostowski W, Koros E. Ethanol-reinforced behaviour in the rat: effects of naltrexone. Eur J Pharmacol. 1999;374(3):321-7.
455. Heinz A, Reimold M, Wrase J, Hermann D, Croissant B, Mundle G, et al. Correlation of stable elevations in striatal mu-opioid receptor availability in detoxified alcoholic patients with alcohol craving: a positron emission tomography study using carbon 11-labeled carfentanil. Arch Gen Psychiatry. 2005;62(1):57-64.
456. Mitchell JM, O'Neil JP, Janabi M, Marks SM, Jagust WJ, Fields HL. Alcohol consumption induces endogenous opioid release in the human orbitofrontal cortex and nucleus accumbens. Sci Transl Med. 2012;4(116):116ra6.
457. Davidson D, Swift R, Fitz E. Naltrexone increases the latency to drink alcohol in social drinkers. Alcohol Clin Exp Res. 1996;20(4):732-9.
458. Volpicelli JR, Rhines KC, Rhines JS, Volpicelli LA, Alterman AI, O'Brien CP. Naltrexone and alcohol dependence. Role of subject compliance. Arch Gen Psychiatry. 1997;54(8):737-42.
459. Da Silva Santos R, Galdino G. Endogenous systems involved in exercise-induced analgesia. J Physiol Pharmacol. 2018;69(1):3-13.

460. Di Chiara G, Acquas E, Tanda G. Ethanol as a neurochemical surrogate of conventional reinforcers: the dopamine-opioid link. Alcohol. 1996;13(1):13-7.
461. Boecker H, Sprenger T, Spilker ME, Henriksen G, Koppenhoefer M, Wagner KJ, et al. The runner's high: opioidergic mechanisms in the human brain. Cereb Cortex. 2008;18(11):2523-31.
462. Veliz P, Boyd CJ, McCabe SE. Nonmedical Prescription Opioid and Heroin Use Among Adolescents Who Engage in Sports and Exercise. Pediatrics. 2016;138(2).
463. Torregrossa MM, Isgor C, Folk JE, Rice KC, Watson SJ, Woods JH. The delta-opioid receptor agonist (+)BW373U86 regulates BDNF mRNA expression in rats. Neuropsychopharmacology. 2004;29(4):649-59.
464. Torregrossa MM, Jutkiewicz EM, Mosberg HI, Balboni G, Watson SJ, Woods JH. Peptidic delta opioid receptor agonists produce antidepressant-like effects in the forced swim test and regulate BDNF mRNA expression in rats. Brain Res. 2006;1069(1):172-81.
465. Zhang H, Torregrossa MM, Jutkiewicz EM, Shi YG, Rice KC, Woods JH, et al. Endogenous opioids upregulate brain-derived neurotrophic factor mRNA through delta- and micro-opioid receptors independent of antidepressant-like effects. Eur J Neurosci. 2006;23(4):984-94.
466. Zhang H, Shi YG, Woods JH, Watson SJ, Ko MC. Central kappa-opioid receptor-mediated antidepressant-like effects of nor-Binaltorphimine: behavioral and BDNF mRNA expression studies. Eur J Pharmacol. 2007;570(1-3):89-96.
467. Pandey SC. A Critical Role of Brain-Derived Neurotrophic Factor in Alcohol Consumption. Biol Psychiatry. 2016;79(6):427-9.
468. Logrip ML, Barak S, Warnault V, Ron D. Corticostriatal BDNF and alcohol addiction. Brain Res. 2015;1628(Pt A):60-7.
469. Jamal M, Van der Does W, Elzinga BM, Molendijk ML, Penninx BW. Association between smoking, nicotine dependence, and BDNF Val66Met polymorphism with BDNF concentrations in serum. Nicotine Tob Res. 2015;17(3):323-9.
470. Ozan E, Okur H, Eker C, Eker OD, Gonul AS, Akarsu N. The effect of depression, BDNF gene val66met polymorphism and gender on serum BDNF levels. Brain Res Bull. 2010;81(1):61-5.
471. Lotrich FE, Albusaysi S, Ferrell RE. Brain-derived neurotrophic factor serum levels and genotype: association with depression during interferon-alpha treatment. Neuropsychopharmacology. 2013;38(6):985-95.
472. Moreira FP, Fabiao JD, Bittencourt G, Wiener CD, Jansen K, Oses JP, et al. The Met allele of BDNF Val66Met polymorphism is associated with increased BDNF levels in generalized anxiety disorder. Psychiatr Genet. 2015;25(5):201-7.
473. Vinberg M, Bukh JD, Bennike B, Kessing LV. Are variations in whole blood BDNF level associated with the BDNF Val66Met polymorphism in patients with first episode depression? Psychiatry Res. 2013;210(1):102-8.
474. Kirkcaldy BD, Shephard RJ, Siefen RG. The relationship between physical activity and self-image and problem behaviour among adolescents. Soc Psychiatry Psychiatr Epidemiol. 2002;37(11):544-50.
475. Hallgren M, Romberg K, Bakshi AS, Andreasson S. Yoga as an adjunct treatment for alcohol dependence: a pilot study. Complement Ther Med. 2014;22(3):441-5.
476. Moonat S, Sakharkar AJ, Zhang H, Pandey SC. The role of amygdaloid brain-derived neurotrophic factor, activity-regulated cytoskeleton-associated protein and dendritic spines in anxiety and alcoholism. Addict Biol. 2011;16(2):238-50.

477. Tambour S, Brown LL, Crabbe JC. Gender and age at drinking onset affect voluntary alcohol consumption but neither the alcohol deprivation effect nor the response to stress in mice. Alcohol Clin Exp Res. 2008;32(12):2100-6.
478. Sommer WH, Rimondini R, Hansson AC, Hipskind PA, Gehlert DR, Barr CS, et al. Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. Biol Psychiatry. 2008;63(2):139-45.
479. Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC. Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav. 2005;84(1):53-63.
480. Franklin KBJ, Paxinos G. The Mouse Brain in Stereotaxic Coordinates, Third Edition. 3rd ed. San Diego: Academic Press; 2008.
481. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
482. Fernandez GM, Lew BJ, Vedder LC, Savage LM. Chronic intermittent ethanol exposure leads to alterations in brain-derived neurotrophic factor within the frontal cortex and impaired behavioral flexibility in both adolescent and adult rats. Neuroscience. 2017;348:324-34.
483. Darcq E, Warnault V, Phamluong K, Besserer GM, Liu F, Ron D. MicroRNA-30a-5p in the prefrontal cortex controls the transition from moderate to excessive alcohol consumption. Mol Psychiatry. 2015;20(10):1219-31.
484. Briones TL, Woods J. Chronic binge-like alcohol consumption in adolescence causes depression-like symptoms possibly mediated by the effects of BDNF on neurogenesis. Neuroscience. 2013;254:324-34.
485. Hauser SR, Getachew B, Taylor RE, Tizabi Y. Alcohol induced depressive-like behavior is associated with a reduction in hippocampal BDNF. Pharmacol Biochem Behav. 2011;100(2):253-8.
486. Solomon MG, Griffin WC, Lopez MF, Becker HC. Brain Regional and Temporal Changes in BDNF mRNA and microRNA-206 Expression in Mice Exposed to Repeated Cycles of Chronic Intermittent Ethanol and Forced Swim Stress. Neuroscience. 2019;406:617-25.
487. Crews FT, Nixon K, Wilkie ME. Exercise reverses ethanol inhibition of neural stem cell proliferation. Alcohol. 2004;33(1):63-71.
488. Koob GF. Neurobiological substrates for the dark side of compulsivity in addiction. Neuropharmacology. 2009;56 Suppl 1:18-31.
489. Becker HC, Diaz-Granados JL, Hale RL. Exacerbation of ethanol withdrawal seizures in mice with a history of multiple withdrawal experience. Pharmacol Biochem Behav. 1997;57(1-2):179-83.
490. Bobadilla AC, Garcia-Keller C, Chareunsouk V, Hyde J, Camacho DM, Heinsbroek JA, et al. Accumbens brain-derived neurotrophic factor (BDNF) transmission inhibits cocaine seeking. Addict Biol. 2018.
491. Funk CK, Koob GF. A CRF(2) agonist administered into the central nucleus of the amygdala decreases ethanol self-administration in ethanol-dependent rats. Brain Res. 2007;1155:172-8.
492. Peters J, Dieppa-Perea LM, Melendez LM, Quirk GJ. Induction of fear extinction with hippocampal-infralimbic BDNF. Science. 2010;328(5983):1288-90.
493. Collins MA, Corso TD, Neafsey EJ. Neuronal degeneration in rat cerebrocortical and olfactory regions during subchronic "binge" intoxication with ethanol: possible explanation for olfactory deficits in alcoholics. Alcohol Clin Exp Res. 1996;20(2):284-92.

494. Grant BF, Dawson DA. Age of onset of drug use and its association with DSM-IV drug abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. J Subst Abuse. 1998;10(2):163-73.
495. Cazorla M, Premont J, Mann A, Girard N, Kellendonk C, Rognan D. Identification of a low-molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice. J Clin Invest. 2011;121(5):1846-57.
496. Beroun A, Nalberczak-Skora M, Harda Z, Piechota M, Ziolkowska M, Caly A, et al. Generation of silent synapses in dentate gyrus correlates with development of alcohol addiction. Neuropsychopharmacology. 2018;43(10):1989-99.
497. Liu C, Chan CB, Ye K. 7,8-dihydroxyflavone, a small molecular TrkB agonist, is useful for treating various BDNF-implicated human disorders. Transl Neurodegener. 2016;5:2.
498. Simon-O'Brien E, Alaux-Cantin S, Warnault V, Buttolo R, Naassila M, Vilpoux C. The histone deacetylase inhibitor sodium butyrate decreases excessive ethanol intake in dependent animals. Addict Biol. 2015;20(4):676-89.
499. You C, Vandegrift BJ, Zhang H, Lasek AW, Pandey SC, Brodie MS. Histone Deacetylase Inhibitor Suberanilohydroxamic Acid Treatment Reverses Hyposensitivity to gamma-Aminobutyric Acid in the Ventral Tegmental Area During Ethanol Withdrawal. Alcohol Clin Exp Res. 2018;42(11):2160-71.
500. Dencker D, Molander A, Thomsen M, Schlumberger C, Wortwein G, Weikop P, et al. Ketogenic Diet Suppresses Alcohol Withdrawal Syndrome in Rats. Alcohol Clin Exp Res. 2018;42(2):270-7.