Title
Brain-derived neurotrophic factor, dynorphin and the homeostatic regulation of ethanol intake

Author
Logrip, Marian Lee

Publication Date
2007-12-13

Peer reviewed|Thesis/dissertation

Brain-derived neurotrophic factor, dynorphin and the homeostatic regulation of ethanol intake

by

Marian Lee Logrip

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

UMI Number: 3289315

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

For my parents

ACKNOWLEDGEMENTS

First I would like to thank my advisors, Patricia Janak and Dorit Ron, for their continual mentorship and training, which have profoundly and permanently shaped my scientific thinking. I am most grateful that they indulged my desire to employ a multidisciplinary approach in my research, the product of which is presented herein. Their support of and excitement about my work has been a source of encouragement, and their faith in me has afforded me more opportunities for scientific presentation than are available to many graduate students, and for these things I will forever be indebted to them both. I hope that my future success will reflect well on the education I have received during my stay in their labs.

For additional contributions to my scientific training I would like to thank my thesis committee – Robert Messing, Steve Finkbeiner and Fernando Valenzuela. I am most appreciative of both their insightful comments over the years, which helped me refine my experimental design, and their assistance in revision of this document. In particular, I would like to thank my committee chair, Robert Messing, whose door was always open and who provided a reliable source of helpful advice and positive reinforcement.

By virtue of the fact that I have worked in two labs as well as in a highly collaborative environment throughout graduate school, it would take volumes to properly thank everyone who has contributed to my life in the lab and my formation as a scientist. However, there are several members of the Janak and Ron labs who deserve individual mention for their involvement in my research. I would like to thank Nancy Chen and Khanhky Phamluong for imparting on me their technical wisdom, as well as for helpful

discussions regarding both methodology and experiment planning and execution. In addition, I am grateful for the mentorship and assistance provided by Mike Gill, who was always willing and able to answer any question. Finally, I would like to thank those in the lab who have been involved either directly or indirectly in various aspects of my project and thus provided me with fruitful discussions about my experimental design or technical assistance: Jerome Jeanblanc, Sebastien Carnicella, Peter Traum and Sweta Bodepudi.

My path to science was not entirely straight, and thus I am indebted to those who helped steer me in the right direction. In particular, I owe a profound debt of gratitude to John Trueswell, who had faith that the girl with the economics degree would make a good research assistant. I would also like to thank Vijaya Ramesh, in whose lab I was first able to explore my interest in diseases of the nervous system.

I am most appreciative for the friends who have worked hard to keep me sane throughout my time in graduate school. In particular, I would first like to thank those among my friends who have also been my roommates for tolerating my quirks and providing me with hours of entertainment: Somayeh Ahmadiantehrani, Peter Castro, Jennifer Hsu and Mercedes Paredes have been an invaluable support system. My life has been greatly enriched by those who have made my life in and out of lab enjoyable: Ajay Ravindranathan and Doug Meckelson have given me a home away from home; Andrew Lee, David Kapfhamer and Chris Walker have been a bountiful source of food and friendship over the years; and Steve Shabel has been not only a welcome companion during many a late night in lab but also a constant morale booster. Finally, I thank

Rachelle Yuvienco, who has been my best friend and an invaluable source of support not only during graduate school but during my entire adult life, for which I am most grateful.

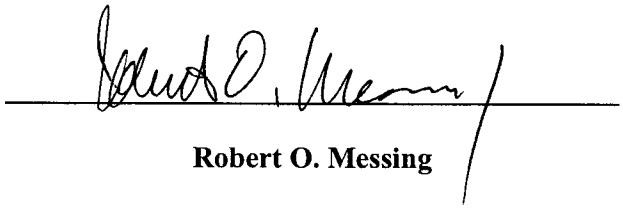
Last, but most certainly not least, I would like to thank my family for the unconditional love and support they have provided me throughout my life. From an early age, my parents instilled in me the importance of education and a stubborn perseverance which have bolstered me throughout this academic journey, and at each step along the way there has been no shortage of pride and encouragement from them as well as from my sister, Alison. I regret that my father could not share in the joy of this accomplishment, but am thankful that he was able to witness the beginning of this journey. In his absence, my mother has provided enough enthusiasm and support for both of them. For this and her endless curiosity about my work I am eternally grateful. Without my family, I would not be where I am today – this work is as much theirs as it is mine.

This research was supported by funds from the State of California for medical research on alcohol and substance abuse through the University of California, San Francisco (P.H.J. & D.R.), and by a grant from the National Institute on Alcohol Abuse and Alcoholism 1 F31 AA015462-01 (M.L.L.).

Brain-derived neurotrophic factor, dynorphin and the homeostatic regulation of ethanol intake

by

Marian Lee Logrip



Robert O. Messing

Thesis Committee Chair

ABSTRACT

Alcoholism is a devastating disease afflicting roughly 17 million Americans. While this figure is staggering, it is all the more striking that 87% of the population can drink without developing dependence, given the addictive potential of alcohol. This suggests the existence of mechanisms which curb excessive alcohol intake and prevent the majority of alcohol drinkers from developing dependence. Previously we identified a homeostatic pathway in the dorsal striatum in which the brain-derived neurotrophic factor (BDNF) is upregulated by ethanol and subsequently acts to reduce ethanol intake (McGough et al., 2004). The central aim of this dissertation is the elucidation of the molecular mechanism underlying striatal BDNF homeostasis, as well as the determination of the functional significance of BDNF homeostasis with respect to ethanol consumption and the progression to addiction.

Here I demonstrate a complete signaling pathway in which ethanol treatment of striatal neurons increases BDNF protein, resulting in the phosphorylation of its receptor TrkB and subsequent activation of the MAPK pathway. Importantly, I show that ethanol increases production of the downstream effector, *preprodynorphin*, via a BDNF- and MAPK-dependent mechanism.

In addition, I show that *in vivo* conditional deletion of BDNF increases ethanol intake. Conversely, increasing BDNF expression in brain regions including the striatum decreases ethanol intake via a dynorphin-dependent mechanism. I also demonstrate that activation of dynorphin's receptor, the kappa opioid receptor, blocks acquisition of ethanol conditioned place preference, indicating that the BDNF/dynorphin pathway may inhibit the formation of ethanol-related memories.

Finally, I reveal a role for the BDNF homeostatic pathway in the progression to addiction. Specifically, I show that BDNF homeostasis breaks down throughout the corticostriatal circuit after extensive experience with ethanol. Critically, BDNF homeostasis does not recover following two weeks of abstinence from ethanol, indicating that inhibition of corticostriatal BDNF may be a hallmark of the addictive process which underlies the propensity to relapse.

Taken together, these data establish an integral role of BDNF in the dorsal striatum, via its downstream effector dynorphin, in regulating ethanol intake and preventing the progression to addiction.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX

CHAPTER 1

Introduction

Alcohol dependence and addiction are pervasive problems in our society, affecting the lives of millions of Americans. According to the National Institute on Alcohol Abuse and Addiction's National Epidemiologic Survey on Alcohol and Related Conditions conducted in 2001-2002, 4.65% of Americans were classified as alcohol abusing while 3.81% demonstrated alcohol dependence (Grant et al., 2004). This represents roughly 8.5% of the population, or 17 million people, having alcohol-related illnesses. As alcohol-related health care costs were estimated at $148 billion in 1992 (Harwood et al., 1999) and rates of alcoholism have not decreased over the ensuing 15 years, alcoholism and alcohol dependence create significant financial strain on our society. Thus research addressing the neurobiological basis of alcohol dependence is vital to finding a solution to this grave societal problem.

Nonetheless, the 8.5% of the population dependent on alcohol pales in comparison to the vast number of "social drinkers" in our society – those who are able to drink moderate quantities of alcohol on an occasional basis but never develop dependence. According to Gallop Poll surveys conducted over the past 65 years, the proportion of the population reporting to be alcohol consumers (as opposed to complete abstainers) has remained consistently between 60% and 70% of those surveyed, about eight times the number of alcoholics in our society (The Gallup Organization, 2007). While the development of alcohol addiction in 13% of all drinkers is a staggering metric for the addictive properties of alcohol, the ability of 87% of the alcohol-consuming population to safely consume alcohol socially throughout their lifetimes without developing dependence points to the existence of mechanisms which function to counteract the development of alcohol addiction. This suggests the existence of

homeostatic mechanisms which curb excessive ethanol intake to prevent the majority of drinkers from developing dependence. Indeed, at the synaptic level homeostatic signaling has been suggested as a mechanism by which neurons retain responsiveness in the face of altered synaptic activity or efficacy (Turrigiano, 2007). Accordingly, this homeostatic pathway would be activated by acute exposure to alcohol – for instance, a single drink – and function to return the system to its baseline status, perhaps by promoting decreased subsequent ethanol intake.

One such homeostatic pathway triggered by acute ethanol exposure commences with translocation of the receptor for activated C kinase 1 (RACK1) from the cytoplasm to the nucleus upon ethanol treatment (Ron et al., 2000). This translocation triggers increased gene expression (He et al., 2002), and one of the genes downstream of the nuclear translocation of RACK1 is the brain-derived neurotrophic factor (BDNF) (Yaka et al., 2003). Expression of RACK1 as a tat-fusion protein (Tat-RACK1) imparts on RACK1 the ability to cross the blood-brain barrier and gain access to brain cells (Schwarze et al., 1999). Systemic administration of Tat-RACK1 increases *BDNF* expression in the dorsal striatum and decreases ethanol intake, while ethanol intake itself also increases *BDNF* expression in the dorsal striatum (McGough et al., 2004). Thus, we proposed the following homeostatic model, in which ethanol acutely increases dorsal striatal BDNF expression, initiating a cascade of events which are ultimately responsible for inhibiting further ethanol consumption (Figure 1.1A).

Given this model, the central goal of the thesis work described herein was first to elucidate downstream signaling events and effector proteins required for BDNF regulation of ethanol intake, as well as to determine whether the progression towards

more compulsive ethanol intake would coincide with breakdown of BDNF signaling (Figure 1.1B). The integral components of the homeostatic pathway elucidated in this dissertation are BDNF, the mitogen-activated protein kinase (MAPK) pathway and the downstream effector protein dynorphin, which regulate ethanol consumption via their action in the dorsal striatum.

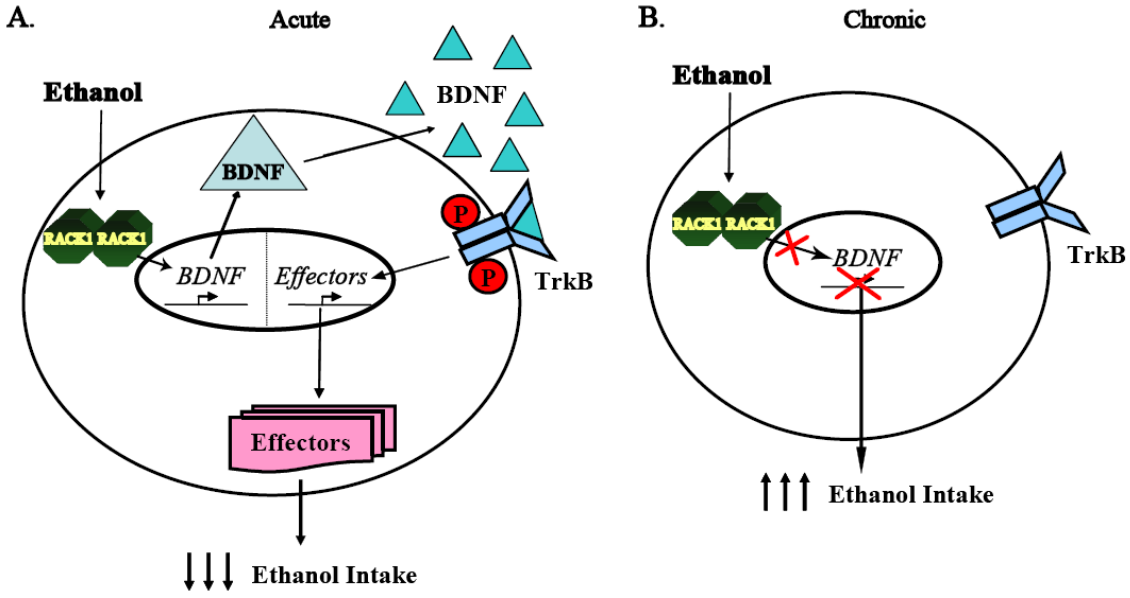


Figure 1.1. Homeostatic control of ethanol intake by BDNF signaling.
(A) Ethanol intake acutely increases BDNF expression via nuclear translocation of RACK1, triggering downstream events which modulate ethanol intake. (B) After chronic exposure to ethanol, RACK1 becomes unresponsive to ethanol, resulting in an inability of ethanol to increase BDNF expression. This breakdown of the BDNF homeostatic response to ethanol causes a concomitant failure of the regulation of ethanol intake by BDNF and its downstream effectors.

THE DORSAL STRIATUM AND ALCOHOL ADDICTION

Cellular composition, circuit connectivity and function

The dorsal striatum is a central component of the basal ganglia, receiving glutamatergic projections from the cortex and thalamus which are modulated by

dopaminergic inputs from the substantia nigra pars compacta (SNc) (Surmeier et al., 2007). The striatum is composed primarily of GABAergic medium-sized spiny neurons (MSNs) which have classically been subdivided into two types based on their efferent projections, dopamine receptor expression and neuropeptide content. The neurons belonging to the direct pathway, which project directly to the basal ganglia output regions, the substantia nigra pars reticulata (SNr) and the internal segment of the globus pallidus, express dopamine D1 receptors as well as the neuropeptides substance P and dynorphin. The indirect pathway neurons, which must relay information via the external segment of the globus pallidus and the subthalamic nucleus before reaching the basal ganglia output regions, express dopamine D2 receptors and enkephalin (Steiner & Gerfen, 1998).

The segregation of D1- and D2-class dopamine receptor expression into specific MSN subtypes is not without controversy, however. Several reports have demonstrated colocalization of D1 and D2 receptor expression in 20 to 50 percent of MSNs analyzed (Lester et al., 1993; Surmeier et al., 1996; Deng et al., 2006) , while others have demonstrated complete coexpression in striatal primary neuronal cultures (Aizman et al., 2000; Falk et al., 2006). Interestingly, additional data suggests that coexpression of D1 and D2 receptor mRNA in the rat is minimal but that a subset of neurons in a restricted region of the striatum express D1 receptors together with the D2-like D3 receptor (Curran & Watson, 1995). Dopamine D1 and D2 receptor co-expression in primary striatal cultures was not reflected in overlapping expression of substance P/dynorphin and enkephalin, while D1 and D2/D3 receptor colocalization in adult animals was consistently reflected in coexpression of the neuropeptides. Thus *in vivo* dopamine

receptor subtype coexpression may have functional consequences not observed *in vitro*. However, it is unknown whether neurons co-expressing D1 and D2/D3 receptors are distributed evenly throughout the striatal projection systems or restricted to either the direct or indirect pathway. Finally, it should be noted that some researchers continue to refute the existence of substantial dopamine receptor colocalization (Le Moine & Bloch, 1995; Aubert et al., 2000) and thus the topic remains a point of contention in the field.

Divergence in dopamine receptor subtype and neuropeptide expression is not the only source of diversity in the dorsal striatum, however. Its functional heterogeneity stems in large part from the topography of the cortical efferents, which impart different behavioral functions on their striatal targets. Cortical input to the dorsal striatum forms a ventromedial to dorsolateral gradient, with the most ventromedial portions receiving more limbic input and the most dorsolateral receiving only sensorimotor projections. Thus the dorsal striatum can be primarily subdivided into two parts: the medial portion, which synthesizes inputs from various association cortices, the dorsal prefrontal cortex and the amygdala, and the lateral portion, which receives afferent projections from sensorimotor cortex (Voorn et al., 2004). This variety in circuit connectivity underlies the functional heterogeneity of the region.

Dorsal striatum-dependent learning and implications for addiction

As mentioned above, the two main subdivisions of the dorsal striatum, due to different afferent connectivity, appear to regulate different forms of learning. The dorsolateral striatum (DLS), which is connected to the sensorimotor cortices, has been suggested to be the seat of habits (Yin & Knowlton, 2006). Habitual actions are

described as inflexible stimulus-response reactions, in which the occurrence of a well-known cue drives a motor behavior which is insensitive to manipulations of the value of the reinforcer. This may be analogous to drug-seeking in addicts, in which drug-associated cues (i.e., the stimulus) serve as a trigger to perform a habitual pattern of actions to obtain drugs (i.e., the response). Unlike the dorsolateral striatum, the dorsomedial striatum (DMS) receives input from various association cortices, the dorsal regions of the prefrontal cortex (PFC) and the amygdala. Because of this circuit connectivity, the DMS maintains sensitivity to reward values and thus subserves a more flexible type of behavior, referred to as response-outcome (Yin & Knowlton, 2006), in which the behavioral response is performed specifically to obtain a reinforcer (or drug). The key difference between DLS- and DMS-dependent memories is the sensitivity of the learned behaviors to reinforcement value. Thus while addiction research has traditionally focused on the nucleus accumbens (NAc), or ventral striatum, as it receives inputs and integrates information from various limbic brain regions, attention has recently shifted to the dorsal striatum as a locus for the maladaptive habit formation which may underlie addiction (Robbins & Everitt, 2002; Gerdeman et al., 2003). The fact that actions become habitual following extensive training suggests that the entire dorsal striatum – in a progression from dorsomedial (drug-sensitive) to dorsolateral (compulsive/habitual) – participates in the various stages of development of drug dependence. Importantly, the BDNF homeostatic pathway, which is selectively activated in the dorsal striatum, may function to negatively regulate that progression.

MOLECULAR COMPONENTS OF HOMEOSTASIS

Brain-derived neurotrophic factor

When originally isolated from the brain, BDNF was noted for its neurotrophic abilities – namely, its capacity to support cellular survival and neurite outgrowth of sensory neurons (Barde et al., 1982). BDNF is widely expressed throughout the nervous system, with highest expression in the cortex and hippocampus but detectable levels in all brain regions tested (Hofer et al., 1990). BDNF belongs to the nerve growth factor family of neurotrophic factors which, upon release from neurons, signal via binding to receptor tyrosine kinases of the tropomyosin-related kinase (Trk) class (Barbacid et al., 1991) or the p75 neurotrophin receptor (Chao & Hempstead, 1995). Neurotrophins are initially synthesized as proproteins, which are proteolytically processed to yield the mature form of the neurotrophin (Lu, 2003). Proneurotrophins bind preferentially to the p75 neurotrophin receptor, while mature neurotrophins selectively bind to their cognate Trks (Chao & Bothwell, 2002). BDNF acts by binding to the receptor tyrosine kinase TrkB (Klein et al., 1991; Soppet et al., 1991), triggering receptor dimerization and autophosphorylation (Kaplan & Miller, 1997, 2000). TrkB can be expressed in either of two isoforms, with its full-length 145 kDa isoform (TrkB-FL) containing an intracellular signal transduction domain which is lacking in the 95 kDa truncated isoforms (TrkB.T1 and TrkB.T2) (Klein et al., 1990; Middlemas et al., 1991). Like BDNF, TrkB is widely expressed throughout the nervous system, with TrkB-FL showing neuron-specific expression while the truncated TrkB appears in neurons as well as in glia and astrocytes (Altar et al., 1994). Thus the neuron-specific TrkB-FL confers on a wide variety of neurons the capacity to initiate BDNF-dependent signaling cascades.

TrkB Signaling

Following BDNF binding, TrkB is autophosphorylated at multiple tyrosine residues within the intracellular tail of the receptor, activating signaling via the MAPK, phosphoinositide 3-kinase (PI3K) and phospholipase C-γ (PLC–γ) signaling cascades (Yuen & Mobley, 1999). The most membrane-proximal phosphorylation site, Tyrosine 484, interacts with Shc and provides the initiation site for both the MAPK and PI3K pathways (Atwal et al., 2000), while Tyrosine 785 directly interacts with PLC-γ to activate its signaling cascade (Middlemas et al., 1994). Tyrosines 670/674/675 comprise the autocatalytic domain of TrkB (Guiton et al., 1994; Middlemas et al., 1994) and phosphorylation of all three of these tyrosines is required for complete activation of downstream signaling events (McCarty & Feinstein, 1998).

Activation of TrkB increases neuronal survival and neurite outgrowth principally through activation of the PI3K pathway, although MAPK signaling can be activated to promote survival following an insult (Kaplan & Miller, 2000). However, activation of the MAPK or PLC-γ pathway primarily functions to increase downstream gene transcription, with PLC-γ triggering the release of intracellular calcium stores and activating calcium-responsive downstream signaling partners such as calcium and calmodulin-dependent kinase and protein kinase C δ, which can in turn activate MAPK signaling (Huang & Reichardt, 2003). As a major regulator of gene transcription downstream of TrkB, we have chosen to focus on the MAPK pathway as a locus for ethanol-induced BDNF signaling.

MAPK signaling & regulation of BDNF-induced gene expression

TrkB activates MAPK signaling via activation of upstream adaptor proteins, which in turn initiate signaling via the MAPK pathway. TrkB directly interacts with and phosphorylates Shc, which then activates Ras via guanine nucleotide exchange factors (GEFs) such as son of sevenless (SOS), which binds to Shc via the adaptor protein growth factor receptor-bound protein 2 (Grb2) (Egan et al., 1993). Following activation by Shc, Ras GEFs promote the conversion of Ras from its GDP-bound to its GTP-bound, or active, state, in which it can activate downstream signaling via the MAPK pathway (Quilliam et al., 2002). The initial phosphorylation acceptor in the MAPK cascade is the MAPK kinase kinase Raf. Upon activation by Ras, Raf phosphorylates the MAPK kinases MAPK and ERK kinases 1/2 (MEK1/2), which in turn phosphorylate the MAPKs extracellular signal-regulated kinases 1/2 (ERK1/2) (Huang & Reichardt, 2003). Note that variation has been found in the mechanisms of MAPK activation by Trks, including the substitution of Shc with Frs-2 as the initial phosphorylation acceptor, as well as use of Rap in place of Ras (Segal, 2003). Activated ERKs can then translocate to the nucleus or activate ribosomal S6 kinases (RSKs), which also move to the nucleus, where both can activate transcription factors to alter gene expression. Thus, a primary function of BDNF-induced MAPK signaling is regulation of gene expression. One such gene whose expression can be altered by BDNF is *preprodynorphin* (*Pdyn*) (Croll et al., 1994), the precursor of the dynorphin protein, which will be discussed in detail below.

Synaptic plasticity and memory formation

Aside from its well-defined roles in development and trophic support, one of the central functions of BDNF in the adult brain is regulation of synaptic strength, or long-term potentiation (LTP). This process of synaptic adaptation has been suggested to be the molecular correlate of memory formation (Teyler & Discenna, 1984; Bliss & Collingridge, 1993; Miyamoto, 2006). A majority of the research detailing the mechanisms of BDNF-dependent LTP has been carried out in hippocampal systems, in part due to the tractability of the hippocampal network, with its well-defined cellular layers and synaptic inputs. In hippocampal preparations, *BDNF* is upregulated following LTP-inducing stimuli (Patterson et al., 1992), and BDNF is required for synaptic potentiation, as reduction of BDNF blocks development of LTP (Korte et al., 1995; Patterson et al., 1996; Korte et al., 1998). Importantly, while BDNF alone is not sufficient to induce LTP, it is permissive, as addition of BDNF specifically potentiates activated and not neighboring synapses (Gottschalk et al., 1998) and co-application of BDNF with subthreshold stimuli results in LTP (Kovalchuk et al., 2002). As BDNF-dependent LTP has been observed in cortical synapses as well (Aicardi et al., 2004), it is likely that BDNF may function as a modulator of synaptic efficacy in multiple brain regions, including the striatum.

BDNF modulation of synaptic plasticity requires TrkB receptor activation and signaling (Kang et al., 1997), in particular via activation of the MAPK cascade (English & Sweatt, 1997). Importantly, both hippocampal-dependent behaviors (Alonso et al., 2002a; Alonso et al., 2002b; Gooney et al., 2002) and amygdala-dependent memory formation (Ou & Gean, 2006) have been shown to be both BDNF- and MAPK-

dependent. Taken together, these data indicate that BDNF signaling via the MAPK pathway plays a crucial role in the synaptic plasticity underlying certain forms of learning and memory. As memory formation is central to the development of addiction (Berke & Hyman, 2000; Hyman et al., 2006), a major focus of this dissertation is the delineation of ethanol-induced BDNF signaling pathways which may be crucial for inhibiting the neuroadaptations underlying alcohol addiction.

BDNF & Addiction

Due to its functions both in regulating neuronal plasticity as well as in providing trophic support against insults (Huang & Reichardt, 2003), BDNF has been explored as a means of regulating drug response for well over a decade now; however, the results have been somewhat conflicting. Initial studies demonstrated that administration of BDNF to the substantia nigra (SN) via minipump for 2 weeks reduced amphetamine-induced locomotion despite increasing basal locomotor activity (Martin-Iverson et al., 1994). This same treatment regimen applied to the ventral tegmental area (VTA), the brain region which sends dopaminergic projections to the NAc, successfully blocked biochemical changes normally resulting from chronic morphine and cocaine treatments (Berhow et al., 1995), such that VTA tyrosine hydroxylase (TH) levels remained unchanged, as did cyclic AMP-dependent protein kinase (PKA) and adenylyl cyclase (AC) activities in the NAc. As adaptations in VTA TH levels and NAc PKA activity are hallmarks of chronic drug administration for psychostimulants, opiates and ethanol (Beitner-Johnson et al., 1992; Ortiz et al., 1995; Self et al., 1995), these data indicate a

role for BDNF in protection against certain chronic drug-induced molecular and behavioral adaptations.

In contrast to these studies, subsequent work found that continuous administration of BDNF into the NAc over a two-week period increased locomotor activation by cocaine (Horger et al., 1999), although blocking endogenous BDNF in either the VTA or NAc by infusion of BDNF neutralizing antibodies had no effect on locomotor sensitization to cocaine (Freeman & Pierce, 2002). Interestingly, in both cases NAc infusions altered locomotor responses to novelty, with activity increased by BDNF infusion and decreased following injection of neutralizing antibodies. Thus the discrepancy between these two data sets does not appear to be the result of an acute difference in mechanism of action at the time of sensitization testing and may illustrate a difference between the capacity of the system to respond to exogenously applied BDNF as compared with intrinsic BDNF. Recent work has demonstrated a role for endogenous BDNF transcription (Filip et al., 2006) and signaling in the shell region of the NAc in response to acute cocaine injection as well as cocaine self-administration (Graham et al., 2007). While Graham and colleagues (2007) demonstrate a role for BDNF in the NAc shell in increasing cocaine self-administration, our previous results have demonstrated a dorsal striatum-specific increase in BDNF after acute ethanol exposure (McGough et al., 2004). Activation of the PLC-γ pathway by cocaine in the NAc shell suggests that it triggers LTP (Minichiello et al., 2002) and thus may indicate a functional dissociation between NAc shell and dorsal striatal BDNF responses to drugs of abuse. Specifically, as potentiation of cocaine self-administration by BDNF is specific for the NAc shell but not the NAc core or dorsal striatum (Graham et al., 2007), it is possible that cocaine-induced

BDNF potentiates synaptic transmission in the NAc shell while in the dorsal striatum BDNF likely inhibits potentiation via increased production of its downstream effector dynorphin, which inhibits LTP (Simmons & Chavkin, 1996).

Dynorphin

Dynorphin is the endogenous ligand for the kappa opioid receptor (KOR) (Chavkin et al., 1982). Both dynorphin and the KOR are expressed in multiple brain regions (Civelli et al., 1985; DePaoli et al., 1994), particularly throughout the striatum (Fallon & Leslie, 1986; Mansour et al., 1994). Maximal KOR expression is observed in the midbrain striatal afferent projection regions, the substantia nigra pars compacta (SNc) and VTA (Mansour et al., 1994). Within the striatum, KORs localize mainly to presynaptic axon terminals (Meshul & McGinty, 2000), and activation of striatal KORs reduces neurotransmitter release (You et al., 1999). In the hippocampus, dynorphin has been localized to dendritic vesicles and has been suggested to function as a retrograde inhibitory neurotransmitter at dentate gyrus synapses (Drake et al., 1994). In light of the presynaptic localization of KORs in the striatum, these data suggest that local dynorphin signaling within the striatum serves to reduce presynaptic transmission and regulate striatal plasticity.

Given the central role of BDNF in synaptic plasticity, and since alteration of gene expression is an integral function of BDNF signaling, proteins regulating synaptic efficacy present attractive targets as downstream effectors of BDNF signaling. *Pdyn*, the mRNA precursor of dynorphin, has been shown previously to be regulated by prolonged infusion of BDNF into the dorsal striatum (Croll et al., 1994). This likely occurs

following coincident activation of both activator protein 1(AP-1) and cyclic AMP response element (CRE) sites in the *Pdyn* promoter (Douglass et al., 1994; Messersmith et al., 1994; Messersmith et al., 1996), as BDNF has been shown to regulate both AP-1 and CRE-dependent transcription (Gaiddon et al., 1996). Importantly, while BDNF increases *Pdyn* in the dorsal striatum, it decreases *Pdyn* levels in the hippocampus (Croll et al., 1994). The underlying cause of this brain region-specific responsiveness of *Pdyn* to BDNF signaling is unknown; however, it may be due to differential epigenetic regulation (reviewed in Tsankova et al., 2007), thus yielding differential sensitivity of *Pdyn* to BDNF signaling. Alternatively, endogenous regulation of *Pdyn* levels by serotonin has been shown to occlude the ability of cocaine to increase *Pdyn* in the hippocampus but not the striatum (D'Addario et al., 2007), and thus serotonin-dependent regulation of hippocampal, but not striatal, *Pdyn* expression may alter its responsiveness to BDNF signaling. Regardless of the source of this differential responsiveness of *Pdyn* to BDNF signaling, it indicates that BDNF may subserve differential mnemonic functions in these two brain regions. Dynorphin blocks hippocampal-dependent spatial memory formation (McDaniel et al., 1990; Sandin et al., 1998), and since BDNF modulates LTP in the hippocampus, its downregulation of hippocampal *Pdyn* may serve to prevent synaptic depression by *Pdyn*. Conversely, since BDNF treatment increases *Pdyn* in the striatum, this suggests a role for BDNF, via dynorphin, in decreasing striatal activation. Interestingly, striatal dynorphin expression is reduced in mice expressing half as much BDNF (Saylor et al., 2006), indicating that endogenous BDNF regulates dynorphin expression. Taken together, these data suggest that BDNF in the striatum may function to inhibit synaptic potentiation via increased expression of *Pdyn*.

Dynorphin & Addiction

Due to its ability to dampen dopamine release (You et al., 1999) and induction of its expression by multiple drugs of abuse (Hurd & Herkenham, 1992; Gulya et al., 1993; Helton et al., 1993; Smith & McGinty, 1994; Wang et al., 1995; Turchan et al., 1997; Adams et al., 2000; Beadles-Bohling et al., 2000; Zhou et al., 2002), dynorphin has long been considered a modulator of drug response (Herz, 1998), including for ethanol (Spanagel, 1996). KOR agonists have been shown to increase the threshold for intracranial self-stimulation, indicating an interference with its rewarding properties (Todtenkopf et al., 2004). In addition, KOR agonists block morphine and cocaine place preference (Suzuki et al., 1992; Funada et al., 1993) as well as self-administration of multiple drugs of abuse (Kuzmin et al., 1997; Negus et al., 1997; Schenk et al., 1999, 2001), including alcohol (Lindholm et al., 2001). Importantly, both dynorphin and the KOR have been implicated in human alcohol abuse. A polymorphism in the *Pdyn* promoter consisting of multiple copies of a 68 base pair repeat, which contains an additional AP-1 binding site and thereby alters stimulus-induced *Pdyn* transcription, has been suggested to modulate human propensity for addiction (Zimprich et al., 2000) and may contribute to vulnerability to codependency on cocaine and alcohol (Williams et al., 2007). Genetic analyses of *Pdyn* and *OPRK1*, the gene encoding the KOR, from alcohol dependent families have shown associations between multiple regions of *Pdyn* and chromosome 2 of *OPRK1*. Taken together these results indicate that dynorphin plays a vital role in the regulation of drug responsiveness, not only in animal models but in humans as well, thus suggesting a mechanism by which dynorphin, as a downstream

effector of BDNF, could modulate the protective effects of BDNF in the dorsal striatum against the development of alcohol addiction.

SUMMARY

The reduction of ethanol intake by BDNF, in combination with the induction of *BDNF* expression by ethanol, suggests a centrality of BDNF signaling mechanisms in the dorsal striatum in the regulation of ethanol intake. The work detailed in this dissertation first characterizes the signaling pathway and a downstream effector regulated by ethanol-induced BDNF (Chapter 2). It further demonstrates a behavioral function of this pathway in controlling ethanol intake and suggests putative mechanisms underlying this regulation of ethanol intake (Chapter 3). Finally, a breakdown in homeostasis is demonstrated, which may function as the molecular underpinning of addiction and the tendency to relapse (Chapter 4). The sum of these data, as viewed within the framework delineated in this chapter, shed light on important neuronal adaptations to alcohol involved in the regulation of ethanol intake. It is my hope that this work may serve as a foundation to launch new avenues of research in the quest to find a treatment for alcoholism.

CHAPTER 2

Ethanol activates BDNF signaling via the MAPK cascade to increase production of its downstream effector, *preprodynorphin*

ABSTRACT

We recently identified brain-derived neurotrophic factor (BDNF) in the dorsal striatum to be a major component of a homeostatic pathway controlling ethanol consumption (McGough et al., 2004; Jeanblanc et al., 2006). We hypothesized that ethanol-mediated activation of the BDNF signaling cascade is required for the ethanol-related function of the neurotrophic factor. Here we demonstrate that exposure of striatal neurons to ethanol results in the activation of the BDNF receptor TrkB, leading to the activation of the mitogen-activated protein kinase (MAPK) signaling pathway and the subsequent increase in the expression of *preprodynorphin* (*Pdyn*). Importantly, we show that ethanol-induced *Pdyn* expression is both BDNF- and MAPK-dependent, indicating that *Pdyn* is a downstream effector of the BDNF homeostatic response to ethanol. Taken together, these results suggest that BDNF regulates ethanol drinking behavior by initiation of MAPK signaling and the ensuing production of downstream gene products, including *Pdyn*.

INTRODUCTION

Alcoholism is a devastating disease, with 8.5% of the American population – over 17 million adults – categorized as alcohol abusing or dependent (Grant et al., 2004). Yet 60 to 70% of the population has admitted to consuming alcohol (ethanol) in self-report surveys taken annually over the past 65 years (Gallup Poll, surveys conducted annually between 1939 and July 12-15, 2007(The Gallup Organization, 2007)). The discrepancy between these two statistics suggests the existence of mechanisms which curb excessive ethanol intake such that the majority of social drinkers do not develop dependence.

The neurotrophic factor BDNF is a likely candidate for inhibiting the transition to addiction, as in humans chromosomal markers flanking the *BDNF* gene (Uhl et al., 2001) have been associated with the development of addiction to multiple drugs of abuse, including alcohol (ethanol). A polymorphism altering a single residue in the BDNF propeptide (Val66Met) has also been linked to earlier onset of alcoholism (Matsushita et al., 2004). In addition, much evidence exists suggesting a role for BDNF in regulating neuronal responses to drugs of abuse, at both the molecular and behavioral levels (Pierce & Bari, 2001; Bolanos & Nestler, 2004). BDNF infusion into the ventral tegmental area (VTA) blocks the development of molecular adaptations to chronic morphine and cocaine treatment (Berhow et al., 1995), and acute administration of (Meredith et al., 2002; Zhang et al., 2002; McGough et al., 2004; Le Foll et al., 2005; Liu et al., 2006) or withdrawal from (Tapia-Arancibia et al., 2001; Grimm et al., 2003) multiple drugs of abuse has been shown to increase *BDNF* levels in various brain regions. Importantly, reduction in BDNF expression results in increased ethanol intake (Hensler et al., 2003; Pandey et al., 2004; Pandey et al., 2006). For instance, in the central and medial amygdala reduction of

BDNF by antisense oligonucleotides increases both ethanol intake and anxiety-like behaviors, indicating a possible role for amygdalar BDNF in drinking associated with anxiety relief. In addition, the same treatment with BDNF antisense, which increased ethanol intake, also reduced phosphorylation levels of proteins known to be downstream of BDNF signaling (Pandey et al., 2006), demonstrating that BDNF may regulate anxiety and ethanol intake via activation of a downstream signaling cascade. Recently cocaine has also been shown to increase BDNF levels and signaling in the nucleus accumbens (NAc), although in contrast to the attenuation of ethanol intake by dorsal striatal and amygdalar BDNF, blockade of BDNF in the NAc decreased cocaine self-administration (Graham et al., 2007).

Previously, we found in the dorsal striatum that BDNF participates in a homeostatic pathway triggered in response to ethanol and which, in turn, regulates behavioral responses to ethanol (McGough et al., 2004; Jeanblanc et al., 2006). Specifically, we found that ethanol exposure increased *BDNF* expression in the dorsal striatum, and that increased *BDNF* expression resulted in decreased ethanol intake (McGough et al., 2004). In addition, mice expressing half as much BDNF ($BDNF^{+/-}$) displayed increased sensitivity to ethanol in multiple paradigms, showing elevated preference for an ethanol-paired environment, increased locomotor sensitization to ethanol and higher ethanol intake following a period of deprivation as compared with wild-type (WT) mice (McGough et al., 2004). Subsequently, we found that inhibition of BDNF signaling using the Trk inhibitor K252a increased ethanol intake, and this effect was abolished in $BDNF^{+/-}$ mice, in which BDNF signaling is reduced compared with WT mice, due to expression of half as much BDNF protein (McGough et al., 2004). Thus, we

hypothesized that ethanol treatment activates the BDNF-mediated signaling cascade, leading to the upregulation of downstream proteins whose activities are responsible for BDNF's ability to control ethanol consumption.

BDNF initiates signaling by binding to its receptor, TrkB, resulting in receptor autophosphorylation and subsequent activation of several signaling cascades, including the MAPK cascade (Huang & Reichardt, 2003). As BDNF-dependent activation of MAPK in neurons results in increased gene transcription (Marsh et al., 1993) and acute administration of cocaine, morphine or nicotine has been shown to increase striatal MAPK activity (Valjent et al., 2004), we hypothesized that ethanol treatment would result in increased activation of the MAPK pathway and subsequent upregulation of genes encoding downstream effectors of BDNF signaling. In addition, we hypothesized that the protein products of these downstream effector genes would be responsible for decreasing ethanol intake.

Because ethanol intake selectively increases BDNF levels in the dorsal striatum (McGough et al., 2004), we sought a downstream effector whose expression in the dorsal striatum could be increased in a BDNF-dependent fashion. As detailed in Chapter 1, extended administration of BDNF into the striatum has been shown to increase the expression of *Pdyn* (Croll et al., 1994), and mice heterozygous for BDNF also show reduced striatal *Pdyn* expression (Saylor et al., 2006). Additionally, striatal MAPK activation is required for induction of *Pdyn* expression by amphetamine (Shi & McGinty, 2006). Interestingly, multiple drugs of abuse, including ethanol, have been shown to increase dynorphin expression in the striatum (Spangler et al., 1993; Lindholm et al., 2000; Fagergren et al., 2003). Increasing *Pdyn* expression in the nucleus accumbens by

infusion of HSV-CREB resulted in place aversion to subthreshold doses of cocaine which was dependent on dynorphin signaling (Carlezon et al., 1998). In addition, dynorphin expression is higher in the striatum of DBA/2 mice, who drink lower quantities of alcohol, than in the high-drinking C57BL/6 strain (Jamensky & Gianoulakis, 1997). Additional evidence has implicated dynorphin and its receptor, the kappa opioid receptor (KOR) (Chavkin et al., 1982), in modulation of ethanol intake. Systemic administration of U50,488H, a KOR agonist, decreased two-bottle choice ethanol intake in rats (Lindholm et al., 2001), whereas systemic administration of nor-binaltorphimine, a KOR antagonist, increased two-bottle choice ethanol intake in rats (Mitchell et al., 2005). Importantly, polymorphisms in the KOR have been associated with increased risk of alcoholism in humans (Xuei et al., 2006), indicating that dynorphin may be directly involved in protection against the development of alcohol addiction. In light of these data, we hypothesized that ethanol treatment activates BDNF signaling via the MAPK pathway to increase the expression of dynorphin.

METHODS

Reagents

Recombinant human BDNF, TrkB-Fc and the Fc fragment of human IgG were purchased from R&D Systems, Inc. (Minneapolis, MN). U0126 was purchased from Cell Signaling (Danvers, MA). Primary antibodies were obtained as follows: TrkB and phosphotyrosine antibodies from Upstate (Temecula, CA), phospho-Trk and phospho-ERK1/2 antibodies from Cell Signaling (Danvers, MA), GAD-67, BDNF and MAP2 antibodies from Chemicon (Temecula, CA) and normal rabbit IgG from Santa Cruz

Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies for Western blot detection were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). AlexaFluor-conjugated secondary antibodies and 4',6-Diamidino-2-phenyindole, dilactate (DAPI), as well as all culture media, B-27 and GlutaMAX supplements, protein G agarose beads and Nu-PAGE gels, were supplied by Invitrogen (Carlsbad, CA). VectaShield mounting medium was obtained from Vector Laboratories (Burlingame, CA). Serum extender was obtained from BD Biosciences (San Jose, CA). Protease and phosphatase inhibitors were obtained from Roche (Basel, Switzerland). TRIzol and the Reverse Transcription System were purchased from Promega Corporation (Madison, WI). All real-time PCR reagents, including TaqMan Gene Expression Assays, were obtained from Applied Biosystems, Inc. (Foster City, CA). DNase was obtained from Sigma Aldrich (St. Louis, MO).

Animals

Sprague Dawley pups or pregnant female rats were obtained from Harlan (Indianapolis, IN) for primary neuronal culture. Animals were housed under a 12 h light/dark cycle, with lights on at 7:00 a.m. and lights off at 7:00 p.m., and were provided with continuous *ad libitum* access to food and water. All animal procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council (1996).

Primary striatal neuronal culture

A litter of Sprague Dawley rats was obtained between the day of birth and the first postnatal day (P0 - P1), sacrificed by rapid decapitation, and the striata dissected out, pooled and digested in a papain solution for 35 minutes. Following inhibition of digestion in media containing bovine serum albumin and trypsin inhibitor, cells were mechanically dissociated using pulled glass pipettes in Minimum Essential Media containing 22 mM D-glucose, 5% fetal bovine serum and serum extender. Neurons were plated on poly-D-lysine-coated plates or CC2-coated chamber slides in Neurobasal-A media (NB-A) containing B-27 and GlutaMax supplements, as well as penicillin and streptomycin. Cultures were maintained for 11 days in vitro (DIV), with 50% of the media changed on DIV1 and DIV7. For phosphorylation studies, media was changed to Basal Medium Eagle containing penicillin and streptomycin on DIV10; otherwise, cells remained in NB-A and 50% of the media was changed 3 hours prior to treatment on DIV11. Additionally, 10 μM cytosine arabinoside (AraC) was added on DIV1 to inhibit multiplication of glial cells.

Immunoprecipitation

Following treatment, media was removed and cells were harvested in RIPA buffer containing: 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid and 2 mM EDTA, as well as protease and phosphatase inhibitor cocktails (Roche). Cell lysates were precleared for 1 hour with protein G agarose beads (Invitrogen). Precleared lysates were then incubated overnight with either TrkB antibodies or normal rabbit IgG. The following day, protein G agarose beads were added

for 2 hours' incubation, then beads were washed thoroughly prior to addition of sample loading buffer. Samples were resolved on a 4-12% gradient Nu-PAGE gel in reducing conditions and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% nonfat dry milk prepared in PBS-0.1% Tween (5% milk) prior to overnight incubation with phosphotyrosine antibodies diluted 1:200 in 5% milk. Secondary antibodies were prepared in 5% milk at a dilution of 1:1000. Blots were stripped briefly in 0.2 M sodium hydroxide and reprobed for TrkB as a loading control.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Primary striatal neurons were homogenized in TRIzol and mRNA isolated according to standard protocol. Samples were treated with DNase prior to reverse transcription using the Reverse Transcription System. The resulting cDNA samples were amplified by TaqMan quantitative PCR using commercially available primer/probe kits. In all cases, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as an internal control.

Immunofluorescence

Subsequent to treatment on DIV11, primary striatal neuronal cultures were washed briefly in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. The cells were then washed twice with PBS and once for 10 minutes with PBS containing 0.1% Triton X-100 (PBS-Triton). Cells were blocked for a minimum of 4 hours with 3% normal donkey serum in PBS-Triton. Cells were incubated in primary antibodies overnight at 4ºC; the antibodies were diluted in PBS-Triton containing 1%

normal donkey serum at the following dilutions: GAD-67 1:1000; BDNF 1:250; pTrk 1:100; TrkB 1:100; phospho-ERK1/2 1:200; substance P 1:10,000; MAP2 1:2000. The slides were then washed three times in PBS-Triton, followed by incubation with AlexaFluor-conjugated secondary antibodies for 2 hours in the dark. The slides were then washed in PBS twice, incubated in 300 nM DAPI for two minutes if nuclear demarcation required, and washed twice more in PBS. Slides were mounted in VectaShield and coverslipped prior to visualization with a laser-scanning confocal microscope (LSM 510 Meta; Zeiss).

Image Analysis

Neurons were located using microtubule-associated protein 2 (MAP2) staining and selected at random for imaging. Quantified images represent the 1 micron-thick plane parallel to the coverslip and midway from the top and bottom of the nucleus. Care was taken to determine the exact center of the nuclear span based on the extent of DAPI staining prior to imaging. While the experimenter was not blind to treatment at the time of imaging, the phospho-ERK1/2 channel was never observed so that selection of neurons for imaging was not biased towards expected response. Thus, the extent of phospho-ERK1/2 fluorescence was unknown prior to capturing each neuron's final image, and no images were discarded from quantification. In addition, all images were quantified after removal of treatment labels and quantification was confirmed by two independent observers who were blind to treatment conditions. Images were processed using Zeiss LSM Image Browser and average fluorescence intensity over the entire span

of the nucleus, as selected based on colocalization with DAPI staining, was analyzed using Adobe Photoshop (Adobe Systems).

Statistical Analysis

Data were analyzed using one-way ANOVAs followed by post-hoc Bonferroni (vs. control) and Student-Neuman-Keuls (differences between treatment groups) tests where appropriate. As data from primary culture treatments were found to be not normally distributed in some cases, dependent upon the between-experiment variability in basal gene expression levels as observed in control cells, these data were transformed using the natural log prior to analysis by ANOVA as described above, where indicated. In addition, primary culture data expressed as percent control were also analyzed by one sample t-test (GraphPad Software, Inc., http://www.graphpad.com/quickcalcs/OneSampleT1.cfm), which confirmed the results of the ANOVAs without requiring transformation. As percent control was calculated individually for each replicate of a given treatment prior to determining the treatment mean, the control population lacked variance and thus has been omitted from the figures. The one-sample t-test compares a given mean with a specific variability (the test data) against a hypothesized control value (for example, 100%) and extrapolates variability in the control based on the variability among the treatment population, thus satisfying the statistical requirement for equal variance in the control population. Note that the conversion to percent control rendered the test data normally distributed, thus satisfying the requirement for normality in the test population for use of the one sample t-test.

RESULTS

Ethanol treatment of primary striatal neurons increases BDNF protein levels and TrkB receptor phosphorylation

Previously, we found that ethanol treatment of dorsal striatal slices increased *BDNF* mRNA levels, and that *in vivo* ethanol increased *BDNF* expression in the dorsal striatum (McGough et al., 2004). We hypothesized that this ethanol-mediated increase in *BDNF* mRNA expression would lead to an increase in the BDNF protein and the consequent activation of the BDNF signaling pathway. To test this hypothesis we established primary striatal neurons as a model system. As shown in Figure 2.1, most primary striatal neurons in culture display characteristics of medium spiny neurons, with medium sized cell bodies and multiply branching dendrites (Figure 2.1A), and express the 67 kDa isoform of glutamic acid decarboxylase (GAD-67) (Figure 2.1B), an enzyme required for GABA synthesis that therefore identifies GABAergic neurons (Panula et al., 1981). In addition, all cells show high expression of the TrkB receptor (Figure 2.1C), as seen in striatal neurons *in vivo* (Altar et al., 1994), thus making the culture system a suitable model for studying the role of BDNF signaling in the striatal response to ethanol.

We first set out to determine whether BDNF protein increased following ethanol treatment in striatal primary neurons, since the expression profiles of BDNF mRNA and protein are not always identical (Nawa et al., 1995). Because ethanol treatment increased *BDNF* mRNA expression after 30 minutes (McGough et al., 2004), we hypothesized that BDNF protein expression would increase as well, but at a later time point due to the temporal constraints of protein synthesis. As predicted and shown in Figure 2.2, we

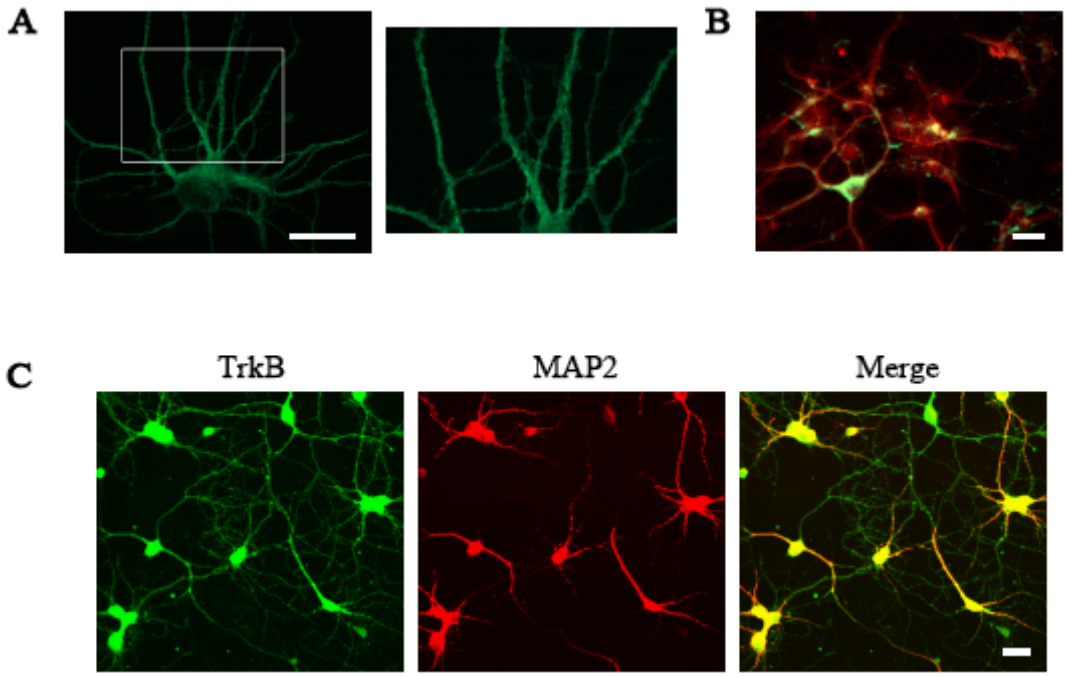


Figure 2.1. Establishment of a primary striatal culture model.
Primary striatal neurons were obtained from pooled striata of P0 Sprague Dawley rats and maintained in Neurobasal-A media for 11 days *in vitro*. (A) Neurons display a medium spiny neuron-like morphology. Neuronal morphology was visualized by staining with MAP2. (B) Primary striatal neurons are GABAergic. Neurons were co-stained with MAP2 (red) to show processes and GAD-67 (green), demonstrating that majority of the cultured neurons are GABAergic. GAD-67 was co-expressed in 88% of MAP2-positive neurons (n = 119 of 139 neurons counted from 8 fields selected at random from 4 separate cultures). (C) Striatal primary neurons express TrkB receptors. Primary neurons were co-stained with TrkB (Green) and MAP2 (red) antibodies. TrkB receptors were expressed in 100% of MAP2-positive neurons (n = 78). Scale bars represent 20 μm.

observed that 2 hours ethanol exposure increased BDNF protein expression in striatal neurons.

Next we determined whether ethanol treatment would also result in the activation of the BDNF receptor, TrkB. Binding of BDNF to the TrkB receptor results in receptor dimerization and autophosphorylation of tyrosine residues on the intracellular tail of the receptor (Huang & Reichardt, 2003). In order to determine the level of TrkB activation following ethanol treatment, TrkB was immunoprecipitated (IPed) and Western blot membranes were probed with anti-phosphotyrosine antibodies, then stripped and

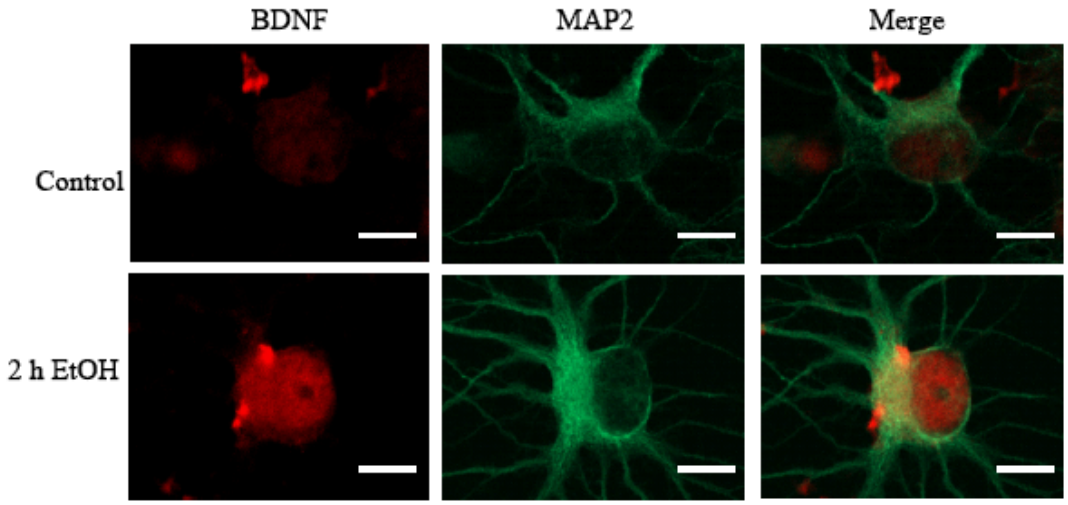


Figure 2.2. Ethanol increases BDNF protein levels in primary striatal neurons. Primary striatal neurons treated without or with 100 mM ethanol (EtOH) as indicated. Fixed cells were stained with anti-BDNF (red; left panel) and anti-MAP2 (green; middle panel). Right panel shows merged images. Visible anti-BDNF fluorescence was seen in 16% of control neurons (n = 9 of 58 over 2 fields) and 85% of ethanol-treated neurons (n = 64 of 75 over 2 fields). Scale bars represent 10 μm.

reprobed for total TrkB levels. As shown in Figure 2.3A&B, treatment of primary striatal neurons with ethanol for 4 hours resulted in a significant increase in the fraction of TrkB receptors activated within the 4-hour ethanol incubation period (main effect of Treatment, $F(1,17) = 5.566$; $p < 0.05$). This was confirmed by immunofluorescent analysis of Trk phosphorylation, as ethanol treatment increased pTrk signal (Figure 2.3C). These results suggest that exposure of striatal neurons to ethanol leads to the activation of TrkB.

Ethanol treatment of striatal neurons leads to the activation of the MAPK pathway via BDNF

BDNF-mediated activation and thus phosphorylation of the TrkB receptor activates MAPK signaling by recruiting and phosphorylating the adaptor protein Shc which interacts with the adaptor protein Grb-2. Grb-2 then recruits a guanine nucleotide

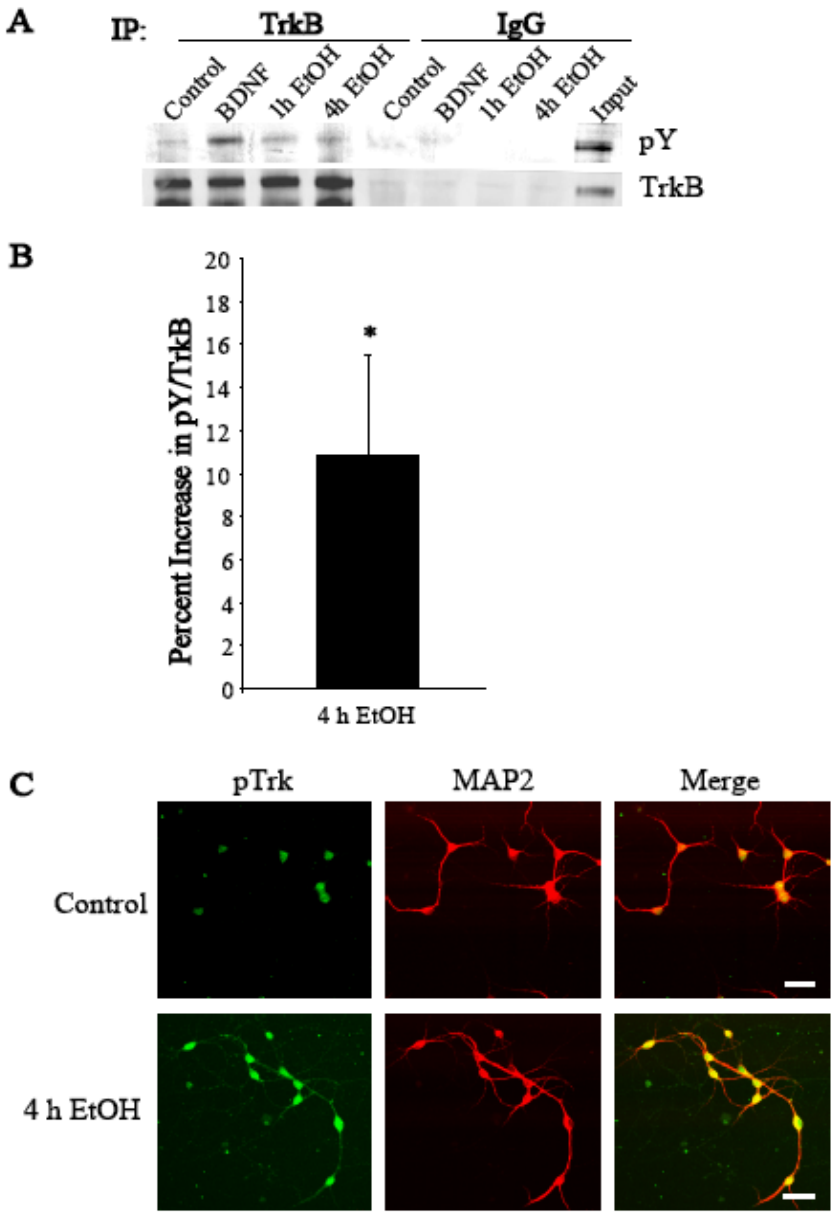


Figure 2.3. Ethanol activates TrkB receptors in primary striatal neurons.
Primary striatal neurons were treated with 100 mM ethanol (EtOH) as indicated or 500 pg/mL BDNF as a positive control. TrkB was immunoprecipitated from total cell lysate and membranes were probed for tyrosine phosphorylation with anti-p-Tyrosine antibodies (pY, top panel). Membranes were stripped and reprobed for TrkB (bottom panel). (A) Representative image showing increased TrkB phosphorylation following BDNF or 4 h ethanol treatment. (B) Quantitation of change in percent tyrosine phosphorylation of TrkB receptors as shown in A. Data are presented as mean +/- S.E.M. * $p < 0.05$, one-sample t-test. n=9. (C) Representative images demonstrating Trk phosphorylation in control as compared to 4 h ethanol-treated neurons. Fixed cells were stained with anti-pTrk(Y490) (green; left panel) and anti-MAP2 (red; middle panel). Right panel shows merged images. pTrk fluorescence was visualized in 90% of ethanol-treated neurons counted (n = 36 of 40 in a single 10X field) as compared to 26% of control neurons (n = 7 of 34 in a single 10X field). Scale bar represents 10 μm.

exchange factor for Ras, resulting in the activation of this small G protein (Grewal et al., 1999). Activated Ras triggers a cascade of phosphorylation by activating the MAPK kinase kinase Raf, which in turn phosphorylates MAPK and ERK kinases 1 and 2 (MEK1/2). MEK1/2 then phosphorylates extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Huang & Reichardt, 2003), which can translocate to the nucleus and activate transcription factors to alter gene expression (Davis, 1995). BDNF signaling via the MAPK cascade has been shown to play a role in downstream gene expression after BDNF treatment of primary neurons (Barnea & Roberts, 2001; Sato et al., 2001; Alonso et al., 2004). As ethanol treatment resulted in TrkB receptor phosphorylation, we hypothesized that the activation of the TrkB receptor would lead to the activation of the MAPK signaling cascade and thus to an increase in ERK1/2 phosphorylation and nuclear localization. To test this possibility, we treated primary striatal neurons with ethanol for 4 hours and visualized changes in the amount and nuclear localization of phospho-ERK1/2 using confocal microscopy. We quantified the level of phospho-ERK1/2 in the nucleus, which we isolated using DAPI as a nuclear marker. As shown in Figure 2.4, ethanol treatment increased the level of phospho-ERK1/2 in primary striatal neurons (Figure 2.4A left, top row (control) vs. bottom row (ethanol), with phospho-ERK1/2 shown in green). Importantly, we found that this phosphorylation of ERK1/2 by ethanol was BDNF-dependent, as addition of TrkB-Fc, a fusion protein that competes with endogenous TrkB receptors for BDNF binding (Shelton et al., 1995), blocked the ethanol-induced ERK phosphorylation (Figure 2.4A, right bottom panel (ethanol + TrkB-Fc) vs. left bottom panel (ethanol alone)). Quantitation of nuclear phospho-ERK1/2 levels revealed a main effect of treatment ($F(3,309) = 4.000$, $p = 0.008$), which was

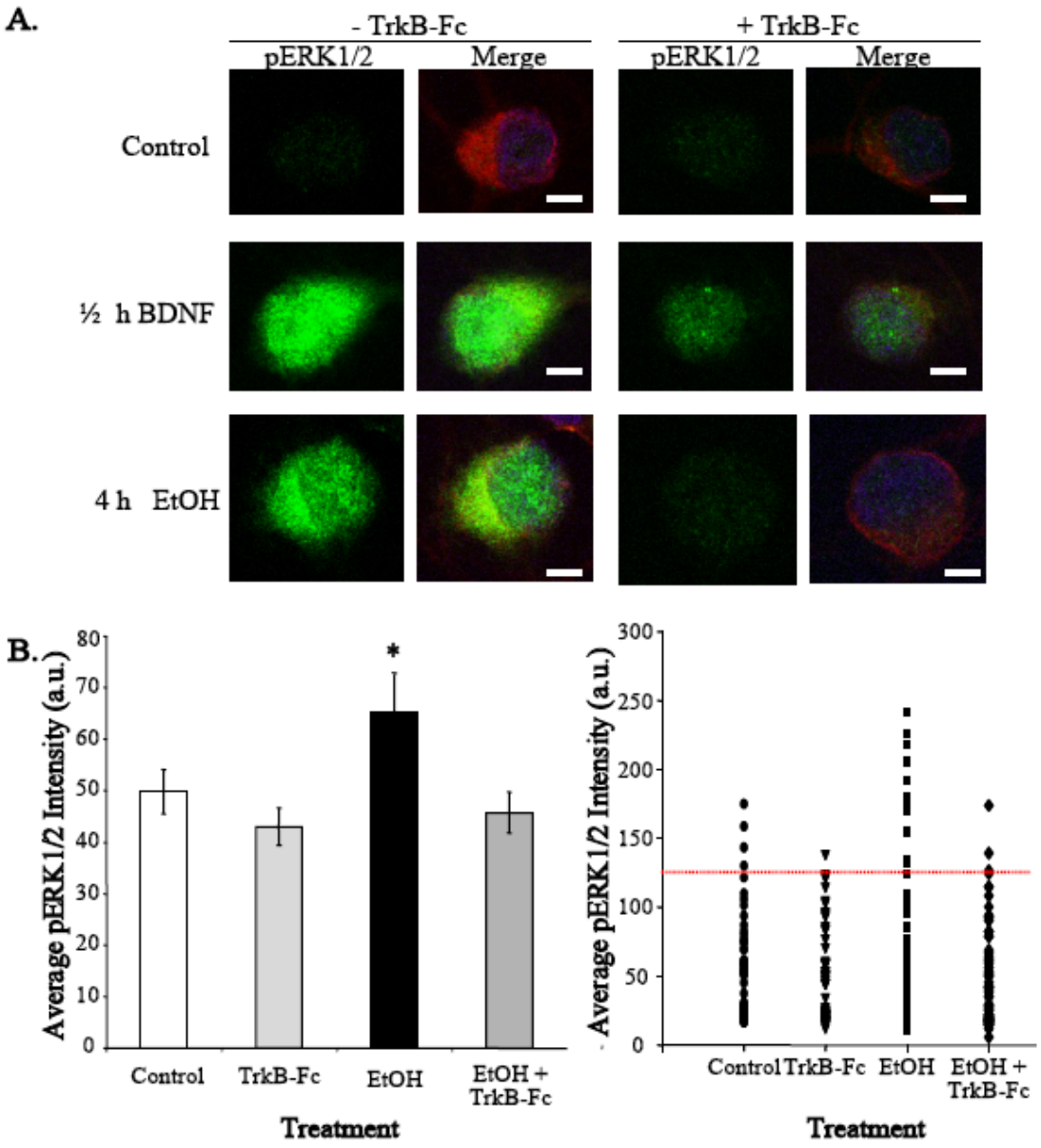


Figure 2.4. Ethanol increases ERK1/2 phosphorylation via BDNF.

(A) Representative confocal images showing the 1 μm plane midway between the top and bottom of the nucleus of primary striatal neurons treated without or with 100 mM ethanol (EtOH) for 4 h or 25 ng/mL BDNF for ½ h as a positive control, in the absence or presence of the BDNF inhibitor TrkB-Fc (250 ng/mL). Fixed cells were stained with anti-phospho-ERK1/ 2 (green, left panel), anti-MAP2 (red) and DAPI to mark nuclei (blue); right panel for each treatment shows merged image. Scale bar represents 2.5 μm. (B) Quantitation of average nuclear phospho-ERK intensity. (left panel) Histogram displays mean phospho-ERK intensity by treatment +/- S.E.M. * $p < 0.05$ vs. all other treatments. n = 71 for control, n = 82 for TrkB-Fc, n = 77 for ethanol, n = 80 for ethanol + TrkB-Fc. Nuclear images were obtained from 19-24 fields per condition from 2 independent experiments. (right panel) Spread of data for individual neuronal phospho-ERK1/2 levels. Red line indicates 2 standard deviations above the mean for control cells, or the 95% confidence interval. Note that only ethanol treatment results in a significant number of cells responding above this threshold.

accounted for by increased ERK1/2 phosphorylation in ethanol-treated cells as compared to all other groups (Figure 2.4B, left panel). Additional analysis demonstrated a significant elevation in phospho-ERK1/2 intensity in 20% of ethanol-treated neurons, as determined by phospho-ERK1/2 intensity of 2 standard deviations above the mean of control cells (Figure 2.4B, right panel, red dashed line), a criterion reached by less than 6% of cells in all other conditions. Taken together, these results show that ethanol exposure of striatal neurons leads to the activation of the MAPK signaling cascade via BDNF, yielding an increase in nuclear phospho-ERK1/2, suggesting that ethanol via BDNF may alter transcription of downstream genes.

BDNF increases *Pdyn* expression in primary striatal neurons

Thus far, we have demonstrated that ethanol exposure of striatal neurons increases BDNF protein levels, resulting in activation of the TrkB receptor, which leads to increased levels of phospho-ERK1/2 in the nucleus of striatal neurons, including those that express dynorphin. Because infusion of BDNF into the striatum increases *Pdyn* expression (Croll et al., 1994), and we observed that primary striatal neurons express high levels of TrkB receptors (Figure 2.1) and respond to ethanol via activation of BDNF signaling (Figure 2.2), we hypothesized that treatment of primary striatal neurons with BDNF would, similarly to the dorsal striatum *in vivo*, increase production of *Pdyn*. In order to test this, primary neurons were treated with or without BDNF for ½ hour, total RNA isolated and *Pdyn* expression levels determined by quantitative real-time PCR. As demonstrated in Figure 2.5, BDNF treatment significantly increased *Pdyn* expression in striatal medium spiny neurons (main effect of Treatment, $F(5,11) = 26.191$, $p < 0.005$). Thus our cell

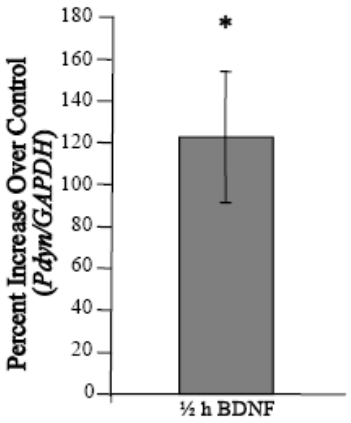


Figure 2.5. BDNF increases *Pdyn* expression in primary striatal neurons.
Primary striatal neurons were treated with 25 ng/mL BDNF for ½ hour. Data are expressed as mean percent increase over control *Pdyn/GAPDH* expression +/- S.E.M. * p <0.05. n=6.

culture model demonstrates remarkable similarity to the striatum *in vivo* in that BDNF signaling results in increased production of *Pdyn*.

Ethanol increases *Pdyn* expression in striatal primary neurons

As we previously found that ethanol increases BDNF levels (Figure 2.2) and BDNF increases *Pdyn* expression (Figure 2.5) in striatal primary neurons, we hypothesized that ethanol treatment of primary striatal neurons would increase *Pdyn* expression. To test this, cells were exposed to ethanol for 2 or 4 hours, as indicated, total RNA isolated and *Pdyn* expression quantified as above. As predicted and shown in Figure 2.6, both 2 and 4 hour ethanol treatments significantly increased *Pdyn* expression as compared to control (main effect of Treatment, $F(5,23) = 12.211$, $p < 0.001$). Thus ethanol treatment triggers increased *Pdyn* expression at time points shown to be relevant to BDNF expression (2 hours, Figure 2.2) and BDNF signaling, including MAPK activation (4 hours, Figures 2.3 & 2.4). This suggested that the increased *Pdyn*

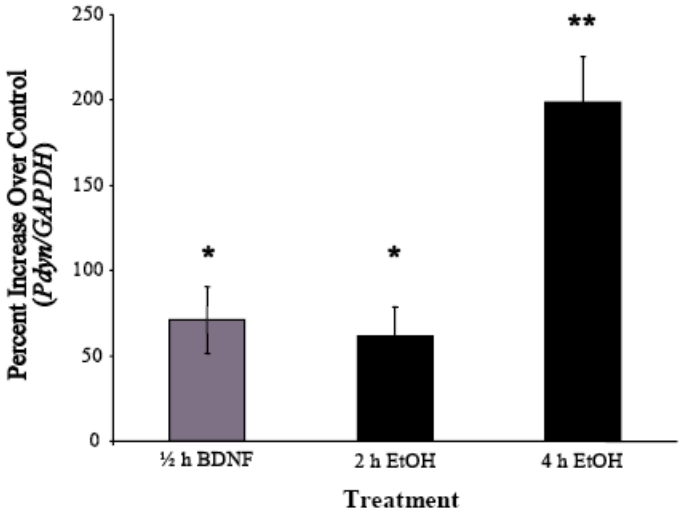


Figure 2.6. Ethanol increases *Pdyn* expression in primary striatal neurons.
Primary striatal neurons were treated without or with 100 mM ethanol (EtOH), as indicated, or with 5 ng/mL BDNF as a positive control. Data are expressed as mean percent increase over control *Pdyn/GAPDH* expression +/- S.E.M. * $p < 0.05$, ** $p < 0.001$. n=6.

expression following ethanol treatment may be due to ethanol-induced BDNF expression and signaling.

Ethanol increases *Pdyn* expression in primary striatal neurons via a BDNF- and MAPK-dependent mechanism

Thus far, we have demonstrated that ethanol exposure of striatal neurons increases BDNF protein levels (Figure 2.2), resulting in activation of the TrkB receptor (Figure 2.3), which leads to increased levels of phospho-ERK1/2 in the nucleus (Figure 2.4), and that the same ethanol treatments significantly increase *Pdyn* mRNA expression (Figure 2.6). Because BDNF can increase expression of downstream genes via activation of the MAPK pathway (Marsh et al., 1993; Barnea & Roberts, 2001), and we observed increased *Pdyn* expression following BDNF treatment (Figure 2.5), we hypothesized that ethanol, via BDNF-mediated activation of ERK1/2, would increase *Pdyn* expression in

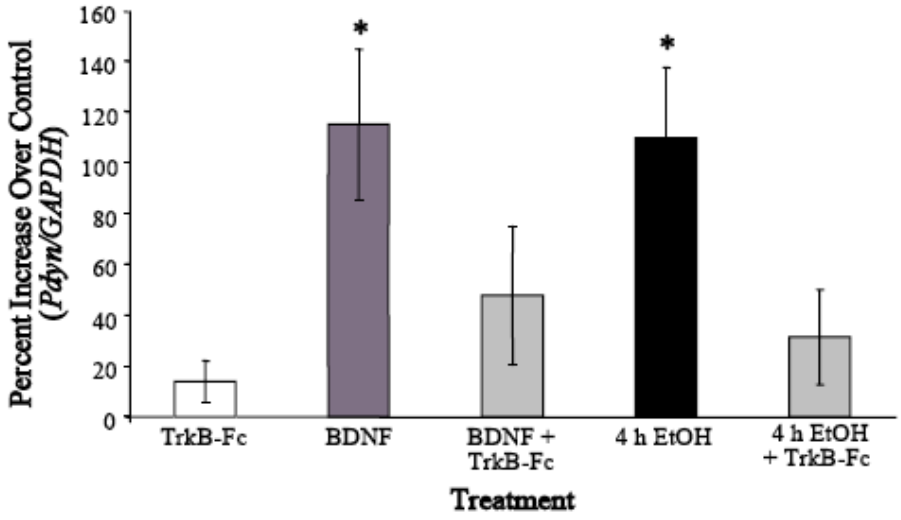


Figure 2.7. Ethanol increases *Pdyn* expression via BDNF.
Striatal primary neurons were treated without or with 100 mM ethanol (EtOH) or with 25 ng/mL BDNF in the absence or presence of the BDNF inhibitor TrkB-Fc (250 ng/mL). Data are expressed as mean percent increase over control *Pdyn/GAPDH* expression +/- S.E.M. * $p < 0.05$. n=6.

primary striatal neurons. To test this, primary striatal neurons were treated with ethanol in the presence or absence of the BDNF inhibitor TrkB-Fc, to block BDNF signaling, or in the presence or absence of the MEK inhibitor U0126, to block activation of ERK1/2. Following treatment, total RNA was isolated from cells and mRNA expression assessed using quantitative RT-PCR. As shown in Figure 2.7, we found a significant increase in *Pdyn* expression following ethanol treatment that was blocked by the addition of TrkB-Fc ($F(5,35) = 10.628$; $p < 0.001$ for BDNF and 4 hours EtOH vs. Control), demonstrating that ethanol treatment increases *Pdyn* via BDNF. In addition, we found that ethanol-induced increases in *Pdyn* expression were also blocked by the MEK inhibitor U0126 (Figure 2.8, $p < 0.05$, for BDNF and 4 hours EtOH vs. Control; $p < 0.05$ for BDNF+U0126 vs. BDNF alone and vs. Control; no significant difference between 4 hours EtOH + U0126 and Control), indicating that ethanol treatment increases *Pdyn* by activating MAPK signaling.

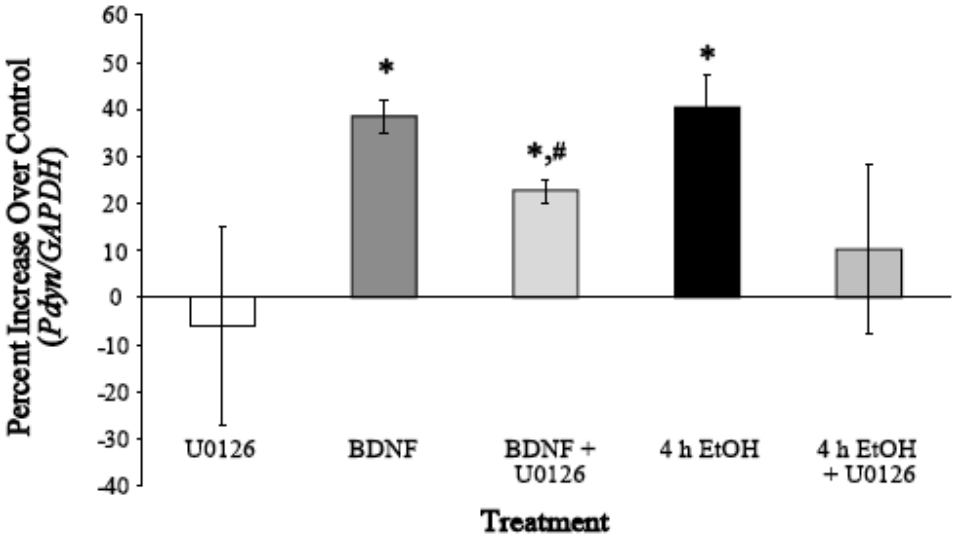


Figure 2.8. Ethanol increases *Pdyn* expression via the MAPK pathway.
Striatal primary neurons were treated without or with 100 mM ethanol (EtOH) or with 25 ng/mL BDNF in the absence or presence of the MEK inhibitor U0126 (20 μm, ½ h pretreatment). Data are expressed as mean percent increase over control *Pdyn/GAPDH* expression +/- S.E.M. * $p < 0.05$ vs. control, # $p < 0.05$ vs. BDNF. n=3.

DISCUSSION

Here we show that ethanol treatment of primary striatal neurons results in activation of the BDNF receptor, TrkB, and subsequent activation of the MAPK cascade, in a BDNF-dependent fashion. We also identify dynorphin as a downstream effector of BDNF signaling in striatal neurons, as ethanol treatment results in increased production of *Pdyn* via a BDNF- and MAPK -dependent mechanism. Taken together, these results indicate that the BDNF homeostatic response to ethanol triggers activation of MAPK signaling, initiating production of the downstream effector dynorphin, whose expression is responsible for the reduction of ethanol intake by BDNF (Figure 2.9).

Ethanol increases BDNF protein levels and TrkB receptor phosphorylation

While we have previously demonstrated increased striatal *BDNF* mRNA expression

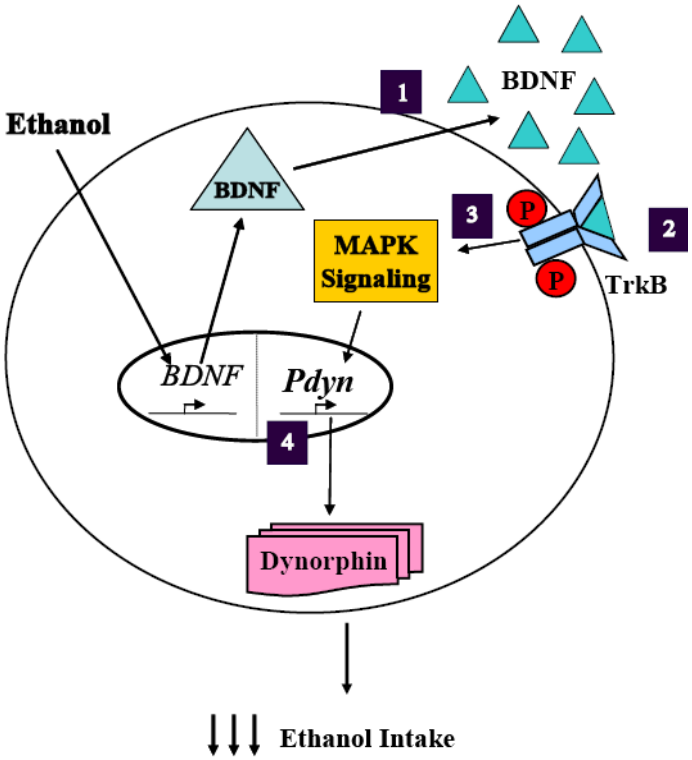


Figure 2.9. Model of the ethanol-induced BDNF signaling cascade.
The striatal response to ethanol results in the following cascade: (1) Ethanol increases BDNF expression. (2) BDNF binds to the TrkB receptor, resulting in autophosphorylation and activation of downstream signaling, including (3) activation of the MAPK signaling pathway. This increases transcription of downstream genes (4), including *Pdyn*. Following translation, dynorphin is released and acts to decrease ethanol intake.

following acute ethanol treatment (McGough et al., 2004) and provided *in vivo* evidence to suggest the involvement of TrkB signaling in the control of ethanol intake by BDNF (Jeanblanc et al., 2006), here we demonstrate that ethanol exposure increases BDNF protein levels and consequently increases activation of the TrkB receptor via a direct effect upon striatal neurons. While others have demonstrated an involvement of striatal BDNF signaling in response to drugs of abuse (Le Foll et al., 2005; Jeanblanc et al., 2006), as well as changes in cocaine self-administration in response to increases or decreases in BDNF (Graham et al., 2007), to our knowledge this is the first demonstration that exposure to any drug of abuse triggers activation of striatal TrkB by

BDNF produced within the striatum. Specifically, these data demonstrate a role for locally-produced BDNF in activation of striatal TrkB signaling, since primary striatal cultures are devoid of cortical input, a major source of BDNF for the striatum *in vivo* (Altar et al., 1997). The observed effect is modest (10.8%); nonetheless it is statistically significant and is in line with the previously reported *in vivo* activation of TrkB signaling by cocaine in the NAc (Graham et al., 2007).

Ethanol exposure leads to the activation of the MAPK signaling cascade

Acute exposure to multiple drugs of abuse activates MAPK signaling in the striatum (Valjent et al., 2004), and exposure to cocaine-related cues in withdrawal increases BDNF and MAPK activation (Lu et al., 2005). Additional evidence demonstrating the involvement of MAPK signaling downstream of BDNF in the central and medial nuclei of the amygdala in response to acute ethanol (Pandey et al., 2006) indicates that BDNF may act via MAPK signaling in the striatum to control ethanol intake. We provide here direct evidence that ethanol exposure leads to the activation of the MAPK signaling cascade in striatal neurons in a BDNF-dependent fashion.

Interestingly, we saw only a partial increase in ERK1/2 phosphorylation, similar to the results obtained for TrkB phosphorylation. This suggests several possibilities. First, ethanol-induced BDNF might act selectively on the striatonigral projection neurons, which express dynorphin (Gerfen, 1988). Thus investigation of a subpopulation-selective striatal neuronal activation by BDNF would be an enlightening future study. However, striatal BDNF infusion increases both *Pdyn* and *proenkephalin* (Croll et al., 1994), indicating that both medium spiny neuron subtypes are responsive to BDNF stimulation.

Thus while a subtype-specific response is an attractive explanation for the moderate level of TrkB and ERK1/2 phosphorylation observed in the current study, a more probable cause is the quiescent nature of striatal cultures. Specifically, striatal cultures devoid of cortical input display limited network activity (Segal et al., 2003). At present it is unknown whether ethanol-treated striatal cultures release BDNF via the constitutive (activity-independent) or regulated (activity-dependent) release pathway. BDNF release via the regulated release pathway would be minimal given the low level of spontaneous activity in primary striatal cultures (Segal et al., 2003), suggesting that the moderate levels of TrkB and ERK1/2 phosphorylation observed may be due to limited release of the ethanol-induced BDNF. Therefore determination of the mechanism of BDNF release following ethanol treatment would be an informative future study. Alternatively, it is possible that striatal afferents are not only necessary for proper striatal synapse formation (Segal et al., 2003) but also play an integral role in mediating the BDNF response to ethanol. Thus the absence of cortical and/or nigral input in primary striatal cultures may blunt the BDNF response to ethanol. Finally, it should also be noted that these cultures are derived from early postnatal rat brains and therefore the observed responses to both ethanol and BDNF may differ from those observed in intact adult animals. For example, repeated forced ethanol ingestion in postnatal rats decreases total brain size and cortical *BDNF* expression (Fattori et al., 2007), while chronic ethanol treatment of primary cortical neurons derived from embryonic rat brain results in elevated cell death (Mooney & Miller, 2003). These data demonstrate the toxicity of prolonged or repeated exposure to ethanol during development of the cortex, and while our treatment is both significantly shorter than those shown to cause toxicity to primary neurons and in the striatum, whose

neuronal composition differs significantly from that of the cortex, we cannot at this time exclude either mild toxicity or developmental stage as contributing factors in the moderate level of response observed. Thus investigation of ethanol-induced alteration of BDNF signaling *in vivo*, in particular regarding the involvement of specific afferent inputs in the BDNF response, will be a critical future study.

Increased expression of *Pdyn* by ethanol via BDNF & MAPK

Striatal dynorphin expression can be upregulated by cocaine (Spangler et al., 1993; Mathieu-Kia & Besson, 1998; Turchan et al., 1998; Fagergren et al., 2003; Schlussman et al., 2005) and amphetamine (Smith & McGinty, 1994; Wang et al., 1995; Turchan et al., 1998); however, responsiveness of striatal dynorphin expression to ethanol treatment has been mixed, with some reports showing increased expression (Gulya et al., 1993) and others showing no change (Mathieu-Kia & Besson, 1998; Cowen & Lawrence, 2001). This may be due in part to differences in ethanol administration protocols and time points of analyses with respect to the final ethanol administration. In addition, some data have shown increases in dynorphin levels only upon ethanol withdrawal (Przewlocka et al., 1997; Beadles-Bohling et al., 2000; Lindholm et al., 2000), but see also (Seizinger et al., 1983; Ploj et al., 2000)); however, these changes were all found outside the dorsal striatum, and thus may demonstrate intrinsic brain region-specific differences in regulation of dynorphin expression. We found that acute ethanol treatment increases *Pdyn* expression via a BDNF- and MAPK-dependent mechanism. Importantly, this induction requires time, as ethanol must first increase *BDNF* mRNA and protein production and release to trigger the signaling cascade which culminates in increased

Pdyn expression. It should be noted that our cultures do not specifically differentiate between dorsal and ventral striatum (NAc), and a rapid increase in dynorphin release in the NAc shell has been demonstrated following ethanol treatment (Marinelli et al., 2006). Nonetheless, the existence of a rapid release mechanism in no way precludes the existence of a second longer-acting mechanism, as described here. In addition, as we observed ethanol-induced BDNF expression specifically in the dorsal, and not ventral, striatum following ethanol intake (McGough et al., 2004), it is likely that the increased *Pdyn* expression we observed, which was dependent on ethanol-induced BDNF signaling and peaked after a 4-hour ethanol treatment, models the homeostatic response of the dorsal striatum following ethanol intake.

Possible mechanism of altered *Pdyn* expression by BDNF and ethanol

As detailed above, multiple drugs of abuse increase both phosphorylation of ERK1/2 (Valjent et al., 2004) and expression of *Pdyn* (Spangler et al., 1993; Turchan et al., 1997; Mathieu-Kia & Besson, 1998; Zhou et al., 2002; Fagergren et al., 2003; Schlussman et al., 2005) in the striatum. However, the time scale for induction of ERK activation and *Pdyn* expression by drugs other than ethanol is much more rapid, with increases in signaling observed in as little as 5 minutes after cocaine injection (Valjent et al., 2000) and increased *Pdyn* expression observed within 2 hours of cocaine or amphetamine treatment (Hurd & Herkenham, 1992). Interestingly, with ethanol treatment we observed an initial increase in *Pdyn* which was significantly elevated as compared to control after 2 hours of ethanol exposure, but which increased even more dramatically after 4 hours treatment. This suggests that there may be two mechanisms

whereby ethanol increases *Pdyn* expression – a more rapid mechanism which may be BDNF-independent and a slower mechanism which, as shown, is BDNF-dependent. Thus the BDNF-dependent alteration in *Pdyn* expression may be mechanistically distinct from the more rapid cocaine- or amphetamine-induced alteration of *Pdyn* levels. The *Pdyn* gene contains both cyclic AMP response element (CRE) and activator protein 1- (AP-1) like domains within its promoter region (Naranjo et al., 1991; Douglass et al., 1994; Messersmith et al., 1994), and amphetamine has been shown to increase *Pdyn* specifically via the CRE sites in a dopamine D1 recpetor-dependent manner (Cole et al., 1995). Importantly, dopamine treatment of striatal neurons – mimicking *in vivo* amphetamine treatment – increased binding of phosphorylated cyclic AMP response element binding protein (CREB) to the three CRE sites of the *Pdyn* promoter within 5 minutes of treatment (Cole et al., 1995). As drugs of abuse, including ethanol, trigger dopamine release (Di Chiara & Imperato, 1985, 1988), and activation of the dopamine D1 receptor increases PKA activity (Simpson & Morris, 1995), this suggests a rapid increase in *Pdyn* expression may be triggered by drug-induced dopamine release and subsequent PKA activation. In particular, this increase in *Pdyn* is likely regulated specifically by activation of the CRE sites, since PKA phosphorylates CREB on serine 133 to activate transcription (Shaywitz & Greenberg, 1999), and PKA and CREB are capable of significantly increasing *Pdyn* expression (Douglass et al., 1994). While dopamine-induced *Pdyn* expression is unlikely in primary striatal cultures in response to ethanol treatment, as they lack dopaminergic input, ethanol has been shown to activate PKA and CREB (Dohrman et al., 1996; Constantinescu et al., 1999). Thus ethanol, like other drugs of abuse, may trigger a fast, BDNF-independent increase in *Pdyn* expression.

Importantly, as demonstrated here, ethanol also activates a slower, BDNF-dependent increase in *Pdyn* mRNA. While CREB is similarly a key target of BDNF (Finkbeiner et al., 1997), unlike dopamine BDNF also stimulates AP-1 activity (Gaiddon et al., 1996), and coactivation of all promoter regions on the *Pdyn* gene may trigger maximal activation, as observed with the 4-hour ethanol treatment. As dynorphin acts at least in part to decrease dopamine release (Reid et al., 1988), both routes of elevated *Pdyn* expression could serve as negative feedback to counteract the acute drug-induced dopamine release. Taken together, these data suggest a dual regulation of *Pdyn* expression in which a partial increase in expression occurs with activation of a subset of promoters whereas a full increase in expression is triggered by complete activation of the CRE and AP-1 promoters. According to this model, dopaminergic activation of striatal neurons induces a partial increase in *Pdyn* sufficient to suppress the acute increase in dopamine release triggered by drug exposure. Conversely, BDNF activates a delayed but heightened *Pdyn* response by activating both the CRE and AP-1 promoter sites, resulting in a further decrease in dopamine release which may be temporally coincident with the withdrawal phase *in vivo*. Heightened dynorphin expression has been suggested to create a dysphoric state in withdrawal (Walker & Koob, 2007) which could provide physiological feedback for the post-ingestive effects of ethanol and decrease subsequent ethanol intake. While this dysphoria is likely subserved by the NAc, increased dynorphin in the dorsal striatum may instead act to weaken the stimulus-response association between the act of ethanol consumption and its rewarding effects (Yin et al., 2004; Yin et al., 2005), thereby preventing the escalation of ethanol intake and the development of the ethanol addiction habit.

CHAPTER 3

BDNF modulates behavioral responses to ethanol via its downstream effector dynorphin

ABSTRACT

Previously we identified a homeostatic pathway whose activity may account for protection against the development of addiction, in which acute ethanol exposure increases expression of brain-derived neurotrophic factor (BDNF), signaling via the mitogen-activated protein kinase (MAPK) pathway and production of the downstream effector *preprodynorphin* (*Pdyn*). In addition, acute ethanol increased *BDNF* specifically in the dorsal striatum *in vivo*, and that elevated *BDNF* expression resulted in decreased ethanol intake, while reduction of BDNF or blockade of BDNF signaling increased both ethanol sensitivity in multiple behavioral paradigms and ethanol consumption (McGough et al., 2004; Jeanblanc et al., 2006). Taken together, these data indicate that BDNF, via the downstream effector dynorphin, may act to reduce acute responses to ethanol, thereby decreasing ethanol intake. Using BDNF conditional knockout mice, in which BDNF is deleted from the forebrain postnatally, we show that BDNF deletion increases ethanol intake without altering preference for other tastants. We also demonstrate that application of either BDNF or Tat-RACK1 to the dorsal striatum, treatments which are capable of decreasing ethanol intake, elevates *Pdyn* expression in the dorsal striatum. Importantly, we show that control of ethanol intake by BDNF requires activity of the downstream effector dynorphin, as blockade of dynorphin's receptor, the kappa opioid receptor (KOR), inhibits the ability of Tat-RACK1, which increases *BDNF* expression, to decrease ethanol consumption. Finally, we demonstrate that mimicking the action of dynorphin via administration of a KOR agonist blocks ethanol conditioned place preference. Taken together, these data show that dynorphin is a key downstream effector of BDNF regulation of ethanol-related behaviors, including consumption.

INTRODUCTION

Recently we identified a homeostatic pathway in which ethanol increases BDNF protein levels, BDNF signaling via its receptor TrkB and the MAPK pathway and expression levels of the downstream effector *Pdyn* (Chapter 2). In conjunction with our earlier findings that acute ethanol increases *BDNF* expression in the dorsal striatum, while BDNF levels and signaling regulate ethanol intake and ethanol sensitivity (McGough et al., 2004; Jeanblanc et al., 2006), we suggest that BDNF regulates ethanol-related behaviors, including ethanol consumption, via increasing expression of the downstream effector dynorphin and activation of its receptor, the KOR.

While BDNF regulation of the response to drugs of abuse was first proposed more than ten years ago (Baek et al., 1996; Berhow et al., 1996), until recently much of the attention has focused on the mesolimbic dopaminergic pathway. Specifically, the focus has been on the role of BDNF in the ventral tegmental area (VTA) and ventral striatum, or nucleus accumbens (NAc) (Berhow et al., 1996; Numan et al., 1998; Horger et al., 1999; Meredith et al., 2002; Grimm et al., 2003; Narita et al., 2003; Lu et al., 2004; Le Foll et al., 2005; Pu et al., 2006; Graham et al., 2007). In particular, a majority of the research investigating BDNF involvement in drug-taking and drug-seeking has focused on cocaine (Berhow et al., 1996; Horger et al., 1999; Grimm et al., 2003; Lu et al., 2004; Le Foll et al., 2005; Pu et al., 2006; Graham et al., 2007). BDNF infusion into the NAc increases locomotor sensitization to cocaine as well as cocaine place preference (Horger et al., 1999). Further investigation has demonstrated a role for BDNF in cocaine craving and drug-seeking during withdrawal, as VTA, NAc and amygdala BDNF levels increase in cocaine-withdrawn animals in a time-dependent manner (Grimm et al., 2003).

Interestingly, recent studies have shown that acute cocaine increases BDNF expression and signaling in the NAc, and that this BDNF signaling increases cocaine seeking (Graham et al., 2007). However, these data demonstrate a role for BDNF that contrasts to that seen for ethanol, not just in the dorsal striatum (McGough et al., 2004; Jeanblanc et al., 2006) but in the cortex and amygdala as well (Pandey et al., 1999; Pandey et al., 2006).

Unlike cocaine, multiple lines of evidence indicate that the BDNF response to ethanol functions to decrease ethanol intake. Mice heterozygous for the cyclic AMP response element binding protein (CREB) drink significantly more ethanol than their wild-type (WT) littermates, concomitant with decreased expression of BDNF mRNA and protein in all brain regions tested, including the cortex and amygdala (Pandey et al., 2004). These mice also display increased anxiety-related behaviors (Pandey et al., 2004), and subsequent studies from the same researchers have demonstrated a requirement for central amygdala BDNF in both of these behaviors (Pandey et al., 2006). Importantly, Pandey and colleagues have shown that administration of BDNF antisense oligonucleotides into the central or medial amygdala, a treatment which reduces both *BDNF* expression and activity of downstream signaling partners including extracellular signal-regulated kinase (ERK) and CREB, significantly increases both anxiety-related behaviors and voluntary ethanol consumption (Pandey et al., 2006). Taken together with our previous work, these data indicate that BDNF expression in the dorsal striatum and amygdala function to reduce ethanol intake and ethanol-related behaviors. Critically, both lines of research have demonstrated evidence for alteration in signaling downstream

of BDNF, indicating a requirement for downstream effectors in the regulation of ethanol-related behaviors by BDNF.

Extended BDNF infusion into the dorsal striatum increases the expression of *Pdyn* mRNA (Croll et al., 1994), and we have demonstrated that ethanol treatment of striatal neurons increases *Pdyn* expression via a BDNF-dependent mechanism (Figure 2.7, p. 38). Additionally, activation of dynorphin's receptor, the KOR, decreases ethanol intake (Lindholm et al., 2001), while blockade of the KOR increases ethanol intake (Mitchell et al., 2005), indicating that dynorphin may acutely regulate ethanol intake. Dynorphin and KOR levels are lower in various components of the mesostriatal dopaminergic system of C57BL/6 (C57) mice, which drink more ethanol, than in DBA mice (Jamensky & Gianoulakis, 1997; Winkler & Spanagel, 1998; Ploj et al., 2000), suggesting that dynorphin expression may inversely correlate with ethanol intake. Admittedly much of the difference in intake between C57 and DBA mice results from heightened aversion to the bitterness of ethanol in DBA mice (Grahame & Cunningham, 1997; Blizard, 2007). However, when oral ingestion is bypassed using intravenous self-administration, C57 mice still administer higher amounts of ethanol than DBA mice during the early phase of training (Grahame & Cunningham, 1997), suggesting that decreased dynorphin and KOR expression correlates with increased ethanol intake. Additional work has demonstrated a dysregulation of dynorphin signaling in ethanol dependence, as blockade of the KOR decreases ethanol self-administration only in ethanol-dependent rats (Walker & Koob, 2007). While this effect is opposite from the proposed role of dynorphin in regulation of acute ethanol intake, it suggests that development of physiological ethanol dependence may involve breakdown of an

otherwise protective dynorphin signal. Additional data indicate that withdrawal increases dynorphin signaling such that inhibition of the KOR results in increased dopamine release in dependent but not nondependent animals (Lindholm et al., 2007). Importantly, dopamine release is required for development of conditioned place preference to multiple drugs of abuse (Tzschentke, 1998), and activation of the KOR has previously been shown to blunt place preference to both morphine and cocaine (Funada et al., 1993; Shippenberg et al., 1996; Pliakas et al., 2001; McLaughlin et al., 2006). Taken together, these data indicate that dynorphin may provide acute feedback following ethanol exposure, possibly by reducing ethanol reward, thus decreasing future ethanol intake.

Based on these existing data, we hypothesized that BDNF modulates ethanol intake via dynorphin/KOR signaling, and that one mechanism of dynorphin regulation of ethanol intake is via inhibition of the rewarding properties of ethanol.

METHODS

Reagents

Recombinant human BDNF, DNase, nor-binaltorphimine (nor-BNI) and N-methyl-N-7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-11-4-benzofu ranacetamide (U50,488H) were obtained from Sigma Aldrich (St. Louis, MO). Physiological saline (0.9% sodium chloride) was obtained from Hospira, Inc. (Lake Forest, IL). Tat-RACK1 fusion protein was expressed in *Escherichia coli* and purified as previously described (He et al., 2002). TRIzol and the Reverse Transcription System were purchased from Promega Corporation (Madison, WI). All real-time PCR reagents, including TaqMan

Gene Expression Assays, were obtained from Applied Biosystems, Inc. (Foster City, CA).

Animals

C57BL/6J (C57) mice were obtained at 6-8 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Two founder mating pairs of $BDNF^{Tm3/Jae}$ mice which contain loxP sites surrounding exon 5 of the BDNF gene (floxed BDNF) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in-house. Original mating pairs of mice expressing CaMKIIα under the control of the Cre promoter (B6.Cg-Tg(Camk2a-cre)1Lfr/Mmcd; CaMKIIα-Cre) were obtained from the Mutant Mouse Regional Resource Center at the University of California, Davis (Davis, CA) and bred in-house. Animals were housed under a 12 h light/dark cycle, with lights on at 7:00 a.m. and lights off at 7:00 p.m., and were provided with continuous *ad libitum* access to food and water. All animals purchased just prior to study were given a minimum of 1 week to rest without experimentation following arrival in the animal facility. All animal procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council (1996).

Two-bottle choice intake

Ethanol

Mice were singly housed in double grommet cages at least one week prior to the initiation of ethanol access. Fluid solutions were provided in cylindrical glass bottles

equipped with standard stoppers, placed on the home cage and available 24 hours a day. Mice were given 2 bottles – one containing water and the other containing a solution of 10% ethanol (v/v). While no water deprivation or sucrose fading were used in drinking studies involving C57 mice, the background strain of the BDNF conditional knockout mice (129S4, BALB-c, B6 hybrid) resulted in high sensitivity to the bitterness of ethanol (regardless of genotype) and thus necessitated sweetening with 0.2% saccharin. Bottles were weighed every 1-2 days to monitor consumption and the placement order of the bottles on the home cage was switched to ensure that consumption levels were not due to a side bias. Body weight and physical appearance were monitored every few days throughout the course of the experiment to ensure that all subjects remained in good health.

Saccharin & quinine

In order to measure differences in taste preference in the BDNF conditional deletion mice, two bottles were provided on the home cage as above, except that, rather than ethanol, the second bottle contained a solution of saccharin (0.03% or 0.06%) or quinine (0.015 mM or 0.03 mM). Bottle placement was switched and consumption and body weight monitored as above.

Tat-RACK1 & nor-binaltorphimine injections

For determination of the effects of Tat-RACK1 and nor-BNI on ethanol intake, a single injection containing either or both of the treatments was administered approximately 3.5 hours prior to the beginning of the dark cycle. Intraperitoneal (i.p.)

injections were given at a volume of 0.02 mL per gram body weight; mice were weighed on the day of injection in the morning, approximately 6 hours prior to injection. The injection mixture, by volume, was 50% Tat-RACK1 (4 mg/kg) or vehicle (10% glycerol in PBS) and 50% nor-BNI (30 mg/kg) or vehicle (0.9% saline). Bottles were returned to the cages 3 hours prior to the onset of the dark cycle, or approximately ½ hour after the injection. Intake was measured over the 17-hour period following the injection (i.e. between 4 p.m. and 9 a.m.).

Acute treatment of striatal slices

Sprague Dawley rats, aged 21-28 days at the time of experimentation, were lightly anesthetized and euthanized by rapid decapitation. The brains were rapidly removed and placed immediately in ice cold aCSF containing (in mM): 126 NaCl, 1.2 KCl, 1.2 NaH_2PO_4, 0.01 $MgCl_2$, 2.4 $CaCl_2$, 18 $NaHCO_3$ and 11 Glucose, saturated with 95% O_2/5% CO_2. Coronal slices (175 μm) were cut on a vibratome (Leica, Nussloch, Germany), the dorsal striata dissected out and transferred to rest in saturated aCSF (as above) at 25°C for 90 minutes prior to treatment. At the conclusion of treatment, slices were collected and samples processed for analysis of mRNA levels by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as described below.

Acute surgical infusion of BDNF

Adult Long-Evans rats (weighing 400 – 600 g at the time of surgery) were anesthetized using isoflurane and placed in a standard stereotaxic frame. Single holes were drilled bilaterally to allow access to the dorsal striatum at AP +1.20 mm, ML +/-

3.40 mm, relative to Bregma. Hamilton syringes – one containing 0.25 μg/mL BDNF and one containing vehicle (PBS) – were inserted into stereotaxic microinjection units and lowered to DV -4.20 mm for direct infusion of BDNF or vehicle into the dorsolateral striatum. Rats were infused with 1 μL BDNF in one hemisphere and 1 μL vehicle in the other; the hemisphere receiving BDNF (left or right) and the order of infusion (BDNF or vehicle first) were counterbalanced across subjects. In order to allow time for signaling events downstream of BDNF, rats were maintained under anesthesia for 3 h after the BDNF infusion. This time point, as well as the BDNF dose chosen, had previously been determined to show behavioral efficacy in reducing ethanol intake in a self-administration paradigm (Jerome Jeanblanc, personal communication). At the end of the 3-hour delay, rats were sacrificed by rapid decapitation and striatal tissue punches were taken from the region immediately surrounding the tip of the infusion track. Samples were processed for analysis of mRNA levels by qRT-PCR as detailed below.

Conditioned place preference (CPP)

The place conditioning apparatus consists of three chambers – two conditioning chambers and a central access chamber. The conditioning chambers, which measure 6.6" long by 5" wide by 5" high, have distinct visual and tactile cues – one chamber has white walls with a stainless steel mesh floor while the other has black walls with a stainless steel rod floor. The central access chamber, which measures 2.85" long by 5" wide by 5" high, has gray walls and a matching smooth gray PVC floor. In addition, each chamber is equipped with a house light, and the luminance is adjusted such that the sum total of the environmental (visual and tactile) cues does not result a significant baseline

preference across all mice for a specific chamber. All chambers are equipped with photobeams which span the width of each chamber – 2 in the central chamber and 6 in each of the conditioning chambers. The chambers are equipped with automated guillotine doors which open upon disruption of the photobeam in the central chamber and, if necessary, close upon disruption of two consecutive photobeams in either conditioning chamber, as determined by experimental condition.

The place conditioning procedure is conducted over 10 consecutive days, during which time mice are trained under a Pavlovian conditioning paradigm to associate ethanol (the unconditioned stimulus, or US) with a specific conditioning chamber (the conditioned stimulus, or CS). The alternate conditioning chamber is paired with saline such that mice are equivalently exposed to both chambers but only one is explicitly paired with reward (reviewed in Tzschentke, 1998). On the first, or habituation, day, mice are placed in the central chamber, both doors open and the subjects are allowed free access to all three chambers for the duration of a 30-minute session. The next eight days comprise the conditioning sessions, with one 5-minute session run daily. On conditioning days 2, 4, 6 and 8, mice are injected with ethanol or saline and immediately confined in the appropriate conditioning chamber (the CS for ethanol or the unpaired chamber for saline). On alternate days (conditioning days 3, 5, 7 and 9), mice are injected with saline or ethanol and confined in the unpaired or CS chamber, respectively, for 5 min. On the tenth, or test, day, mice are placed in the central chamber and allowed free access to both conditioning chambers for the duration of a 30-minute session. Both habituation and test sessions are performed in a drug-free state.

To determine the involvement of the KOR in the acquisition of conditioned place preference for ethanol, mice received two i.p. injections on each conditioning day – the first of U50 (1 mg/kg of a 0.1 mg/mL solution) or vehicle, followed 10 minutes later by a second injection of ethanol (2 g/kg of a 20% (v/v) solution of ethanol in saline) or saline. Mice were placed in the central access chamber immediately after the ethanol/saline injection. Drug condition and chamber were counterbalanced across the days as follows: half the animals received drug treatments on days 2, 4, 6 and 8 and vehicle on days 3, 5, 7 and 9, and the other half received vehicle on days 2, 4, 6 and 8 and drug treatments on days 3, 5, 7 and 9. Within these two groups, half received drug treatment paired with the white chamber and half with the black, such that drug treatment day, drug-paired chamber and drug-paired chamber-by-treatment-day were all counterbalanced across the treatment groups.

Conditioned place aversion (CPA) training sessions were performed similarly, except that a single injection of U50 (1 mg/kg, s.c.) or saline was administered immediately before a 30-minute conditioning session, and all mice were conditioned against preference. In addition, CPA conditioning required only 3 training sessions per side.

All data are presented as the average of the CPP Score, which is calculated as the difference in time spent in the CS chamber and time spent in the saline-paired chamber on the test day. Mice that spent more than 50% of the time in a single chamber on habituation day were omitted from conditioning; this severe pre-existing bias occurred in 10-20% of mice depending on group. In addition, a post-hoc analysis was conducted to exclude all mice spending a significantly greater amount of time in any individual

chamber on test or habituation day than the other mice belonging to the same treatment group. A significantly greater amount of time in a given chamber was calculated as greater than 2 standard deviations above the mean time spent in that chamber by the treatment group as a whole. This criterion resulted in the exclusion of 3 mice, or roughly 5% of the subjects.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Brain sections were homogenized in TRIzol and mRNA isolated according to standard protocol. Samples were treated with DNase prior to reverse transcription using the Reverse Transcription System. The resulting cDNA samples were amplified by TaqMan quantitative PCR using previously designed primer/probe kits. In all cases, *GAPDH* was used as an internal control.

Statistical Analysis

Data were analyzed using one-way ANOVAs followed by post-hoc Bonferroni (vs. control) and Student-Neuman-Keuls (differences between treatment groups) comparisons where appropriate. For experiments involving within-subjects designs, repeated measures ANOVAs were used followed by multiple post-hoc Student-Neuman-Keuls paired comparisons. In addition, gene expression data expressed as percent control were analyzed by one-sample t-test (GraphPad Software, Inc., http://www.graphpad.com/quickcalcs/OneSampleT1.cfm). As percent control was calculated individually for each replicate of a given treatment prior to determining the treatment mean, the control population lacked variance. The one-sample t-test compares

a given mean with a specific variability against a hypothesized value of control (for example, 100%) and extrapolates variability in the control based on the variability among the treatment population, thus satisfying the statistical requirement for equal variance.

RESULTS

As we determined previously that ethanol treatment increases BDNF signaling in striatal neurons, resulting ultimately in increased production of *Pdyn* (Chapter 2), we sought to determine the role of BDNF and dynorphin in ethanol intake in mice.

Conditional deletion of BDNF increases ethanol intake

Previously we found that reduction of BDNF in heterozygous mutant mice ($BDNF^{+/-}$), which express approximately half the normal amount of BDNF protein (Kolbeck et al., 1999), resulted in increased measures on several ethanol-related behaviors (McGough et al., 2004). Specifically, $BDNF^{+/-}$ mice showed increased ethanol place preference, increased ethanol-induced locomotor responding – with both an increased acute response to ethanol and increased sensitization after chronic ethanol injections – and a prolonged increase in ethanol intake after a period of deprivation (McGough et al., 2004). However, due to numerous developmental functions of BDNF (Klein, 1994), BDNF homozygous mutant mice are not viable past 2 weeks of age (Ernfors et al., 1994; Ernfors et al., 1995) and there may be compensatory changes in $BDNF^{+/-}$ mice due to reduced developmental BDNF expression that could mask the full function of BDNF in regulating ethanol intake. For instance, we did not observe a difference in baseline ethanol intake between $BDNF^{+/-}$ and their WT littermates, a finding

contrary to our hypothesized function of BDNF as a regulator of ethanol intake. To overcome the limitations of conventional deletion, we utilized a conditional knockout model for spatial and temporal restriction of BDNF expression. Conditional deletion of genes can be achieved via the Cre/loxP system, which uses the P1 bacteriophage Cre enzyme to achieve recombination of genes flanked by loxP sites. Under this system, DNA between loxP sites will be excised from the genome upon Cre-driven recombination (Sauer & Henderson, 1988; Nagy, 2000), providing a means for removal of a gene of interest (or a portion thereof) in the presence of Cre recombinase. As mice have been previously developed expressing BDNF surrounded by loxP sites (floxed BDNF; for gene structure see Figure 3.1A) (Rios et al., 2001), we elected to use these animals as a means of overcoming the developmental lethality of traditional BDNF knockout. In order to achieve deletion of BDNF, Cre recombinase must be introduced into the floxed BDNF mice. Thus the regulation of both spatial and temporal deletion of BDNF can be precisely controlled via restriction of Cre expression. We chose to mate the floxed BDNF line with mice expressing Cre under the control of the calcium and calmodulin kinase IIα (CaMKIIα) promoter (CaMKcre) (Xu et al., 2000). CaMKIIα expression is restricted primarily to the forebrain, and the onset of expression is postnatal, with very low expression at postnatal day (PND) 4 which increases over the next two weeks to reach adult levels around PND21 (Burgin et al., 1990). Through the mating of floxed BDNF and CaMKcre mice, we achieved highly significant BDNF knockdown throughout the forebrain (Figure 3.1B), with *BDNF* levels approaching the lower limits of detection throughout the cortex and striatum and significant knockdown in the

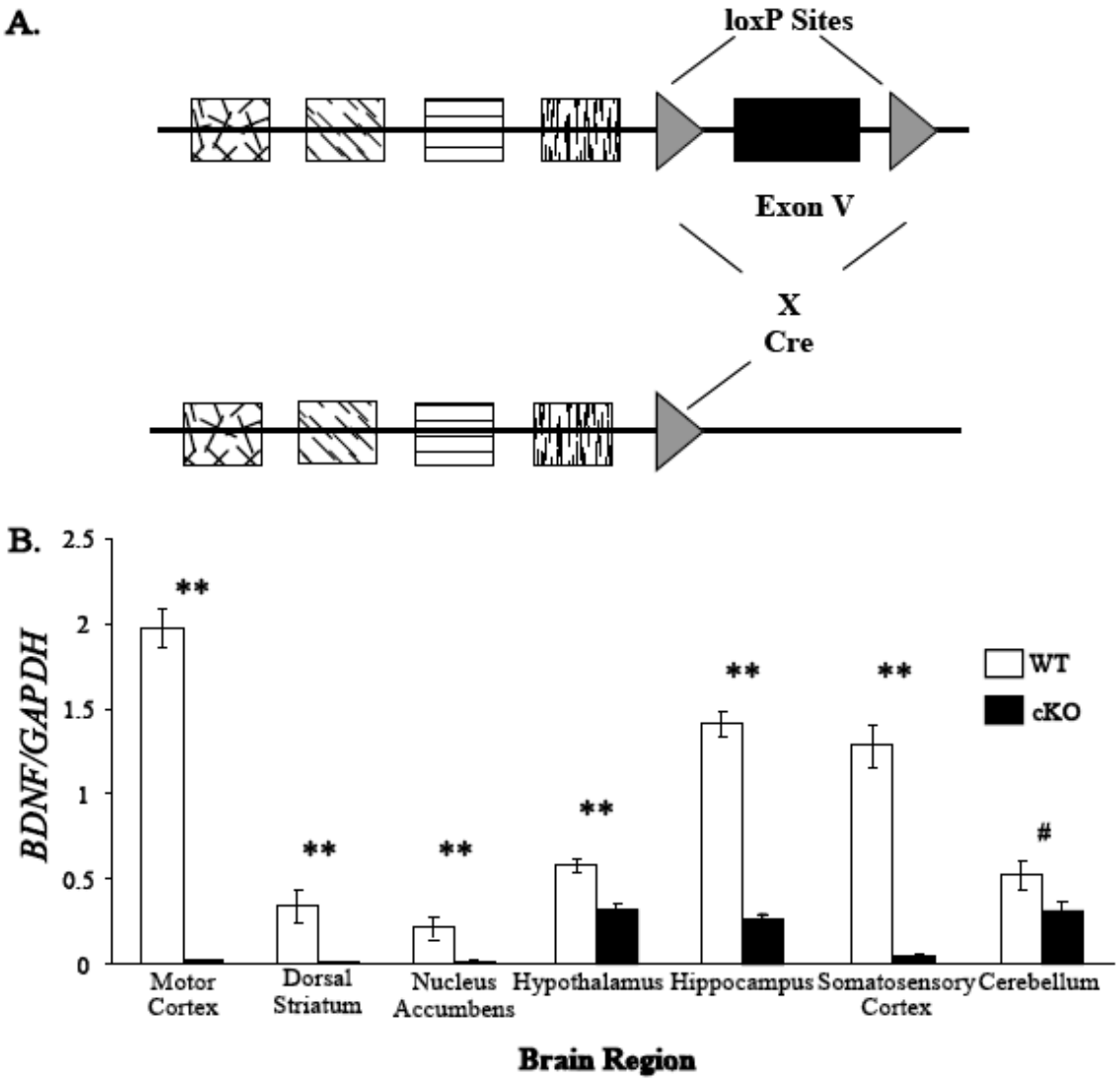


Figure 3.1. Conditional deletion of BDNF under the CaMKIIα promoter is primarily restricted to the forebrain.

(A) Diagram demonstrating the construction of floxed BDNF which provides a template for conditional deletion of the BDNF gene. The single coding exon of the BDNF gene is surrounded by loxP sites and is removed in the presence of Cre. Adapted from Rios et al. (2001). (B) Demonstration of BDNF knockdown in BDNF cKO mice in the presence of CaMKCre, with the highest knockdown occurring in frontal brain regions. Data are presented as mean *BDNF* expressed as a ratio to *GAPDH* +/- S.E.M. ** $p \leq 0.001$; # $p = 0.051$. n = 7 for WT, n = 6 for cKO.

hypothalamus and hippocampus as well ($F(1,90) = 171.806$, $p < 0.001$ for Genotype across all brain regions). The lack of developmental disruption from our knockout was evidenced in part by equivalent survival of both Cre-expressing (BDNF cKO) and Cre-nonexpressing (BDNF WT) mice, indicating that Cre expression did not leak through into

the embryonic period, as this would have resulted in decreased viability of the Cre-expressing mice due to developmental defects, similar to the issues observed with the conventional BDNF knockout mice (Ernfors et al., 1994; Ernfors et al., 1995). In addition, we observed no overt behavioral or physical differences in the mice studied. For instance, unlike previously published data (Rios et al., 2001), the BDNF cKO mice were not obese, but in fact displayed a trend towards decreased weight as compared to the WT mice at the time of testing (BDNF cKO: 25.5 g; BDNF WT: 30.5 g, $p = 0.065$). This may have been due to the fact that the BDNF cKO mice were, on average, 4 weeks younger than the BDNF WT mice (BDNF cKO: average age = 11 weeks at start of experiment, median = 10 weeks and range = 7-17 weeks; BDNF WT: average age = 15 weeks, median = 17 weeks and range = 10-20 weeks); due to breeding difficulties this was the closest age-matched group available.

In order to assess the effect of the conditional deletion of BDNF on ethanol intake, BDNF cKO and age-matched WT littermate mice were provided with two bottles of fluid on the home cage – one containing 10% ethanol and the other containing water. During a pilot study it was determined that both the BDNF cKO and BDNF WT mice were averse to the flavor of the ethanol solution, as all mice regardless of genotype were reluctant to consume unsweetened ethanol (average consumption of 1.6 g/kg/day). This taste aversion could be overcome by the addition of sucrose to sweeten the solution, a procedure which has previously been utilized to increase ethanol self-administration in rats (Samson, 1986). In the sweetened condition, ethanol intake was increased to 7.3 g/kg/day. Because sucrose can interfere with the pharmacological effects of ethanol by decreasing the amount of ethanol absorbed into the bloodstream (Roberts et al., 1999), we

elected to use the noncaloric sweetener saccharin (0.2%) as the sweetener for the ethanol solution. As shown in Figure 3.2A, BDNF cKO mice drank significantly more ethanol than their WT littermates (main effect of Genotype, $F(1,12) = 6.528$; $p < 0.05$). This was not due to genotype differences in taste preference, as we observed no differences in preference for saccharin or quinine solutions at any concentration tested (Figure 3.2B&C; all p's > 0.35). This indicates that the increase in sweetened ethanol intake in BDNF cKO mice is due to an increased drive to consume ethanol in the absence of forebrain BDNF.

Taken together, these results reinforce the hypothesis that ethanol-induced BDNF expression regulates ethanol intake, as postnatal, forebrain-enriched conditional deletion of BDNF significantly increases ethanol intake. Given our previous *in vitro* finding that ethanol increases striatal *Pdyn* expression via BDNF (Chapter 2), we next investigated the involvement of dynorphin in the regulation of ethanol intake by BDNF.

Tat-RACK1 *ex vivo* and BDNF infusion *in vivo* increase *Pdyn* expression in the dorsal striatum

As ethanol increases *BDNF* expression, and increased BDNF levels result in decreased ethanol consumption *in vivo* (McGough et al., 2004), we were interested in determining whether striatal dynorphin might be involved in the control of ethanol consumption since ethanol increases *Pdyn* expression via BDNF (Chapter 2). In order to demonstrate the involvement of *Pdyn* in the regulation of ethanol intake by BDNF, we first set out to demonstrate the capacity of BDNF to increase *Pdyn* expression following treatments known to reduce ethanol intake *in vivo*. First we determined whether an acute infusion of

BDNF would increase *Pdyn* in the striatum *in vivo*. Previously it has been shown that continuous BDNF infusion into the dorsolateral striatum over a two week period resulted in increased *Pdyn* expression (Croll et al., 1994); however, it was unknown whether this altered *Pdyn* expression was a compensatory response to chronic BDNF treatment or one which could be triggered by a single BDNF treatment. As others in the lab recently determined that a single infusion of BDNF into the dorsolateral striatum 3 hours prior to testing significantly reduced ethanol self-administration (Jeanblanc et al., personal communication), we hypothesized that *Pdyn* expression would be increased at the same time point. To test this, we infused BDNF (0.25 μg) into the dorsolateral striatum of rats and assessed changes in gene expression 3 hours later (Figure 3.3A). We found that BDNF infusion significantly increased *Pdyn* expression as compared to vehicle infusion (main effect of Treatment, $F(1,15) = 6.716$; $p < 0.05$), demonstrating that acute activation of BDNF signaling in the dorsal striatum increases *Pdyn* expression. Importantly, this increase in *Pdyn* expression in the dorsal striatum was temporally linked to decreased ethanol intake, as the same BDNF infusion significantly decreased ethanol self-administration in rats (Jerome Jeanblanc, personal communication). This suggested that BDNF could act to control ethanol consumption *in vivo* via increasing dynorphin expression.

While increased *Pdyn* following application of exogenous BDNF demonstrated the capacity of the striatum to respond to BDNF by increasing *Pdyn*, we next wanted to determine whether BDNF produced by the striatum could also increase *Pdyn*. To test this, we increased BDNF expression by treating dorsal striatal slices with Tat-RACK1, a Tat fusion protein which we have previously shown increases *BDNF* expression in the

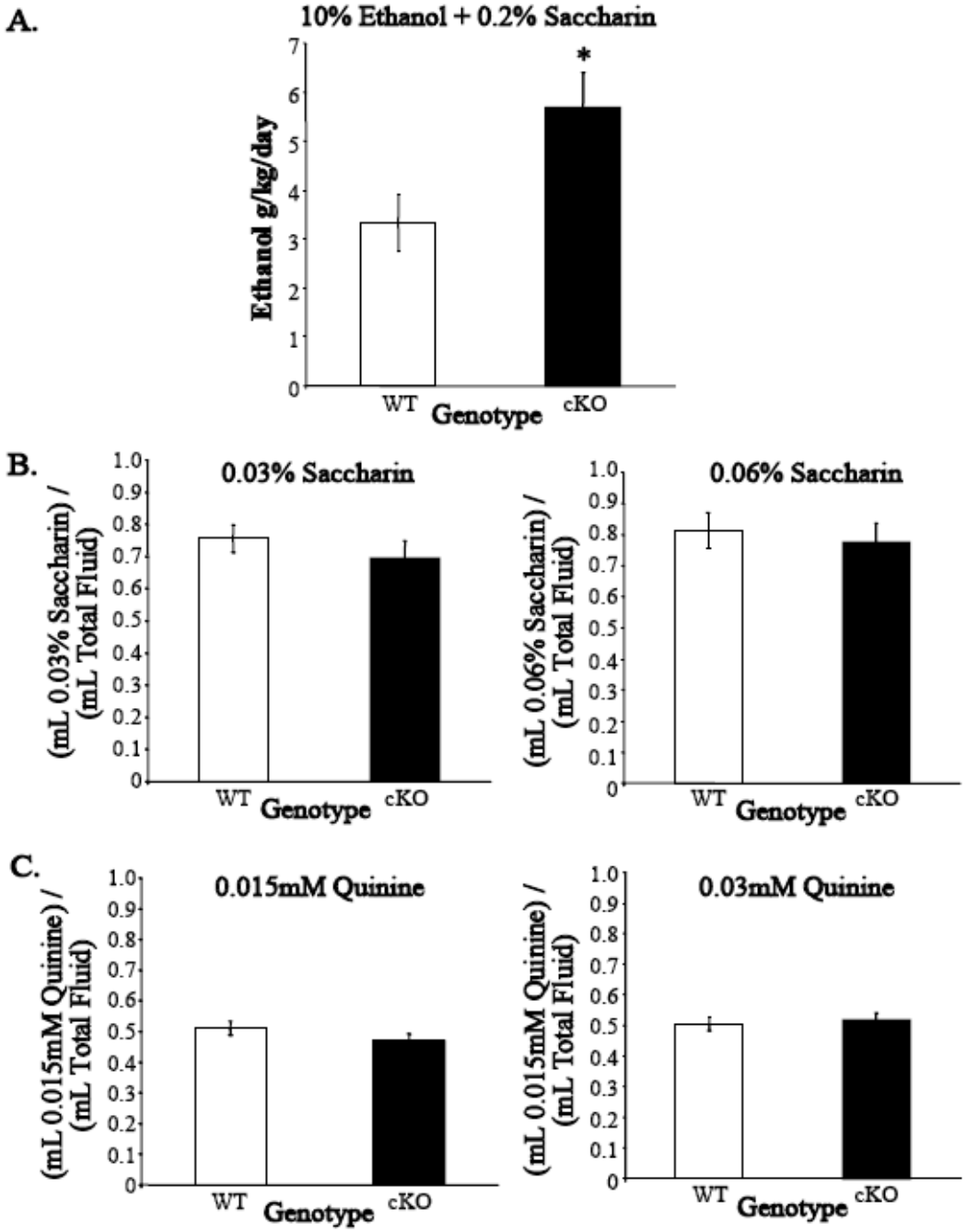


Figure 3.2. Conditional deletion of BDNF specifically reduces ethanol intake without affecting taste preference.

BDNF cKO and WT mice were provided with 24-hour access to 2 bottles of fluid on the home cage, one containing water and the other containing either sweetened ethanol (10% ethanol, 0.2% saccharin), saccharin (0.03% or 0.06%) or quinine (0.015 mM or 0.03 mM), as listed above. Data are averaged over a single week of access. (A) BDNF cKO mice drink more sweetened ethanol than WT littermates. Data are expressed as mean grams ethanol consumed per kg body weight over a 24-h period +/- S.E.M. * $p < 0.05$. (B, C) BDNF cKO mice show no difference in preference for sweet solutions (B) at either of 2 saccharin concentrations tested or for bitter solutions (C) at either of 2 quinine concentrations tested. Data are expressed as the mean ratio of mL flavored solution to mL total fluid consumed over a 24-h period +/- S.E.M. n = 7 for WT, n = 6 for cKO.

striatum when administered systemically (McGough et al., 2004). The receptor for activated C kinase 1 (RACK1) is a scaffolding protein which translocates to the nucleus upon ethanol exposure (Ron et al., 2000), where it triggers increased transcription of genes, including *BDNF* (He et al., 2002; Yaka et al., 2003). By expressing RACK1 as a Tat fusion protein, Tat-RACK1 can cross cell membranes and the blood-brain barrier (Schwarze et al., 1999), such that systemically administered Tat-RACK1 results in increased *BDNF* in the brain via a molecular mechanism similar to that triggered by ethanol (He et al., 2002; McGough et al., 2004). Importantly, we have shown previously that systemic administration of Tat-RACK1 significantly decreases ethanol intake (McGough et al., 2004; Jeanblanc et al., 2006). To test the efficacy of Tat-RACK1 to increase *Pdyn* expression in the dorsal striatum, striatal slices were incubated with 1 μM Tat-RACK1 for 4 hours; this time point was chosen based on previous experience (McGough et al., 2004; Jeanblanc et al., 2006) to allow time for the necessary transcription, translation and signaling cascades upstream of *Pdyn* mRNA production. We found that, as predicted, Tat-RACK1 treatment of dorsal striatal slices significantly increased *Pdyn* expression (Figure 3.3B; main effect of Treatment, $F(1,19) = 5.407$, $p < 0.05$). The fact that two treatments known to decrease ethanol intake also increased striatal *Pdyn* expression strongly implicated dynorphin as a required component in the regulation of ethanol consumption by BDNF signaling. Thus, we theorized that the regulation of ethanol intake by increased BDNF expression would require the expression of dynorphin and subsequent signaling via its receptor, the KOR (Chavkin et al., 1982).

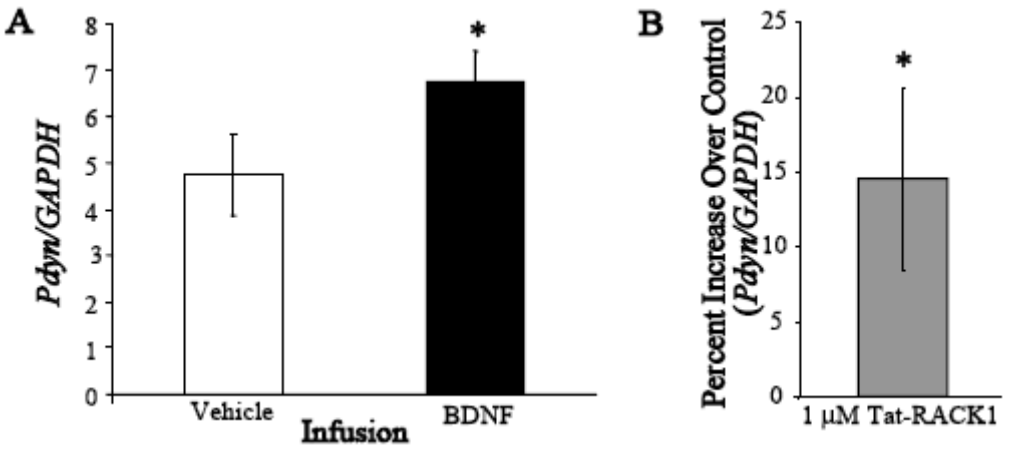


Figure 3.3. Infusion of BDNF into the dorsal striatum and treatment of dorsal striatal slices with Tat-RACK1 increase *Pdyn* expression.

(A) BDNF increases *Pdyn* expression in the dorsal striatum *in vivo*. Infusion of 0.25 μg BDNF into the dorsolateral striatum increases *Pdyn* mRNA expression 3 h post-infusion. Data are expressed as a ratio of *Pdyn* to *GAPDH* expression +/- S.E.M. * $p < 0.05$ as compared to vehicle-infused control. n = 8 per group. (B) Tat-RACK1 increases *Pdyn* expression in striatal slice. Dorsal striatal slices were treated with 1 μM Tat-RACK1 for 4 h. Data are expressed as mean percent increase over vehicle-treated control *Pdyn/GAPDH* expression levels +/- S.E.M. * $p < 0.05$. n = 10.

Reduced ethanol intake following Tat-RACK1 injection requires KOR signaling

Previous work from our lab has demonstrated the ability of Tat-RACK1 to act as a potent inhibitor of ethanol intake (McGough et al., 2004; Jeanblanc et al., 2006), and, as demonstrated above, Tat-RACK1 can increase striatal *Pdyn* expression (Figure 3.3B). Thus we hypothesized that regulation of ethanol intake by Tat-RACK1 would require intact dynorphin signaling via activation of the KOR. To test the requirement of dynorphin for the reduction of ethanol intake by BDNF following Tat-RACK1 treatment, C57 mice consuming ethanol under a standard two-bottle choice paradigm were injected with vehicle or Tat-RACK1 concurrently with vehicle or nor-BNI, an antagonist of the KOR. As most ethanol consumption occurs during the dark cycle, treatments were given 3 hours prior to dark cycle onset in order to allow sufficient time for expression of downstream genes, such as *Pdyn*, and bottles were returned to the cages ½ h after

injection. As previously reported (McGough et al., 2004; Jeanblanc et al., 2006), mice injected with Tat-RACK1 showed a highly significant 61% reduction in ethanol consumption. However, when mice were given nor-BNI along with Tat-RACK1, the ability of Tat-RACK1 to decrease ethanol intake was blunted, as they displayed only a 31% reduction in ethanol consumption (Figure 3.4A, $F(2,31) = 61.690$, $p < 0.001$ for Vehicle vs. Tat-RACK1 alone or Tat-RACK1 + nor-BNI, $p < 0.005$ for Tat-RACK1 alone vs. Tat-RACK1 + nor-BNI). Surprisingly, nor-BNI alone decreased ethanol intake (Figure 3.4B, $F(1,19) = 47.953$, $p < 0.001$ for Vehicle vs. nor-BNI), possibly due to residual antagonism of the mu opioid receptor at the onset of ethanol access (Endoh et al., 1992). This may account for the incomplete antagonism of Tat-RACK1's effects by nor-BNI. Because no treatment affected water intake, changes in total fluid consumption and ethanol preference match those described for ethanol consumption (data not shown). Taken together, these data show that inhibition of the KOR significantly reduced the ability of Tat-RACK1 to alter ethanol intake, indicating that dynorphin is responsible, at least in part, for the ability of BDNF to control ethanol consumption.

Activation of the KOR blocks acquisition of ethanol conditioned place preference

Ethanol consumption is a complex behavior which can be modulated by multiple factors (Samson & Czachowski, 2003). As such, an increase in ethanol intake following genetic or pharmacologic manipulation can indicate that the treatment either increases the rewarding properties of ethanol, thus increasing the motivation to consume, or that the manipulation decreases the unitary reward value of ethanol, thereby increasing the amount of ethanol required to achieve an equivalent amount of reinforcement. Thus

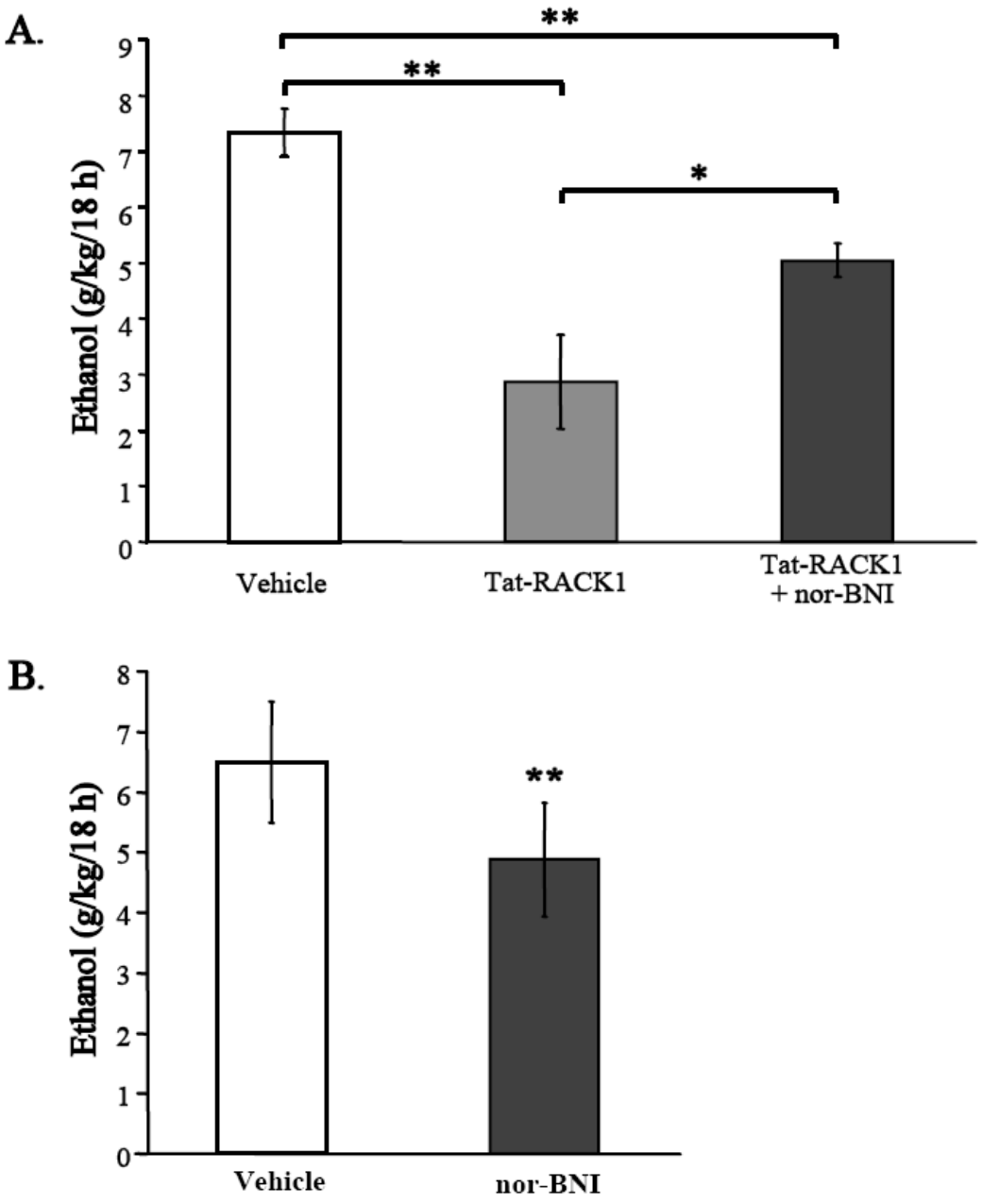


Figure 3.4. Blockade of KOR signaling inhibits the ability of Tat-RACK1 to decrease ethanol intake.

(A) Tat-RACK1 decreases 2-bottle choice ethanol intake via increased dynorphin signaling in C57BL/6J male mice. Mice were treated with vehicle (saline), 4 mg/kg Tat-RACK1 or 4 mg/kg Tat-RACK1 + 30 mg/kg nor-binaltorphimine (nor-BNI). Ethanol intake was recorded over the 18-h period following injection. Histograms depict mean grams ethanol consumed per kg body weight per 18 h +/- S.E.M. * $p <$ 0.005; ** $p < 0.001$. n = 8 per drug group (within-subjects design). (B) Nor-BNI alone decreases 2-bottle choice ethanol intake in C57BL/6J male mice. Mice were treated with vehicle (saline) or 30 mg/kg nor-BNI and ethanol intake was recorded over the 18-h period following injection. Histograms depict mean grams ethanol consumed per kg body weight per 18 h +/- S.E.M. ** $p < 0.001$. n = 10 (within-subjects design).

multiple paradigms exist to address various components of the reinforcing, stimulating and sedative properties of ethanol; these paradigms, in combination with ethanol drinking studies, can provide a more complete picture of the factors influencing changes in ethanol intake.

One such paradigm which can be used to measure the rewarding effects of drugs is conditioned place preference (CPP). CPP utilizes a Pavlovian conditioning mechanism, in which a drug such as ethanol, which serves as the unconditioned stimulus (US), is paired with a specific location during conditioning, which serves as the conditioned stimulus (CS). The subject is subsequently tested in a drug-free state to determine the amount of time spent in that location, as compared to a separate unpaired location. Thus preference for the CS is used as a measure of the reward produced by the US (reviewed in Tzschentke, 1998). We hypothesized that dynorphin might decrease ethanol intake by decreasing its rewarding properties and thus elected to test the ability of KOR agonists to inhibit acquisition of ethanol CPP. DBA mice received either the KOR agonist U50,488H (U50; 1 mg/kg, i.p.) or vehicle 10 minutes prior to ethanol (2 mg/kg, i.p.) on drug conditioning days, and two injections of saline spaced 10 minutes apart on alternate days. As shown in Figure 3.5A, mice administered ethanol developed a significant preference for the ethanol-paired chamber and acquisition of this preference was completely blocked by co-administration of U50 (main effect of Treatment, $F(3,49) = 5.357$, $p < 0.005$). As KOR agonists can trigger conditioned place aversion (Iwamoto, 1985), and the time spent in the CS was slightly although not significantly lower in the U50-treated group as compared to the saline-treated group, we confirmed that the dose we selected lacked such aversive properties in a separate group of mice. As predicted,

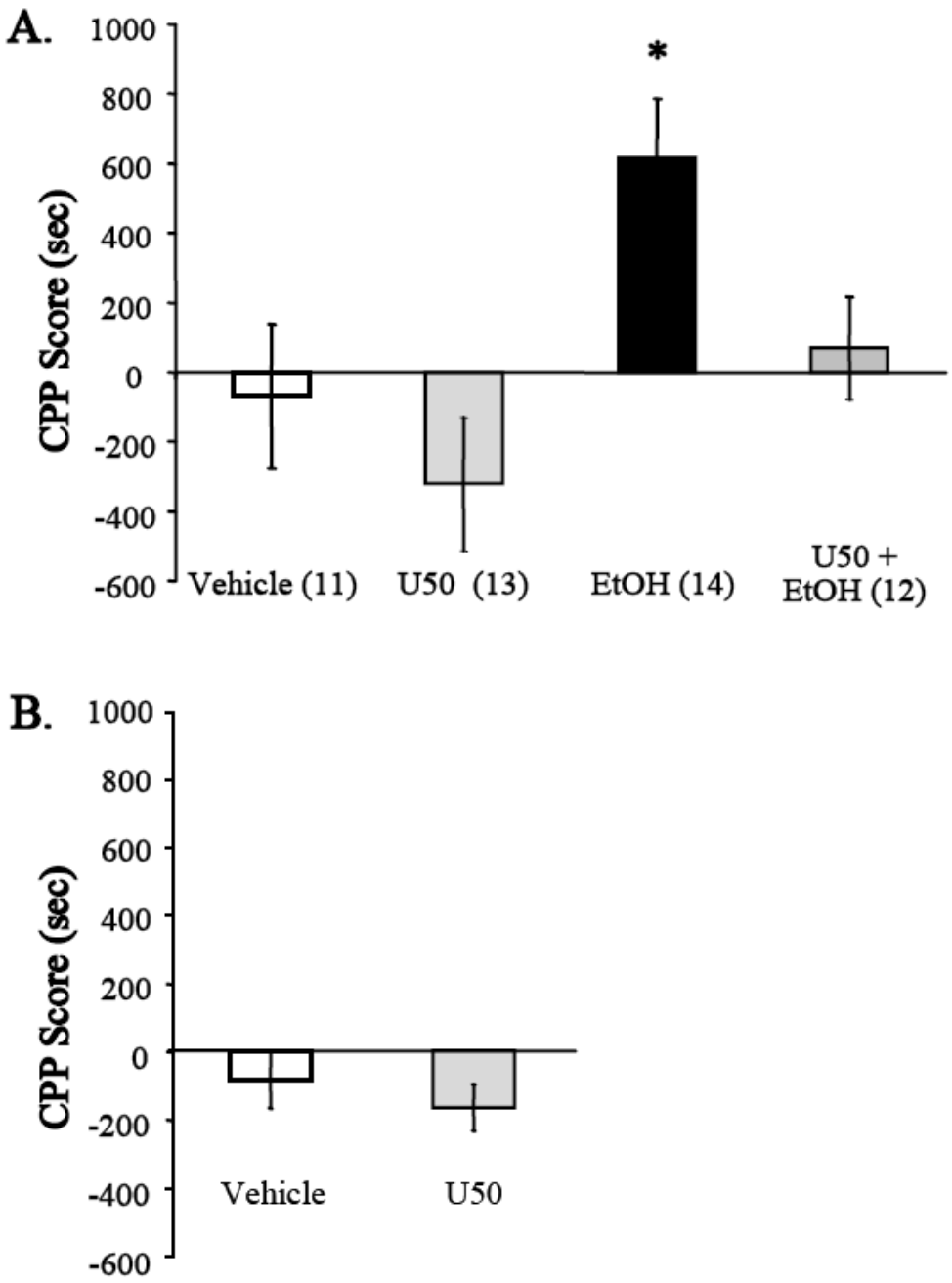


Figure 3.5. The KOR agonist U50,488H blocks acquisition of ethanol conditioned place preference at a nonaversive dose.

(A) Acquisition of ethanol conditioned place preference in DBA/2J mice is blocked by administration of U50,488H (1 mg/kg) 10 min prior to ethanol (2 g/kg) injection. Data are expressed as mean CPP Score, which is the difference between time spent in the drug-paired side and time spent in the saline-paired side on test day, +/- S.E.M. * $p < 0.05$ vs. all other groups. n = 11-14 per group, as indicated. (B) The same low dose of U50,488H (1 mg/kg) which blocks ethanol CPP does not cause conditioned place aversion. Data are expressed as CPP Score +/- S.E.M. n = 6 per group.

the low dose of U50 which effectively blocked ethanol place preference did not, on its own, produce a conditioned place aversion, as no significant differences were observed between the U50 and saline treatment groups (Figure 3.5B; $F(1,11) = 0.583$, $p = 0.463$). Taken together, these results indicate that activation of the KOR reduces the rewarding properties of ethanol and thus blocks acquisition of ethanol CPP.

DISCUSSION

The results presented here demonstrate a role for BDNF, via its downstream effector dynorphin, in the regulation of ethanol intake. We found that conditional deletion of BDNF increased ethanol intake, an effect which did not generalize to other rewarding substances and which was independent of differences in sensitivity to ethanol's bitter taste, as both saccharin and quinine preference were unaltered by genotype. In addition, we show that both BDNF and Tat-RACK1, which increases *BDNF* expression, increase striatal *Pdyn*, and that activation of the KOR by dynorphin is required, at least in part, for the reduction of ethanol intake following Tat-RACK1 injection. Finally, we demonstrate that activation of the KOR blocks the rewarding properties of ethanol and thus inhibits acquisition of ethanol CPP. Taken together, these results indicate that the homeostatic control of ethanol intake via BDNF includes increased production of dynorphin and activation of the KOR, and that this signaling pathway is involved in ethanol reward.

Increased ethanol intake following forebrain BDNF deletion

Previously we found that a reduction of BDNF levels by half resulted in increased behavioral responses to ethanol under multiple paradigms, including locomotor sensitization, ethanol intake after a period of deprivation and conditioned place preference (McGough et al., 2004). However, although increased ethanol intake had previously been demonstrated in female $BDNF^{+/-}$ mice (Hensler et al., 2003), we were unable to detect a similar difference in male mice. While developmental compensations in male mice may mask an effect which conversely may be exacerbated in females due to estrogen regulation of BDNF expression (Sohrabji et al., 1995; Jezierski & Sohrabji, 2000, 2003), it was essential to confirm that ethanol consumption is, in fact, regulated by BDNF regardless of sex. In addition, due to the lethality of germline deletion of BDNF (Ernfors et al., 1994), the effects of total deletion of BDNF on ethanol intake had not been investigated.

To circumvent developmental compensation issues, it was necessary to assess ethanol intake in a mouse model in which BDNF expression was unaltered during the gestational period. We found that postnatal conditional deletion of BDNF throughout the forebrain resulted in increased ethanol intake as compared to WT mice. Importantly, there was no difference in preference for either sweet or bitter solutions, indicating that the increased ethanol intake in BDNF cKO mice was not due to genotypic differences in taste responsiveness or to a generalized increase in intake of rewarding substances, as can be seen with various genetic and pharmacological manipulations (Gabriel & Cunningham, 2005; Kovacs et al., 2005; Blednov et al., 2006; Cai et al., 2006). In combination with the previous data from $BDNF^{+/-}$ mice demonstrating increased

sensitivity to ethanol in multiple paradigms (McGough et al., 2004), these results suggest that forebrain BDNF may regulate ethanol intake by decreasing the rate of adaptation to its stimulating properties. By counteracting neuronal changes responsible for the development of sensitization to ethanol, BDNF may thus function to slow down the progression to neuronal adaptations responsible for addiction, thereby reducing ethanol intake.

Regulation of ethanol intake by BDNF requires dynorphin & the KOR

While numerous studies have investigated the role of the kappa opioid system in modulating ethanol intake, to date results have also been somewhat divergent (Spanagel, 1996; Holter et al., 2000; Lindholm et al., 2001; Kovacs et al., 2005; Mitchell et al., 2005; Blednov et al., 2006; Doyon et al., 2006; Zapata & Shippenberg, 2006; Walker & Koob, 2007). Here we show that blockade of the kappa opioid receptor by the antagonist nor-BNI inhibits the ability of Tat-RACK1 – which increases striatal *BDNF* expression *in vivo* (McGough et al., 2004) and *Pdyn* mRNA expression in striatal slices – to decrease ethanol intake. These results suggest an important role for endogenous dynorphin as key regulator of the homeostatic control of ethanol intake by BDNF in the dorsal striatum. This is in agreement with studies showing decreased voluntary ethanol intake during a 2-hour access period following systemic administration of the KOR agonist U50 in rats (Lindholm et al., 2001), as well as data demonstrating lower expression of dynorphin and the KOR throughout the striatum of C57 mice, which drink more ethanol, as compared to low-drinking DBA mice (Jamensky & Gianoulakis, 1997). It should be noted that recent work indicates that a high taste aversion to ethanol in the DBA mouse strain contributes

significantly to the reduced voluntary ethanol consumption observed in DBA mice, and thus these results should be interpreted cautiously. Nonetheless, taken in combination with both the KOR agonist suppression of ethanol intake and additional evidence showing increased voluntary ethanol intake in rats following KOR antagonist treatment (Mitchell et al., 2005), these data support a role for the dynorphin/KOR system in the negative regulation of ethanol intake.

However, this contrasts with data from both *Pdyn* and KOR knockout mice (Kovacs et al., 2005; Blednov et al., 2006; Zapata & Shippenberg, 2006), in which ethanol intake is decreased in male and female (KOR, Kovacs et al., 2005) or only female (*Pdyn*, Blednov et al., 2006) knockout (KO) mice. It is possible that the effects observed in these knockout mice, which are contrary to our hypothesis, are due to compensatory changes in the remaining components of the opioid system, as the *Pdyn* KO mice appear to have upregulated KOR signaling (Chefer & Shippenberg, 2006) and the KOR KO mice may have similar developmental compensations. In addition, as the homeostatic control of ethanol intake by BDNF is specific to the dorsal striatum, global deletion of dynorphin or the KOR may mask the effects of a dorsal striatum-specific role for these proteins due to alterations in signaling in other brain regions.

Dynorphin and the KOR regulate conditioned place preference: Modulation of drug reward

Activation of KOR signaling has previously been shown to inhibit the acquisition of place preference to multiple drugs of abuse (Funada et al., 1993; Shippenberg et al., 1996; Pliakas et al., 2001; McLaughlin et al., 2006). In particular, morphine conditioned

place preference was blocked by the KOR agonist U50, but this effect could be bypassed by co-administration of a dopamine receptor agonist (Funada et al., 1993), indicating that activation of the KOR inhibited morphine place preference via blockade of morphine-induced dopamine release. This effect was not specific to morphine, as U50 also blocked conditioned preference to cocaine (Shippenberg et al., 1996). Here we demonstrate that U50 blocks ethanol CPP at a dose and time point which do not cause aversion in the absence of ethanol. Interestingly, the ability of KOR agonists to block acquisition of CPP is sensitive to the temporal distance between administration of the KOR agonist and the abused drug, as increased delay between U50 and cocaine injections reverses the effect of KOR agonism on place preference (McLaughlin et al., 2006). As the increased delay between the U50 and cocaine treatments mimics the effect of stress pre-exposure on cocaine CPP (McLaughlin et al., 2003; McLaughlin et al., 2006), these data suggest a mechanism for reconciling the different roles of endogenous dynorphin in response to acute and chronic ethanol treatment discussed above. Specifically, dynorphin may function acutely to block ethanol reward while a chronic dysregulation of the dynorphin/KOR system creates a dysphoric state which can be alleviated by ethanol intake, thus triggering increased ethanol consumption in withdrawal.

Possible mechanism of action for dynorphin-mediated reduction in ethanol consumption

According to our model of homeostatic control of ethanol intake by BDNF and its downstream signaling partner dynorphin (Figure 3.6), we hypothesize that dynorphin modulates ethanol intake by providing post-ingestive feedback to regulate subsequent

ethanol consumption bouts. We theorize that BDNF homeostasis could break down over time, concomitant with the development of an addictive phenotype, such that in a state of ethanol dependence each drinking bout will cease to result in increased BDNF and dynorphin production. This loss of dynorphin feedback could result in oversensitivity of the KOR system, as suggested by Walker and Koob, who demonstrated that the KOR antagonist nor-BNI decreased ethanol intake in ethanol-dependent rats but not in ethanol-

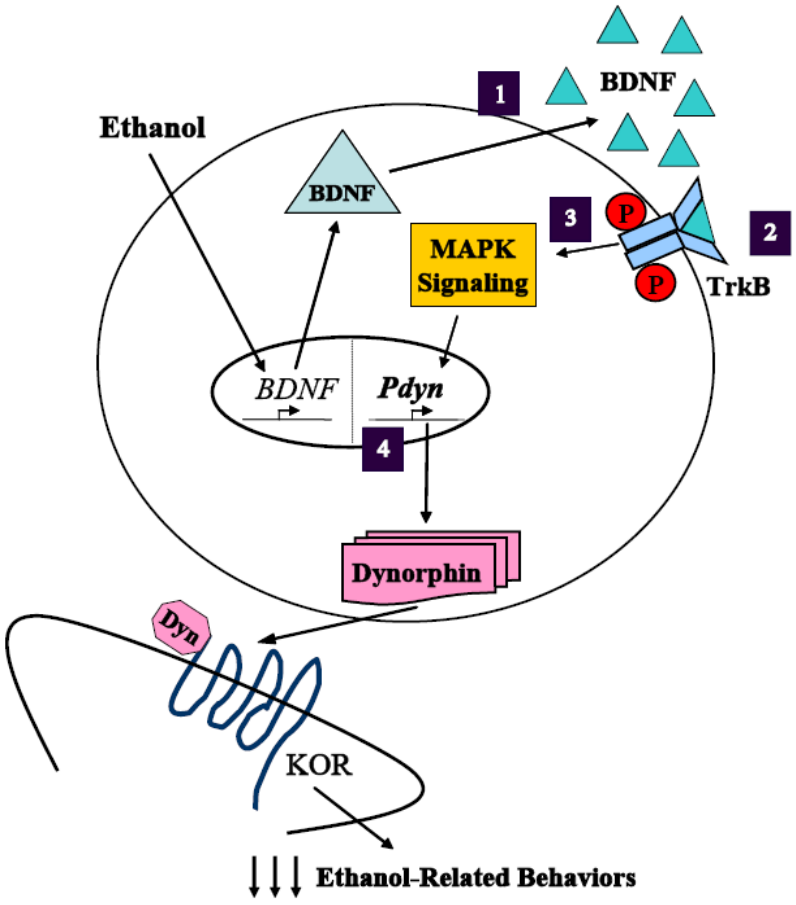


Figure 3.6. BDNF homeostatic regulation of ethanol intake via dynorphin.
Together with the data presented in Chapter 2 and herein, we propose the following model: (1) Ethanol increases BDNF protein production and release. (2) BDNF binds to the TrkB receptor, resulting in receptor dimerization and autophosphorylation. (3) The phosphorylated TrkB receptor activates downstream signaling via the MAPK pathway, resulting in (4) increased production of *Pdyn*. Following translation, dynorphin is released and acts via (5) activation of the KOR, which in turn decreases ethanol -related behaviors, including ethanol intake.

experienced, nondependent rats (Walker & Koob, 2007). The inability of nor-BNI to increase ethanol intake in nondependent animals, as observed by ourselves and others (Williams & Woods, 1998; Doyon et al., 2006), even after a period of alcohol deprivation (Holter et al., 2000), supports a role for dynorphin in between-bout rather than within-bout regulation of ethanol intake. Research from Mitchell and colleagues (Mitchell et al., 2005) showing an increase in ethanol intake several days after nor-BNI administration may add additional support for this hypothesis, as the blockade of dynorphin feedback may require multiple ethanol intake sessions in nondependent animals to impact consumption. Interestingly, while we saw an initial decrease in ethanol intake immediately after nor-BNI treatment, we also observed a dramatic but short-lived increase in consumption only on the 4^{th} day after nor-BNI injection (60.5% mean increase in ethanol consumption, main effect of Treatment, $F(1,19) = 16.895$, $p < 0.005$ for 1 day before vs. 4 days after nor-BNI injection). As changes in consumption this distant from drug treatment are often missed and difficult to rationalize, this may explain the discrepancy between the lack of effect of nor-BNI generally seen in nondependent animals and the report from Mitchell et al. (2005). Nonetheless, taken together these data suggest a dynamic regulation of ethanol consumption by dynorphin.

How dynorphin functions to decrease ethanol intake is as yet unknown; however, the reduction of neurotransmitter release following activation of KOR signaling presents one possible mechanism (Spanagel et al., 1992; You et al., 1999). In the NAc, the KOR localizes to presynaptic terminals of both glutamatergic and dopaminergic inputs (Svingos et al., 1999; Svingos et al., 2001), and activation of the KOR in the NAc has previously been shown to decrease dopamine (Spanagel et al., 1990) as well as glutamate

and GABA (Hjelmstad & Fields, 2003) release. Interestingly, repeated administration of KOR agonists blocks cocaine-induced elevation of accumbal dopamine release (Thompson et al., 2000), indicating that regulation of dopamine release may underlie the ability of the dynorphin/KOR system to regulate drug intake. Like all drugs of abuse, acute ethanol induces dopamine release in the NAc (Di Chiara & Imperato, 1985), and this dopamine release is increased by nor-BNI administration despite the fact that this same nor-BNI treatment does not alter ethanol intake (Doyon et al., 2006). As expected, KOR knockout mice show elevated ethanol-evoked dopamine release in the NAc (Zapata et al., 2006). Because acute ethanol treatment increases dynorphin release in the NAc (Marinelli et al., 2006), and dynorphin, via activation of KOR signaling, decreases neurotransmitter release in the NAc and striatum (Spanagel et al., 1990; Zhang et al., 2004), one function of the BDNF/dynorphin homeostatic feedback could be to protect against dysregulation of dopamine release following ethanol exposure, as ethanol-dependent rats show increased dopamine release while intoxicated but reduced basal dopamine tone in withdrawal (Hunt et al., 1979). While acute ethanol induces dopamine release in the dorsal striatum as it does in the NAc (Signs et al., 1987; Di Chiara & Imperato, 1988), it is as yet unknown whether dopamine release in the dorsal striatum is altered in ethanol-dependent animals. Of particular interest is the possible progression to alteration of basal and/or stimulus-evoked dopamine release following extensive ethanol experience, which would be in line with both our hypothesis and the dysregulation of dynorphin signaling observed by Walker and Koob in dependent rats (Walker & Koob, 2007).

Altered neurotransmitter release and concomitant mitigation of neuronal adaptations to ethanol is an attractive hypothesis, and one in line with a role of BDNF in the dorsal striatum regulating ethanol intake (McGough et al., 2004; Jeanblanc et al., 2006). In particular, since the dorsal striatum is involved in habit formation (Yin & Knowlton, 2006) and addiction is considered a maladaptive habit (Gerdeman et al., 2003; Everitt & Robbins, 2005), inhibition of ethanol-induced neuronal adaptations in this brain region by BDNF could hinder the development of the addictive habit. However, as alterations in BDNF expression were not limited to the dorsal striatum, an alternative explanation for the ability of BDNF to regulate ethanol intake involves the function of amygdalar BDNF in anxiety and ethanol intake.

A key contributor to the complex regulation of ethanol intake is the bipartite physiologic response to ethanol – namely that it can produce both rewarding effects, subjectively experienced as a "high", as well as anxiolytic effects, observed in humans as the tendency to drink as a means of alleviating stress or anxiety in social situations (Pohorecky, 1981). The withdrawal state following chronic ethanol exposure is characterized by enhanced anxiety in animal models (Rasmussen et al., 2001) and in humans (Hall & Zador, 1997). Withdrawal from ethanol is accompanied by increased production of anxiogenic factors such as corticotrophin releasing factor (Lack et al., 2005) and decreased expression of anxiolytic factors such as neuropeptide Y (Zhang & Pandey, 2003) in the central nucleus of the amygdala. Importantly, reversal of these withdrawal-induced molecular adaptations or lesion of the central nucleus of the amygdala results in decreased ethanol intake (Rassnick et al., 1993; Moller et al., 1997; Zhang & Pandey, 2003). Recently, it has been suggested that BDNF in the amygdala

functions to control both anxiety levels and ethanol intake, as knockdown of amygdalar BDNF by infusion of antisense oligonucleotides increased both anxiety and ethanol intake (Pandey et al., 2006). Similar to striatal BDNF, decreasing amygdalar BDNF concomitantly downregulated MAPK signaling, indicating a role for BDNF signaling and production of downstream effectors in the control of anxiety-modulated ethanol intake by the central nucleus of the amygdala. As recent data have shown dynorphin expression in a subset of CRF-positive central amygdala neurons (Marchant et al., 2007), dynorphin may also play a role in mediation of ethanol withdrawal-related anxiety, and thus may be involved in the regulation of ethanol intake by amygdalar BDNF.

Taken together, the data presented here suggest an extension of the model describing the homeostatic control of ethanol intake by BDNF (Figure 3.6). Ethanol intake elevates BDNF production in the dorsal striatum, and this BDNF acts to decrease ethanol intake, as removal of forebrain BDNF results in elevated ethanol consumption. Ethanol-induced BDNF production next triggers increased production of dynorphin, which activates signaling via the KOR. This activation of the KOR may provide feedback regarding the previous ethanol consumption bout, possibly by overriding the learned associations of consumption and the rewarding properties of ethanol via decreasing presynaptic release of dopamine and/or glutamate in the dorsal striatum during a post-consumption consolidation period and thus counteract the development of addiction. As both BDNF (Uhl et al., 2001; Matsushita et al., 2004) and the KOR (Xuei et al., 2006; Williams et al., 2007) have been associated with development of drug and alcohol addiction in humans, further research to address the function of dynorphin in providing this type of feedback, as well as determination of a mechanism by which this

homeostatic system fails to control ethanol intake over time, show promise not only for furthering our understanding of the neuronal changes underlying the development of addiction but also for finding a possible treatment for alcoholism – and possibly addiction to other drugs of abuse.

CHAPTER 4

Breakdown of BDNF homeostasis following extended limited access to ethanol

ABSTRACT

Alcoholism is a chronically relapsing condition (O'Brien, 1994), indicative of long-term neuronal adaptations maintaining the addiction even after prolonged periods of abstinence. In order to more appropriately model patterns of consumption which lead to the development of ethanol dependence, we have implemented a model of home cage limited access to ethanol which results in high ethanol intake in a short period of time, thus causing daily intoxication followed by withdrawal. Here we demonstrate that a single acute bout of ethanol intake increases *brain-derived neurotrophic factor* (*BDNF*) mRNA expression in the dorsal striatum. Importantly, this ethanol-induced increase in dorsal striatal *BDNF* is not detected after 6 weeks of daily ethanol consumption under the limited access paradigm. In addition, 6 weeks' limited access results in a decrease in cortical *BDNF*. Changes in *BDNF* expression after extended limited access relate directly to the immediately preceding consumption bout, as no difference is seen following 24 hours' withdrawal as compared with ethanol-naïve controls. Significantly, these ethanol-induced changes in *BDNF* levels following consumption are not ameliorated by 2 weeks' abstinence, since this abstinence period does not render the dorsal striatum *BDNF* expression ethanol-responsive, nor does it reverse the ethanol-induced reduction in cortical *BDNF*. Taken together, these data indicate that the BDNF homeostatic pathway is activated following a single bout of ethanol drinking, breaks down by the end of 6 weeks of limited access and does not recover its protective function after a 2-week deprivation period. These results have significant implications for the treatment of alcoholism, as the persistence of the altered BDNF signaling may contribute to the inflexibility of the addictive behavior.

INTRODUCTION

Alcohol abuse and addiction are complex traits, as evidenced by the fact that human alcoholics are often subdivided into multiple subtypes based on variation in drinking triggers and consumption patterns (Allen et al., 1994; Del Boca, 1994; Epstein et al., 1994; Vrublevsky, 1994). Additionally, alcoholism is a chronically relapsing condition (O'Brien, 1994; Oroszi & Goldman, 2004), and factors prompting relapse are equally varied as those central to alcohol drinking prior to recovery (Meyer, 1994). Due to the complexity of the human disease, one of the consistent challenges for alcohol research has been the design of animal models of alcohol intake that best replicate the human condition (Altshuler, 1981). In particular, mice generally do not self-administer intoxicating quantities of ethanol (Dole & Gentry, 1984) and thus are unlikely to develop physiological dependence on ethanol (Dole et al., 1985). In order to address mechanisms which may regulate dependence – and thus present useful targets for treating human alcoholics – researchers have developed a variety of paradigms to create physiological dependence (Samson & Falk, 1975; Falk & Tang, 1988; Grahame & Grose, 2003; Becker & Lopez, 2004; Finn et al., 2005; Lopez & Becker, 2005; Rhodes et al., 2005; Szumlinski et al., 2007). Traditionally, ethanol dependence has been initiated through forced ethanol exposure, either via inclusion in a liquid diet which provides the only available food source (Lieber et al., 1989) or by exposure to ethanol vapor for an extended period of time (Crabbe et al., 1983; Le Bourhis & Aufrere, 1983). Although these models consistently produce intoxication, making them reliable paradigms for studying the physiological dependence involved in alcohol addiction, they lack some construct validity in that the progression to addiction in humans is concomitant with the learned

associations governing the acquisition and consumption of drugs of abuse. Thus these paradigms do not address the full spectrum of psychological dependencies which persist for a very long interval and which are responsible for the high rates of relapse in long-time abstinent former drug users (Weiss & Porrino, 2002). Several paradigms have been designed to elevate consumption which more closely mirror the human condition, including schedule-induced polydipsia, in which ethanol is the available liquid during intermittent food pellet presentation (Samson & Falk, 1975; Tang & Falk, 1983), and the use of sweetened ethanol solutions in the initial phases of ethanol exposure, which has been shown to increase ethanol self-administration even after removal of the sweetener (Samson, 1986). However, while these can yield pharmacologically relevant blood ethanol levels (Falk et al., 1972; Grant & Samson, 1985), the pairing of ethanol with food or sugar may confound the interpretation of the results as the second (natural) reinforcer, rather than the ethanol itself, may drive the self-administration behavior (Altshuler, 1981).

Due to its face validity and ease of use, a majority of ethanol intake studies in the mouse have utilized the standard two-bottle choice paradigm, in which two bottles are available on the home cage for 24 hours per day, with one containing water and the other containing ethanol, usually at a concentration within the range of 6% to 20% (v/v). While this model allows free choice in the decision to drink – thus reproducing one psychological element lacking in the forced ethanol paradigms – it rarely results in consumption of intoxicating levels of ethanol (Dole et al., 1985). Therefore, in the quest for a more realistic animal model of ethanol dependence and progression to addiction, recent efforts have turned to limited access models which can result in drinking to

intoxication (Finn et al., 2005; Rhodes et al., 2005; Szumlinski et al., 2007). The central tenet of these paradigms is the provision of ethanol for a brief period daily or every few days, resulting in repeated cycles of intoxication and withdrawal. Several variants of this paradigm have been initiated, with variation in both the length of access (½ to 4 hours) and the frequency of access (daily or every few days). Importantly, all variants have been shown to induce intoxication due to significant ethanol intake in a limited period of time, resulting in blood alcohol levels higher than 100 mg% in most C57BL/6 (C57) mice (Rhodes et al., 2005; Sharpe et al., 2005; Szumlinski et al., 2007), as well as locomotor incoordination (Rhodes et al., 2007), phenotypes which are rarely observed in unlimited access two-bottle choice studies. However, while these paradigms produce intoxicating ethanol intake, for the duration of the limited ethanol exposure water is unavailable.

To address the function of BDNF homeostasis in both the acute response to ethanol and the progression to ethanol dependence, we established a modified version of the aforementioned limited access paradigms in which the standard two-bottle choice task was provided for a limited epoch daily. Using this behavioral paradigm, we set out to test the hypothesis that the progression to addiction would be concomitant with a breakdown in homeostatic BDNF signaling in the dorsal striatum.

METHODS

Reagents

TRIzol and the Reverse Transcription System were purchased from Promega Corporation (Madison, WI). All real-time PCR reagents, including TaqMan Gene

Expression Assays, were obtained from Applied Biosystems, Inc. (Foster City, CA). DNase was obtained from Sigma Aldrich (St. Louis, MO).

Animals

C57BL/6J mice were obtained at 6 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Animals were housed under a 12-hour reverse light/dark cycle, with lights on at 10:00 p.m. and lights off at 10:00 a.m., and were provided with continuous *ad libitum* access to food and water. Due to the reversal of normal light/dark cycle, animals were given 2 weeks to adjust to the housing conditions prior to performance of any study procedures. All animal procedures were approved by the Gallo Center Institutional Animal Care and Use Committee and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council (1996).

Two-bottle choice ethanol intake

Mice were singly housed in double grommet cages at least two weeks prior to the initiation of ethanol access. Fluid solutions were provided in cylindrical glass bottles equipped with standard stoppers. Mice were given two bottles – one containing water and the other containing a solution of 10% ethanol (v/v) – for 4 hours each day, with access beginning 2 hours into the dark cycle. For the water-only access group, two bottles of water were placed on the home cage during the ethanol access period. A single water bottle was available the other 20 hours each day. Bottles were weighed immediately before and after each 4-hour access period, and the placement order of the bottles on the home cage was switched daily to ensure that consumption levels were not

due to a side bias. Body weights were monitored every 6 days; the final weight was taken 36 hours prior to sacrifice to ensure minimal disruption of consumption due to handling stress.

Gene Expression after Ethanol Consumption

To determine changes in gene expression after ethanol intake, mice were sacrificed immediately after the final ethanol access session and brain regions were microdissected out and flash frozen to minimize RNA degradation. Diagrams depicting the brain regions encompassed by each of the sections can be found in Appendix B. In order to maintain consistency in length of ethanol access, onset of access on the final day was staggered over a 1-hour period. Order of sacrifice was counterbalanced by condition such that no systematic differences existed in circadian timing of sacrifice for water and ethanol groups. Brain sections were processed for quantification of RNA expression levels as described below.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Brain sections were homogenized in TRIzol and mRNA isolated according to standard protocol. Samples were treated with DNase prior to reverse transcription using the Reverse Transcription System. The resulting cDNA samples were amplified by TaqMan quantitative PCR using previously designed primer/probe kits. In all cases, *GAPDH* was used as an internal control.

Statistical Analysis

Gene expression data were analyzed using one-way ANOVAs followed by post-hoc Student-Neuman-Keuls tests where appropriate. In addition, simple pairwise linear regressions were used to analyze correlations between gene expression and ethanol intake. Ethanol intake was analyzed using Student's t-tests, except when expressed as percent change from control, in which case data were analyzed using the one-sample t-test (GraphPad Software, Inc., http://www.graphpad.com/quickcalcs/OneSampleT1.cfm). Given previous data from the lab showing increased *BDNF* expression in the dorsal striatum after both acute ethanol injection and ethanol consumption and no decreases in BDNF expression in any region analyzed (McGough et al., 2004), *BDNF* expression following a single ethanol intake session was analyzed using a one-tailed t-test.

RESULTS

Previously we demonstrated increased *BDNF* expression in the dorsal striatum following ethanol intake under a standard 24-hour two-bottle choice access procedure (McGough et al., 2004). In addition, we found that BDNF negatively regulates ethanol intake (McGough et al., 2004; Jeanblanc et al., 2006) and thus proposed that BDNF functions as a homeostatic regulator of ethanol intake. As such we hypothesized that the progression to addiction would be accompanied by a breakdown of BDNF homeostasis. Unfortunately, ethanol intake under the standard two-bottle choice paradigm fails to result in ethanol dependence (Dole et al., 1985), and thus this paradigm provided an inadequate model for assessing the function of BDNF in the progression to addiction. Therefore we established a limited access paradigm in which mice were provided with

daily limited access to two-bottle choice voluntary ethanol consumption, with ethanol available during hours 2-6 of the dark cycle. This time window was chosen for two reasons: first, as mice are nocturnal, the likelihood that they will drink is higher at night than during the day; second, in order to dissociate ethanol intake from feeding, we delayed the onset of access for 2 hours after lights out. As demonstrated in Figure 4.1A, even a single session of ethanol access under this paradigm results in significant ethanol consumption by C57 mice. Importantly, mice given access to ethanol show highly significant preference for the ethanol bottle over the water bottle, while those with access to two bottles of water show near chance selection of either water bottle (Figure 4.1B). As this level of ethanol consumption is comparable to the values obtained with limited access to forced ethanol resulting in blood ethanol levels of roughly 100 mg/dl (Rhodes et al., 2005), this demonstrated that the limited voluntary ethanol access paradigm was capable of producing ethanol consumption to intoxication. Importantly, C57 mice provided with limited access to 2-bottle choice ethanol consume pharmacologically relevant quantities of ethanol upon their first encounter with ethanol.

A single session of ethanol intake increases *BDNF* expression

Previously we have shown that both ethanol consumption under a standard 2-bottle choice paradigm and acute ethanol injection increase *BDNF* expression in the dorsal striatum (McGough et al., 2004). Therefore we hypothesized that a single session of limited access voluntary ethanol consumption would similarly increase *BDNF* expression in the dorsal striatum. As shown in Figure 4.2A (left panel), we observed an increase in *BDNF* expression in the dorsal striatum immediately following the single

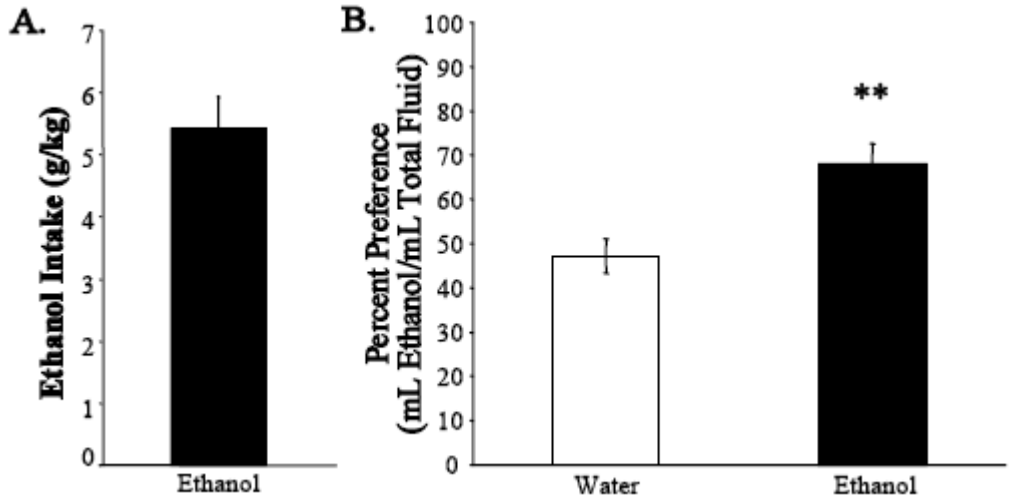


Figure 4.1. Significant ethanol intake and preference demonstrated in a single 4-h free-choice limited access session.

C57BL6/J mice were provided with access to a single, 4-hour two-bottle choice access period beginning 2 h into the dark cycle. (A) C57BL/6J mice show a high level of ethanol consumption in the initial 4-h access period. Data are presented as mean grams ethanol intake per kg body weight per day +/- S.E.M. (B) Mice given access to ethanol show significantly higher preference for the ethanol bottle than seen in the water group for a water bottle in the same position. Data are presented as mean percent preference, calculated as mL ethanol consumption per mL total fluid consumption +/- SEM. ** $p < 0.005$. n = 15 for Water, n = 18 for Ethanol.

4-hour access session ($p < 0.05$ for Water vs. Ethanol, one-tailed t-test), suggesting that a single acute ethanol consumption bout, like both acute ethanol injection and ethanol consumption under the standard 24-hour two-bottle choice access paradigm, stimulates *BDNF* production in the dorsal striatum. Importantly, as seen in previous experiments (McGough et al., 2004), this effect was specific to the dorsal striatum, as there was no difference in NAc *BDNF* expression between ethanol and water access groups (4.2A, right panel; $F(1,32) = 0.117$, $p = 0.734$), although *BDNF* levels appear slightly elevated in all NAc samples as compared to dorsal striatum (Figure 4.2A, right vs. left), perhaps due to a novelty effect of initial exposure to two-bottle access independent of ethanol availability. We also observed no change in cortical *BDNF* expression, as demonstrated by Motor Cortex samples (no effect of Treatment, $F(1,32) = 1.281$, $p = 0.266$),

confirming that increased *BDNF* expression following acute ethanol intake was restricted to the dorsal striatum.

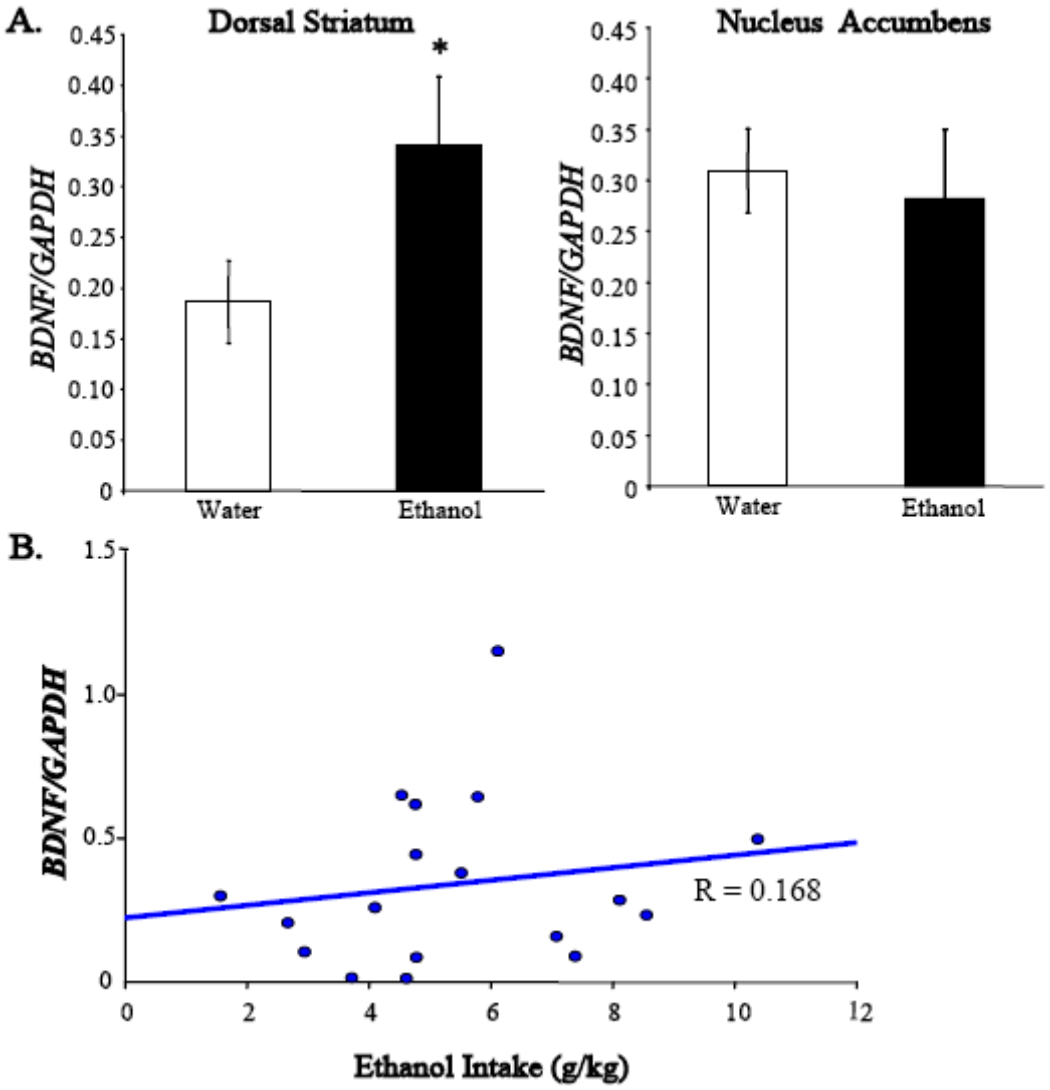


Figure 4.2. Ethanol intake during a single 4-h free-choice ethanol access session increases *BDNF* expression in the dorsal striatum.

C57BL6/J mice were provided with access to a single two-bottle choice access period beginning 2 h into the dark cycle. (A) *BDNF* is increased in the dorsal striatum immediately following 4 h ethanol intake. Data are expressed as mean *BDNF* expression relative to *GAPDH* +/- S.E.M. * $p < 0.05$. n = 15 for Water, n = 18 for Ethanol. (B) *BDNF* expression is not correlated with quantity of ethanol consumed. Graph depicts the pairwise comparisons between *BDNF* expressed as a ratio to *GAPDH* and ethanol intake expressed as grams ethanol consumed per kg body weight. Blue line represents the simple linear regression that best models the data. $p = 0.505$.

In addition, we hypothesized that homeostatic upregulation of BDNF functions to provide a feedback signal based on the level of consumption, and as such dorsal striatal BDNF should be positively correlated with ethanol intake. Unexpectedly, we found no correlation between the quantity of ethanol consumed in a single session and the expression level of *BDNF* in the dorsal striatum (Figure 4.2B, $R = 0.168$, $p = 0.505$).

Extensive intake under limited access results in breakdown of BDNF homeostasis

Given the proposed function of BDNF as a homeostatic regulator of ethanol intake, we wanted to determine whether repeated exposure to ethanol intoxication and withdrawal over an extended period of time would alter the dorsal striatal BDNF response to ethanol intake. To test this, mice were given daily 4-hour limited access sessions for 6 weeks and *BDNF* expression assessed immediately after the final limited access session. As shown in Figure 4.3A, 6 weeks' limited access ethanol intake results in higher preference for the ethanol bottle than a single day of access; in addition, there was also a slight increase in total ethanol intake over this time period. Importantly, ethanol consumption caused no change in dorsal striatal *BDNF* expression following extended exposure to the limited access paradigm (Figure 4.3B; no effect of Treatment, $F(1,14) = 0.00124$, $p = 0.972$). As expected, no changes were observed in the nucleus accumbens (NAc) (no effect of Treatment, $F(1,13) = 0.981$, $p = 0.341$) or hippocampus ($F(1,14) = 0.559$, $p = 0.468$) as well. Unexpectedly, we saw a highly significant decrease in *BDNF* expression throughout the cerebral cortex (Figure 4.3B; main effects of Treatment as follows: Motor Cortex $F(1,14) = 43.430$, $p < 0.001$; Somatosensory Cortex $F(1,14) = 25.486$, $p < 0.001$), a region in which *BDNF* expression is unaffected by acute

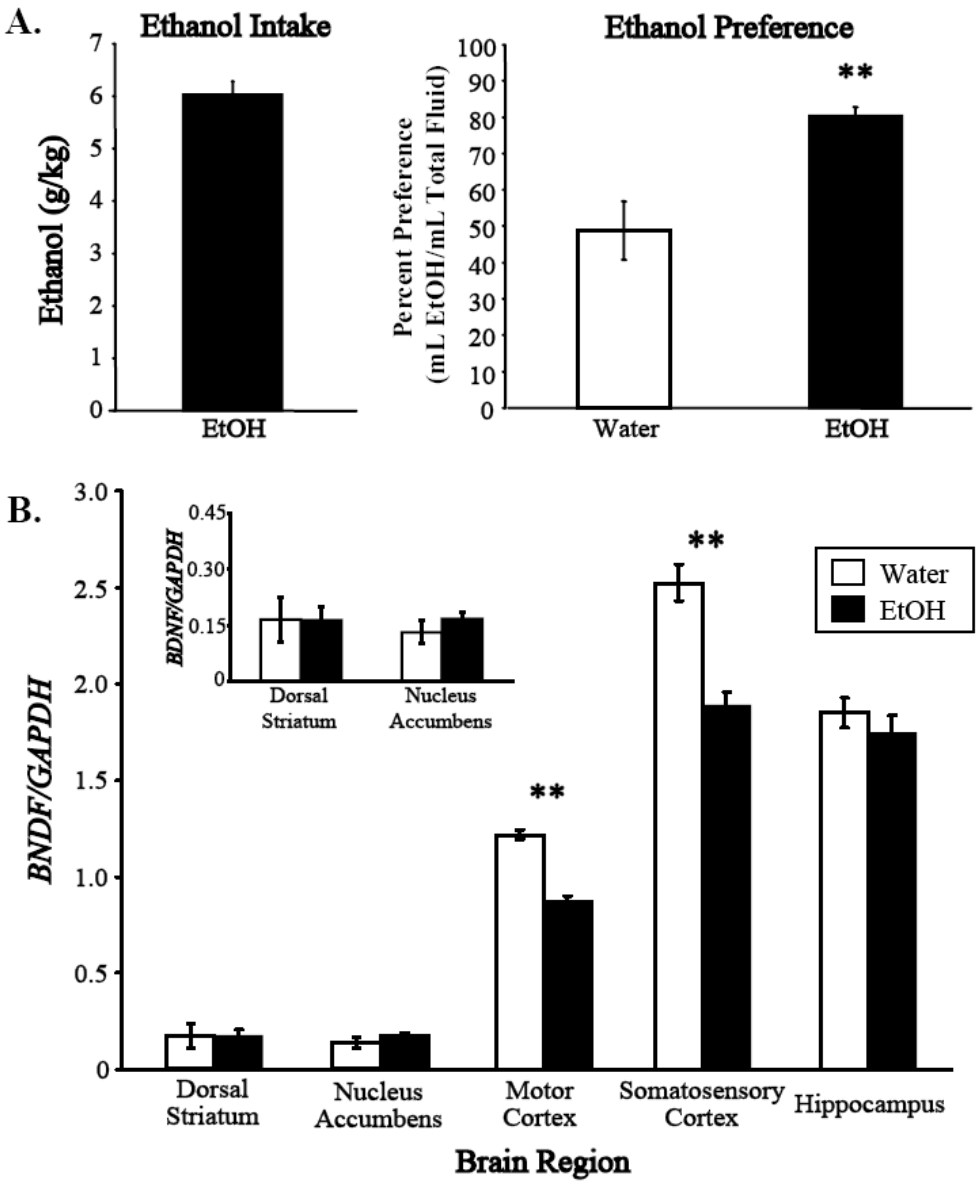


Figure 4.3. Repeated daily 4-h free-choice limited access to ethanol for 6 weeks results in breakdown of homeostatic *BDNF* expression.

C57BL6/J mice were provided with two-bottle choice access to 10% ethanol and water (EtOH) or water alone (Water) for hours 2-6 of the dark cycle daily for 6 weeks. All mice had 1 water bottle for the other 20 h each day. Brain sections were collected immediately after the final access period. (A) Mice drink significant quantities of ethanol (left) and show very high preference for the ethanol bottle (right) when one is available. (left) Data are expressed as mean grams ethanol consumed per kg body weight per 4 h immediately prior to sacrifice +/- S.E.M. n = 10. (right) Data are expressed as mean preference, calculated as mL ethanol (or water from the bottle in the same position) consumed per mL total fluid consumed, +/- S.E.M. ** $p < 0.001$. n = 10 for ethanol, n = 5 for water. (B) *BDNF* levels were determined from total brain RNA obtained from dorsal striatum, nucleus accumbens, motor cortex, somatosensory cortex and hippocampus. Inset: Magnification of striatal subregions. Data are presented as mean *BDNF* expressed as a ratio to *GAPDH* +/- S.E.M. ** $p < 0.001$. n = 10 for Ethanol, n = 5 for Water.

exposure to ethanol. Because the cortex is a significant source of BDNF for the striatum (Kokaia et al., 1998; Baquet et al., 2004), this was an exciting finding in that both local BDNF produced in the striatum and BDNF delivered by the cortex may be involved in the regulation of ethanol intake via TrkB signaling within the striatum. As shown here, both systems are impaired after prolonged experience with limited access ethanol consumption.

In addition, these data raised the question of whether the lack of observable change in *BDNF* expression within the striatum was due to a failure of ethanol to increase *BDNF* levels, or whether the extensive ethanol experience had altered the baseline level of *BDNF* expression. Specifically, if ethanol experience had reduced baseline *BDNF* expression, then each ethanol drinking bout might function to return *BDNF* levels to the ethanol-naïve (control) baseline expression level, rather than increasing *BDNF* expression above control levels, as observed in the single-day exposure animals. In order to test this, we replicated the experiment with the inclusion of a withdrawal group. This group received the same 6 weeks of ethanol access except that the final ethanol access session occurred 24 hours prior to sacrifice. As shown in Figure 4.4, the changes in gene expression observed after 6 weeks of ethanol access, as compared to a single day of ethanol access, occur in the absence of a baseline change in the dorsal striatum (no effect of Treatment, $F(2,28) = 0.222$, $p = 0.803$). Thus in the dorsal striatum the lack of increased *BDNF* expression after intake results from a breakdown in ethanol-mediated *BDNF* expression within the dorsal striatum and is not due to an altered basal set point. Similarly, the reduced *BDNF* expression observed in the cortical regions requires proximal ethanol experience, as a 24-hour withdrawal returns cortical *BDNF* levels to the

same baseline as observed in ethanol-naïve controls (Figure 4.4; main effects of Treatment as follows: Motor Cortex $F(2,28) = 4.956$, $p < 0.05$; Somatosensory Cortex $F(2,29) = 7.419$, $p < 0.005$). Taken together, these data indicate that the breakdown of BDNF homeostasis after 6 weeks' limited access to ethanol does not involve a change in baseline *BDNF* expression in either the cortex or the dorsal striatum, but that each drinking episode triggers a decrease in cortical *BDNF* while failing to alter dorsal striatal *BDNF* expression.

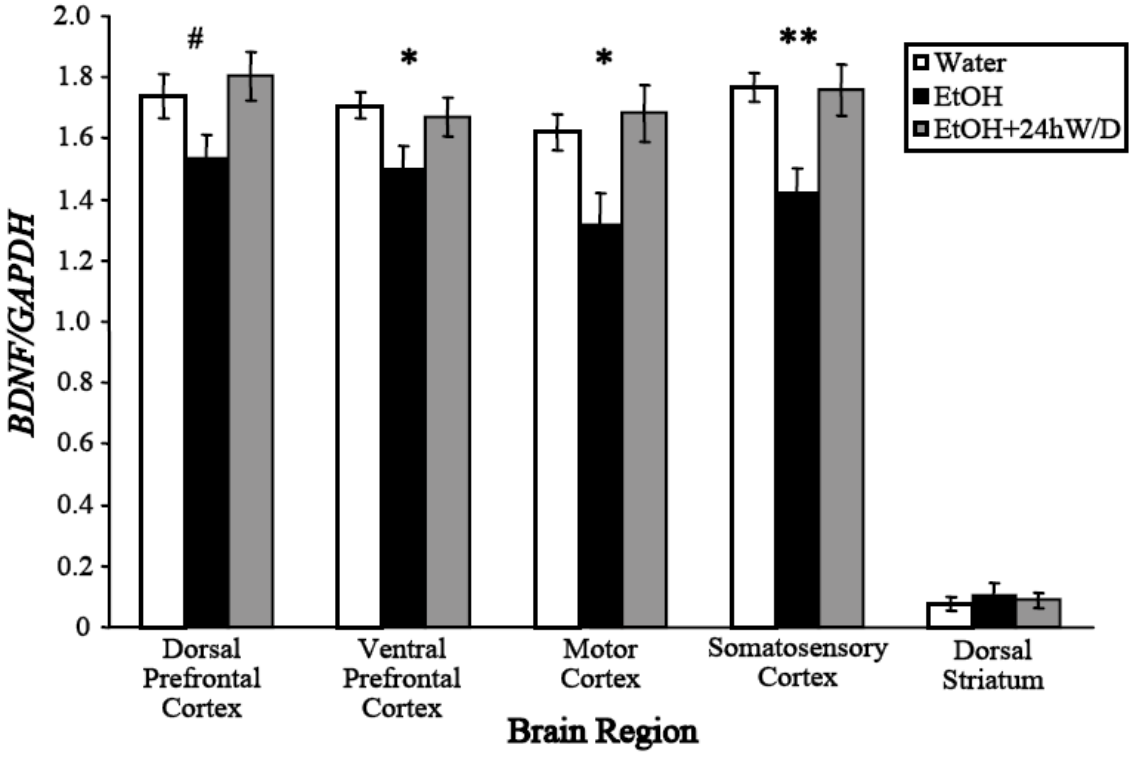


Figure 4.4. Breakdown of homeostatic *BDNF* expression is not due to reduced basal expression levels after extended experience with limited ethanol access.

C57BL6/J mice were provided with two-bottle choice access to 10% ethanol and water (EtOH) or water alone (Water) for hours 2-6 of the dark cycle daily for 6 weeks. All mice had 1 water bottle for the other 20 h each day. For the 24-h withdrawal group (EtOH+24hW/D), the final ethanol access session terminated 24 h prior to sacrifice. Brain sections were collected immediately after the final access period. *BDNF* levels were determined from total brain RNA obtained from dorsal and ventral prefrontal cortex, motor cortex, somatosensory cortex and the dorsal striatum (DS). Data are expressed as mean *BDNF* as a ratio to *GAPDH* +/- S.E.M. # $p < 0.059$, * $p < 0.05$; ** $p < 0.005$, EtOH vs. Water and EtOH+W/D. n = 9-10 per group.

In addition to determining the effect of withdrawal on *BDNF* expression, due to the unexpected reduction in cortical *BDNF* we expanded our analysis to include the prefrontal cortex (PFC) subdivided into dorsal regions, which project mainly to the dorsomedial striatum (DMS), and ventral regions, which project mainly to the NAc. Interestingly, decreased *BDNF* was not restricted to sensorimotor cortex but included both prefrontal regions analyzed, although the effect was slightly more pronounced in dorsal PFC. As shown in Figure 4.4, a significant, ethanol consumption-dependent decrease in *BDNF* was observed in dorsal PFC samples (main effect of Treatment, $F(2,27) = 3.404$, $p < 0.05$), while a strong trend towards decreased *BDNF* was seen in the ventral PFC ($F(2,29) = 3.152$, $p = 0.059$). Thus protracted limited access to ethanol intake results in a global decrease in cortical *BDNF* which is triggered by the proximal ethanol consumption bout.

BDNF homeostatic signaling does not recover following two weeks deprivation

Because alcoholism is a chronically relapsing disease (O'Brien, 1994; Oroszi & Goldman, 2004), understanding the persistence of the molecular underpinnings of alcohol addiction is critical for disease treatment. In animal models, the propensity to relapse, which is usually equated with craving, is frequently demonstrated by increased drug-seeking in instrumental paradigms (Sanchis-Segura & Spanagel, 2006). However, in less behaviorally demanding paradigms such as two-bottle choice, increased craving in withdrawal must be equated with increased drug-taking, as the only measure available is amount of ethanol consumed. Previous experiments using standard 24-hour two-bottle

choice ethanol access have demonstrated, with variable success, increased ethanol intake following a two-week deprivation period, termed the alcohol deprivation effect (McKinzie et al., 1998; Koros et al., 1999; Rodd-Henricks et al., 2000). One reason for the high variation in demonstration of the alcohol deprivation effect may be the lack of physical alcohol dependence produced by 24-hour access to ethanol (Dole et al., 1985). Because mice drink sufficient quantities of ethanol under limited access to become intoxicated (Rhodes et al., 2005), we hypothesized that renewed access to ethanol after a two-week deprivation would result in a significant increase in ethanol intake. Additionally, we hypothesized that this would require lack of recovered functionality of the BDNF homeostatic pathway. As shown in Figure 4.5A, ethanol deprivation results in a moderate 32% increase in ethanol intake, as compared to pre-deprivation intake levels, which is not statistically significant. Importantly, there is no difference in *BDNF* regulation as compared to before deprivation. That is, ethanol intake does not trigger *BDNF* expression in the dorsal striatum (no effect of Treatment, Figure 4.5B; $F(1,14) = 0.0626$, $p = 0.806$) but does cause a decrease in *BDNF* in all cortical regions (Figure 4.5B; main effects of Treatment as follows: Dorsal PFC $F(1,14) = 4.972$, $p < 0.05$; Ventral PFC $F(1,14) = 9.013$, $p < 0.05$; Motor Cortex $F(1,14) = 12.920$, $p < 0.005$; Somatosensory Cortex $F(1,14) = 10.821$, $p < 0.01$). Similarly to the 24-hour withdrawal group, there do not appear to be changes in basal *BDNF* expression in the continued abstinence group (Water, Figure 4.5), as compared to the control and 24-hour withdrawal groups (Figure 4.4). Taken together, these data demonstrate that two weeks of abstinence is insufficient for recovery of BDNF homeostasis following an additional acute bout of

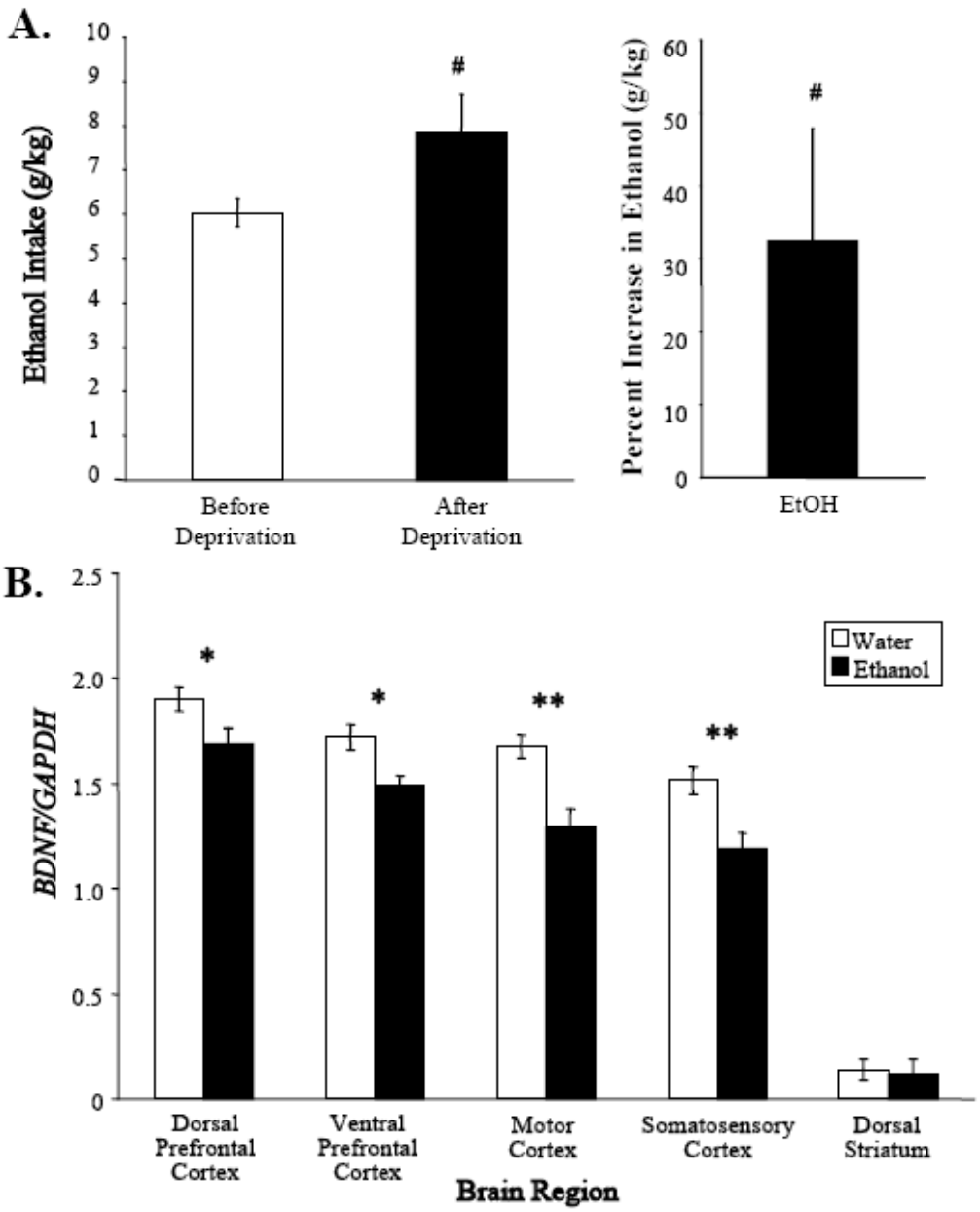


Figure 4.5. A two-week deprivation does not reverse long-term ethanol-induced breakdown of *BDNF* signaling.

C57BL/6J mice were given 4 h limited access to two-bottle choice for 10% ethanol for hours 1-5 of the dark cycle for 8 weeks, then deprived of ethanol access for an additional 2 weeks. Mice were then divided into two groups – one receiving an ethanol renewal session for 4 h prior to sacrifice (EtOH) and one receiving an additional day of deprivation (Water). (A) Ethanol intake is elevated during the renewal session as compared to the last session prior to deprivation. (left) Data are expressed as mean grams ethanol intake per kg body weight per 4 h +/- S.E.M. # $p = 0.09$. (right) Data are expressed as percent increase in ethanol intake after, as compared to before, deprivation +/- S.E.M. # $p = 0.07$. $n = 7$ for Water, $n = 8$ for EtOH. (B) *BDNF* levels were determined from total RNA obtained from dorsal and ventral prefrontal cortices, motor and somatosensory cortices and the dorsal striatum. Data are expressed as mean *BDNF* as a ratio to *GAPDH* +/- S.E.M. * $p < 0.05$, ** $p < 0.01$ Water vs. EtOH by brain region. n=7 for Water, n = 8 for EtOH.

ethanol consumption, and this continued deficiency in corticostriatal BDNF signaling is accompanied by increased ethanol intake upon re-exposure.

Relationship between ethanol intake and cortical *BDNF* inhibition

As the depression in cortical *BDNF* expression required proximal ethanol intake, we hypothesized that *BDNF* expression would be inversely correlated with the quantity of ethanol intake. Thus we performed a regression analysis of the *BDNF* levels in the various cortical regions and ethanol intake, for each of the 6-week ethanol access groups detailed above. Similar to the results discussed above for the dorsal striatal *BDNF* and acute ethanol intake (Figure 4.2B), no significant correlations were found between ethanol intake and cortical *BDNF* as measured immediately afterwards or following 24 hours' withdrawal. For completeness, these analyses have been included in Appendix C.

DISCUSSION

These results demonstrate that dorsal striatal *BDNF* is produced following a single bout of ethanol intake, thus confirming the function we had proposed based on previous results (McGough et al., 2004; Jeanblanc et al., 2006); namely, that the BDNF homeostasic pathway is triggered by individual acute drinking bouts. This pathway becomes unresponsive following repeated daily bouts of ethanol intoxication, suggesting that the progression to addiction is coupled with dysregulation of BDNF homeostasis. Importantly, the breakdown of BDNF homeostasis is not restricted to the dorsal striatum but encompasses the entire cortex, a major source of afferent projections which are thought to deliver BDNF to the striatum (Kokaia et al., 1998; Baquet et al., 2004). The

breakdown of BDNF homeostasis may result from cessation of nuclear translocation of the receptor for activated C kinase 1 (RACK1) upon ethanol intake, which is required for ethanol to increase *BDNF* transcription (McGough et al., 2004). Previously our lab has demonstrated *in vitro* that RACK1 is excluded from the nucleus following a 48-hour (chronic) ethanol treatment (Vagts et al., 2003). Importantly, a prolonged withdrawal period of 24 hours was required to restore ethanol-induced RACK1 nuclear translocation, whereas a 4-hour withdrawal was insufficient (Vagts et al., 2003). These data indicate that prolonged ethanol exposure causes a breakdown of BDNF homeostasis via inhibition of RACK1-mediated *BDNF* expression, and that recovery of RACK1 function requires a considerable duration of ethanol withdrawal. Critically, two weeks' abstinence from ethanol *in vivo* does not lead to a recovery of BDNF homeostatic signaling, perhaps due to continued unresponsiveness of RACK1 to ethanol, and re-exposure to ethanol at this time point yields slightly increased drinking. Taken together, these data suggest the novel idea that BDNF homeostasis may not only control acute ethanol intake but that deficits in BDNF response to ethanol may underlie the propensity to relapse.

A single intoxicating drinking bout activates *BDNF* transcription

Although we previously demonstrated that an acute intoxicating dose of ethanol, administered via i.p. injection, or an acute drinking bout 1.5 months after commencement of 24-hour access could increase dorsal striatal BDNF (McGough et al., 2004), this is the first demonstration that a single bout of ethanol consumption triggers *BDNF* expression in the dorsal striatum. This has significant implications for the function of BDNF as a putative regulator of social drinking in humans since these data demonstrate that dorsal

striatal BDNF provides post-ingestive feedback following only one ethanol drinking bout. Since multiple drinking episodes are not required for BDNF expression, this homeostatic pathway is poised to respond to ethanol drinking in the human social drinker. Data demonstrating a significant correlation between a mutation in the BDNF gene and alcoholism in a panel of Japanese patients (Matsushita et al., 2004) support the theory that BDNF functions as a mediator of ethanol intake in humans, perhaps by inhibiting the progression to addiction.

BDNF expression is triggered by drinking but not correlated with quantity consumed

Since BDNF activates homeostatic signaling in a pathway providing post-ingestive feedback to regulate future ethanol intake, we predicted that the increase in *BDNF* should encode information regarding the dose of ethanol reaching the brain. Surprisingly, following acute ethanol intake, dorsal striatal *BDNF* expression was found to be uncorrelated with the quantity of ethanol consumed. This suggests two alternate possibilities – either ethanol triggers an increase in *BDNF* expression via an all-or-none switch or, alternatively, some mice consume less ethanol upon initial exposure due to preexisting high levels of BDNF expression. Given our previous work showing a dose-dependent increase in *BDNF* in hippocampal primary neurons (McGough et al., 2004), the first hypothesis is unlikely, although regulation of *BDNF* expression in the striatum *in vivo* may be under differential control than that observed in hippocampal primary neurons and thus this possibility cannot be excluded. Nonetheless, the alternative is quite appealing, as, according to our theory, a priori high levels of dorsal striatal BDNF should

reduce ethanol intake. Reduced NAc BDNF levels have been demonstrated previously in a strain of rats bred for high alcohol intake as compared with a strain bred for low alcohol preference (Yan et al., 2005), indicating that BDNF expression in ethanol-naïve animals may inversely correlate with the propensity to consume ethanol. As can be observed in ethanol-naïve mice, baseline *BDNF* expression is quite variable, so the pre-ethanol baseline BDNF levels may, in part, be responsible for variation in ethanol intake in a single day's exposure. Under this mechanism, reaching a certain threshold of *BDNF* expression may result in cessation of drinking. Unfortunately at present there is no means of accounting for animal-by-animal variation in basal expression, as ascertaining neuronal gene expression is a terminal procedure and thus precludes the monitoring of both pre- and post-ethanol *BDNF* in the same animals. In addition, due to the length of the ethanol access period (4 hours), individual animals' consumption patterns may result in earlier or later induction of *BDNF* expression whereas all measures of gene expression occur at the same time point. This may also contribute to the variability observed in *BDNF* expression and the lack of direct correlation with ethanol intake. Regardless, these data demonstrate that a single bout of ethanol drinking causes a striking increase in *BDNF* expression, reinforcing the role of BDNF as a homeostatic regulator of dorsal striatal synaptic plasticity discussed in the preceding chapters.

Breakdown of BDNF homeostasis after repeated episodes of intoxication and withdrawal

Here we provide evidence that following six weeks' repeated daily intake of ethanol at levels sufficient to induce intoxication, not only does dorsal striatal *BDNF*

become nonresponsive to ethanol but each drinking bout triggers a decrease in cortical *BDNF*. As the cortex has been considered a major source of striatal BDNF (Kokaia et al., 1993; Baquet et al., 2004), this suggests that not only does local BDNF fail to cause an increase in signaling but perhaps BDNF delivered by the cortex also decreases, resulting in an even greater depression of striatal BDNF signal transduction. Interestingly, although alterations in mRNA expression were limited to the period immediately following ethanol intake, as we saw no difference following 24 hours' withdrawal, previous data has shown decreased cortical BDNF protein after a 24-hour withdrawal from chronic forced ethanol treatment via liquid diet (Pandey et al., 1999). This suggests that the depression in cortical BDNF protein may be significantly prolonged as compared to the changes in gene expression. One possible explanation for this discrepancy is the difference in length of exposure to high ethanol intake between our limited access paradigm and chronic forced ethanol treatment. Specifically, chronic forced ethanol consumption over a period of days may instigate a protracted dampening of corticostriatal BDNF signaling which fails to return to the ethanol-naïve baseline rapidly upon ethanol removal, whereas discrete consumption bouts provided under our paradigm may reduce the duration of BDNF downregulation. Alternatively, the dynamics of BDNF mRNA and protein expression may be temporally offset, as it has been suggested that the BDNF protein has a fairly long half-life (Nawa et al., 1995). Thus a decrease in mRNA observed immediately post-exposure might not be followed by a decrease in protein levels for several hours, yet this change in protein expression could be more long-lasting than the alteration in mRNA expression. Interestingly, Sanna and colleagues (2002) have demonstrated a prolonged decrease in striatal extracellular signal-

regulated kinase (ERK) activation during withdrawal from chronic intermittent ethanol exposure which lasts at least 7 hours after the cessation of ethanol exposure (Sanna et al., 2002). As demonstrated in Chapter 2, striatal ERK phosphorylation is downstream of BDNF signaling, indicating that the prolonged decrease in ERK activity reported by Sanna et al. (2002) may be the functional consequence of decreased striatal BDNF signaling, which is predicted by the decrease in *BDNF* expression demonstrated here. Thus it will be necessary to determine that the changes in *BDNF* mRNA observed here result in altered BDNF protein expression and signaling pathways as demonstrated *in vitro* in Chapter 2.

BDNF as a negative regulator of associative memory formation in the development of alcohol addiction

As detailed in Chapter 3, BDNF regulates ethanol intake via its downstream effector dynorphin, which has been shown to decrease dopamine release (Spanagel et al., 1990; You et al., 1999). As we demonstrate here, this is an acute effect which ceases after repeated ethanol exposure, putatively concomitant with a progression towards an addictive phenotype. This suggests a functional role for the upregulation of dorsal striatal BDNF/dynorphin signaling by ethanol as a negative regulator of the associative memories underlying ethanol addiction.

While a vast majority of the research into the mechanisms supporting drug addiction has focused on the NAc due to its central role in the mesolimbic reward pathway (Self, 2004), reward learning is not independent of the dorsal striatum, as inactivation of the DMS blocks acquisition of instrumental conditioning (Yin et al.,

2005). Thus the action of BDNF signaling following acute ethanol intake may be to inhibit plasticity in the DMS and thereby diminish the acquisition of the response-outcome contingency, or the learned association linking the factors defining the drug-seeking and drug-taking experience with the physiological effects of alcohol. In support of this hypothesis, alcohol treatment of DMS slices has been shown to reverse the mechanism of neuronal response from long-term potentiation (LTP) to long-term depression (LTD), resulting in weakening of synaptic strength upon administration of normally potentiating stimuli (Yin et al., 2007). This proposed regulation of striatal LTD via BDNF contrasts with its traditional role as a factor promoting LTP, a function which has been extensively studied in the hippocampus (Korte et al., 1995; Patterson et al., 1996; Gottschalk et al., 1998; Korte et al., 1998; Kovalchuk et al., 2002). However, hippocampal BDNF infusion decreases, while striatal BDNF increases, *preprodynorphin* (*Pdyn*) expression (Croll et al., 1994), suggesting that BDNF may have brain region-specific effects on synaptic potentiation via differential regulation of downstream effectors such as *Pdyn*. Taken together, these data suggest that BDNF via its downstream effector dynorphin may act to reduce neurotransmitter release in the DMS, thereby converting the DMS response to stimulation from LTP to LTD.

Reversal of the DMS response to stimulation – from potentiating to inhibiting – confers on the region a dorsolateral striatum (DLS) -like property, as these two subpopulations of the dorsal striatum normally respond antithetically to stimulation (Partridge et al., 2000). The DLS is the central locus for habit formation (Yin & Knowlton, 2006), which has gained increasing attention in the addiction field over the past several years, as human addiction manifests itself as a persistent, habitually relapsing

condition (Gerdeman et al., 2003; Everitt & Robbins, 2005). While it is tempting to think that transformation of DMS plasticity to a DLS-like phenotype might partake in the progression to addiction, an alternate explanation, given the addiction-delaying function of BDNF, is that its functionality may be DLS-specific. Thus the reason for the delayed progression to addiction may be inhibition of DLS plasticity and ensuing postponement of habit formation.

Unfortunately the current data are unable to resolve this debate. The dorsal striatal section assessed encompasses both subdivisions of the dorsal striatum (see Appendix B for schematic diagrams of the brain dissections). Additionally, every region of cortex surveyed displays a significant decrease in *BDNF* following prolonged ethanol intake, thus precluding any region-specific effect in the face of decreased receipt of anterograde BDNF in the striatum. Surprisingly, this decrease in cortical *BDNF* also included the limbic subregions of the prefrontal cortex, indicating that this effect was not specific to dorsal striatal afferents. At present the role of cortical BDNF in regulating ethanol intake is unknown. In light of the current data, activation of BDNF signaling in the striatum via cortically-delivered BDNF presents an attractive hypothesis; however, this can neither be substantiated nor refuted based on the mRNA expression data presented herein. Thus it will be of great interest in the future to determine what role cortical BDNF plays in regulation of ethanol intake, as well as the time course of breakdown of BDNF homeostasis. In particular, elucidating whether BDNF expression and signaling is differentially regulated in striatal subregions or whether differences exist in the order and timing of involvement of the various cortical afferent regions will be enlightening studies.

Taken together, these data support a biphasic function of BDNF homeostasis in the corticostriatal network. Initially, BDNF in the dorsal striatum acts to delay or prevent the onset of addiction via its upregulation subsequent to individual acute ethanol consumption bouts. Once BDNF homeostatic signaling breaks down en route to addiction, however, it becomes a putative marker of recovery, as renewal of ethanol-induced BDNF signaling would indicate a reinstatement of homeostasis. It should be noted that several limitations exist given the current data which present critical future directions in elucidating the role of BDNF in dependence and relapse. First, as mismatch can exist between BDNF mRNA and protein expression (Nawa et al., 1995), a critical next step will be to demonstrate that the observed changes in *BDNF* expression directly correlate with alterations in BDNF protein expression and signaling. Additionally, as the role of cortical BDNF in ethanol intake is unknown, it is possible that the effect of decreased *BDNF* expression may be local (i.e. within the cortex) and have no effect on BDNF signaling in the dorsal striatum. Thus an additional line of future investigation must address the function of cortical BDNF in regulating ethanol intake and its interrelatedness with or independence from the regulation of ethanol intake by dorsal striatal BDNF. Finally, demonstration that ethanol intake under limited access retains sensitivity to increased BDNF expression, via Tat-RACK1 treatment or direct BDNF infusion into the dorsal striatum, will lend support to the hypothesized role of renewed BDNF homeostasis following protracted abstinence in preventing relapse. Refining our understanding of the specific striatal subregions or corticostriatal circuits involved in BDNF homeostasis, as well as the temporal features regulating its breakdown and

possible recovery, present critical future studies, as they will provide crucial information regarding the mechanism by which protective pathways in the brain fail and thus result in addiction. Most importantly, as BDNF mutations and polymorphisms surrounding the gene have been implicated in the human alcoholic condition (Uhl et al., 2001; Matsushita et al., 2004), complete delineation of the route by which BDNF ceases to exercise homeostatic control over ethanol intake may provide valuable targets for the development of medications to treat alcohol dependence.

CHAPTER 5

GENERAL CONCLUSIONS & FUTURE DIRECTIONS

The work presented herein clearly establishes a role for brain-derived neurotrophic factor (BDNF) and its downstream effector dynorphin in the homeostatic control of ethanol intake. As detailed in the preceding chapters, ethanol intake acutely – but not chronically – increases *BDNF* expression in the dorsal striatum. Increased striatal BDNF results in activation of the mitogen-activated protein kinase (MAPK) signaling cascade and subsequent production of *preprodynorphin* (*Pdyn*). Importantly, BDNF is required to mitigate ethanol intake, as deletion of BDNF throughout the forebrain increases ethanol intake. Dynorphin is critically involved in the regulation of ethanol intake by BDNF, as blockade of the kappa opioid receptor (KOR) inhibits BDNF's ability to decrease ethanol intake. Since activation of the KOR blocks the acquisition of ethanol conditioned place preference, we suggest that dorsal striatal BDNF via dynorphin may regulate ethanol intake by inhibiting the acquisition of the action-outcome relationship between the act of ethanol consumption and its rewarding properties, thus delaying the progression to compulsive consumption that characterizes alcohol addiction. Specifically, since activation of the KOR reduces neurotransmitter release, we suggest that BDNF via dynorphin may decrease dopamine and/or glutamate release, thus preventing plastic changes at dorsal striatal synapses involved in the development of alcohol addiction.

Upon chronic ethanol intake, dorsal striatal *BDNF* fails to respond to ethanol consumption bouts. Under our limited access paradigm, the breakdown of BDNF homeostasis – which may be akin to the development of ethanol dependence after a series of ethanol intake bouts and subsequent forced withdrawal periods – occurs within six weeks of daily ethanol consumption. Additionally, each individual drinking bout blunts

BDNF expression throughout the cortex, a major afferent projection to and source of BDNF for the striatum, although baseline cortical *BDNF* levels remain unchanged. Taken together, these results suggest that the BDNF homeostatic pathway provides post-ingestive feedback which serves acutely to reduce ethanol intake via its action in the dorsal striatum. Importantly, this homeostatic function breaks down at a broader circuit level following repeated ethanol intake and withdrawal bouts, encompassing both the dorsal striatum and the projecting cortex. As alcoholism is a chronically relapsing disease, the lack of recovery of BDNF homeostasis after a two-week period of forced abstinence suggests a critical role for corticostriatal BDNF in the propensity to relapse. Therefore these results have important implications regarding the plastic changes within the corticostriatal network which may subserve the development and maintenance of alcohol addiction.

BDNF, DYNORPHIN AND THE HOMEOSTATIC REGULATION OF NEUROTRANSMITTER RELEASE

Exposure to multiple drugs of abuse, including ethanol, results in increased dopamine release in both the ventral and dorsal striata, while aversive substances – including KOR agonists – decrease dopamine release (Di Chiara & Imperato, 1988). Activation of the BDNF homeostatic pathway results in increased dynorphin, which acts on presynaptic neurons to dampen dopamine and glutamate release (Spanagel et al., 1990; Wagner et al., 1993; You et al., 1999; Hjelmstad & Fields, 2003). Recent work has demonstrated a requirement for dopamine signaling during the memory consolidation period in dorsal striatum-dependent tasks (Legault et al., 2006). In addition, while

dorsomedial and dorsolateral striatum respond differentially to high frequency stimulation with long-term potentiation or depression of synapses, respectively (Partridge et al., 2000), both forms of striatal plasticity are dependent on dopamine (Centonze et al., 2001). As the time scale of ethanol-induced BDNF activation parallels that proposed to be involved in striatal memory consolidation (Legault et al., 2006), these data indicate that the BDNF homeostatic pathway may act to prevent changes in synaptic strength by limiting dopamine release during the period of consolidation of learned associations between ethanol and the actions required to procure ethanol, thus slowing the progression to addiction.

INVOLVEMENT OF THE DORSAL STRIATUM IN BEHAVIORAL ADAPTATION AND THE PROGRESSION TO ADDICTION

Much attention has recently focused on the concept of addiction as a maladaptive form of habit learning, in which the addictive substance usurps the natural reward processing mechanism, resulting in striatal hyperactivation and eventual compulsive drug-seeking (Robbins & Everitt, 2002; Gerdeman et al., 2003; Everitt & Robbins, 2005). Habit formation is thought to be regulated by the dorsolateral striatum (DLS) in rodents, which roughly corresponds to the putamen in humans and nonhuman primates. Data from human cocaine addicts has shown that presentation of cocaine-related cues during withdrawal results in striatal dopamine release which is maximal in the caudate/putamen (CPu) and is highly correlated with self-reported craving (Volkow et al., 2006). Unlike cocaine users, dopamine levels in the CPu of human smokers did not correlate with self-reported craving; however, the level of dopamine release in the CPu during smoking was

highly correlated with whether or not the smoking experience was pleasurable (Barrett et al., 2004). Neither study observed any changes in ventral striatal (nucleus accumbens, NAc) dopamine, indicating that having progressed to addiction, drug-related dopamine release shifts to dorsal striatal structures, supporting a role for the dorsal striatum in the habitual behaviors which characterize drug abuse. Taken together, these data suggest that drug-related dopamine release, which in the early phases of drug-taking is most prominent in the NAc (Di Chiara & Imperato, 1988), shifts with extensive experience to the dorsal striatum.

The lateral portion of the dorsal striatum has been shown to be required for habit learning, as lesion or inactivation of this region causes automated, or habitual, responses to regain sensitivity to reward manipulations (Yin et al., 2004, 2006). As the name suggests, habits are inflexible behaviors which were once performed to attain a particular goal but are no longer driven by that goal (Yin & Knowlton, 2006). In the case of addiction, the habit of drug-seeking and drug-taking becomes the goal (Robbins & Everitt, 2002; Everitt & Robbins, 2005). The progression from goal-oriented to habitual behavior is paralleled by a shift in striatal activation from ventral (NAc) and dorsomedial during early training to encompassing more dorsal and lateral portions of the striatum after extensive training (Porrino et al., 2004). Habit learning has been shown to rely on the DLS, as inactivation of the DLS returns otherwise automated responses to their previously goal-oriented state (Yin et al., 2006); that is, the actions become once again sensitive to the value of the reinforcer. Importantly, DLS inactivation blocks both relapse and context- or cue-induced reinstatement of cocaine self-administration in rats (Fuchs et

al., 2006), reinforcing the role of the DLS in the performance of the habitual action underlying the addiction.

This is particularly salient in light of the given data, because acute ethanol experience, by increasing BDNF signaling and subsequent production of dynorphin, likely results in decreased neurotransmitter release within the striatum. Since dopamine release is required for striatal plasticity and consolidation (Partridge et al., 2000; Legault et al., 2006), inhibition of dopamine release could block the progression to habitual drug-taking by preventing maintenance of plastic events in the DLS.

The dorsal striatum does not function solely to control habitual learning, however. The dorsomedial striatum (DMS), which receives projections from various association cortices (McGeorge & Faull, 1989), is integral in the acquisition of behaviors contingent with reinforcement (Yin et al., 2005). Like the DLS, plasticity in the DMS is dependent on intact dopaminergic signaling (Yin et al., 2007), and as such would be inhibited by dynorphin-induced decreases in dopaminergic release. Since a single acute ethanol intake bout increases BDNF signaling – theoretically long before the onset of habit learning – this implicates BDNF/dynorphin modulation of DMS synaptic plasticity as a likely target of the acute response to ethanol.

Regardless, this in no way precludes a function for BDNF in the DLS for several reasons. First, it is possible that the delayed involvement of the DLS in instrumental conditioning may in part be due to the function of homeostatic pathways such as this one. Under this mechanism, DLS responding in conditioning requires a breakdown of homeostatic pathways which normally dampen DLS activity, thus introducing the source of temporal delay in DLS involvement. Alternatively, both pathways may be

equivalently hampered by the BDNF/dynorphin pathway in the striatum prior to its breakdown, and once the inhibition of plasticity wears off, i.e. ethanol stops increasing striatal BDNF, plasticity will, over time, "feed forward" from the DMS to the DLS via interconnections in the substantia nigra which were suggested to exist in rodents and primates some time ago (Joel & Weiner, 1994) and have recently been demonstrated in primates (Haber et al., 2006). Accordingly, incentive-based learning should progressively activate neurons in a ventral-to-dorsolateral striatal gradient.

SUMMARY AND FUTURE DIRECTIONS

As demonstrated here, BDNF and dynorphin in the dorsal striatum function as members of a homeostatic pathway triggered by acute ethanol consumption, which act via a post-ingestive feedback mechanism to dampen future ethanol intake. Following extensive experience with ethanol consumption, this homeostatic pathway breaks down, resulting in no dorsal striatal BDNF response to ethanol intake, with a concurrent decrease in BDNF expression throughout the cortex. Importantly, homeostasis is not restored following two weeks' abstinence, indicating that the alteration in BDNF/dynorphin homeostasis is persistent and may be a component of relapse to ethanol consumption.

These data raise several fascinating lines of future investigation. Although we have suggested a role for this pathway in decreasing presynaptic release, particularly of dopamine, in the dorsal striatum, it is in fact unknown whether ethanol-induced BDNF signaling alters dopamine dynamics, and if so, how. Thus determination of the role of the BDNF/dynorphin pathway in regulating neurotransmitter release and synaptic plasticity

in the dorsal striatum is key to understanding the function of BDNF homeostasis at a synaptic level. Additionally, the progression to breakdown of dorsal striatal BDNF signaling is currently unknown, with the upper limit being 6 weeks of repeated daily ethanol intake/withdrawal bouts. Thus, determination of the time course by which BDNF in the dorsal striatum ceases to respond to ethanol intake is integral to understanding the function of BDNF homeostasis. In particular, studies which determine both the breakdown of this signaling pathway and, in parallel, changes in synaptic plasticity or the dynamics of dopamine release would add significant knowledge to the synaptic mechanism by which BDNF functions to decrease ethanol intake and delay the onset of addiction.

The lack of recovery of BDNF homeostasis after two weeks' abstinence, given the high propensity to relapse in human alcoholics, suggests BDNF homeostasis as an attractive target for determining whether any period of abstinence is sufficiently long to allow the brain to recover to its pre-addicted state, i.e. to be "cured". Specifically, if dorsal striatal BDNF signaling prevents habitual ethanol drinking, then determination of the length of abstinence – if ever – required for the brain to regain its capacity for homeostatic BDNF regulation in response to ethanol is essential to understanding the human recovery process. While the time scale in humans will likely be orders of magnitude longer than that of the rodent, ascertaining *BDNF* expression after a single acute ethanol challenge subsequent to increasing magnitudes of protracted abstinence is essential to understanding the persistence of the addictive habit. If recovery of BDNF homeostasis is possible, it will also be of great interest to determine the difference in temporal dynamics between recovery from a simple behavioral paradigm such as ours –

in which the only ethanol-associated cue is the appearance of the bottle but which reliably produces repeated intoxication and withdrawal – and changes in BDNF expression under an operant procedure, which does not cause intoxication but involves the learned association between more tangible drug-related contextual cues akin to relapse-inducing triggers in human alcoholics.

Understanding the role of dorsal striatal BDNF in the initial acquisition of ethanol self-administration is also of great interest. Our data demonstrate that *BDNF* expression increases following – rather than during – periods of ethanol self-administration. This change in BDNF expression and signaling coincides temporally with a period of post-training memory consolidation, thus suggesting that the BDNF/dynorphin homeostatic pathway may delay the progression to addiction by inhibiting the plasticity underlying dorsal striatum-dependent memory formation. It would be of great interest to test whether interference with BDNF signaling, for instance via administration of inhibitory antibodies, immediately following the initial exposure to limited access ethanol intake would increase ethanol consumption in the subsequent session.

Similarly, improved understanding of the functional role of dynorphin/KOR signaling in regulation of ethanol intake provides an avenue of drug development to treat alcoholism. As detailed above, the changes in BDNF/dynorphin signaling putatively act to dampen dopamine release following ethanol intake. This suggests that increasing dynorphin/KOR signaling after ethanol consumption, thus increasing the level of feedback, could decrease subsequent ethanol intake, particularly after extensive experience, when the feedback ceases to function. Hypersensitivity of the KOR system in ethanol-dependent animals has been demonstrated (Lindholm et al., 2000; Lindholm et

al., 2007; Walker & Koob, 2007), potentially due to a compensation for loss of dynorphin regulation, indicating that dependent animals should be highly responsive to control of ethanol intake by KOR agonists. However, the temporal constraints of these effects are currently unknown. Ideally, the administration of KOR agonists by a route which mimics endogenous homeostatic feedback may be capable of interfering with the memories underlying habitual ethanol intake, thus functionally weakening or breaking the addiction. Thus determination of the ability of KOR agonists to decrease subsequent ethanol intake when administered after the conclusion of an ethanol access session is essential to understanding how manipulations of this system may provide usable targets for drug development.

CONCLUDING REMARKS

As alcohol addiction is a major mental health problem in our society, understanding neuronal mechanisms regulating alcohol intake is vital to the development of treatments. Here we delineate a pathway by which BDNF in the dorsal striatum activates MAPK signaling, increases dynorphin, and thereby provides feedback subsequent to individual alcohol drinking bouts. Importantly, we show breakdown of this pathway, concomitant to the progression to an addictive phenotype, within the corticostriatal circuit, indicating that BDNF may function within this network to prevent formation of the habits underlying addiction. These studies highlight the importance of BDNF as a putative regulator of social, rather than compulsive, drinking and set the stage for future investigations which will not only complete our understanding of BDNF

homeostasis but hopefully yield fruitful new directions for development of treatments for alcoholism.

CHAPTER 6

References

Adams DH, Hanson GR, Keefe KA (2000). Cocaine and methamphetamine differentially affect opioid peptide mRNA expression in the striatum. *J Neurochem* **75**(5): 2061-70.

Aicardi G, Argilli E, Cappello S, Santi S, Riccio M, Thoenen H, Canossa M (2004). Induction of long-term potentiation and depression is reflected by corresponding changes in secretion of endogenous brain-derived neurotrophic factor. *Proc Natl Acad Sci U S A* **101**(44): 15788-92.

Aizman O, Brismar H, Uhlen P, Zettergren E, Levey AI, Forssberg H, Greengard P, Aperia A (2000). Anatomical and physiological evidence for D1 and D2 dopamine receptor colocalization in neostriatal neurons. *Nat Neurosci* **3**(3): 226-30.

Allen JP, Fertig JB, Mattson ME (1994). Personality-based subtypes of chemically dependent patients. *Ann N Y Acad Sci* **708**: 7-22.

Alonso M, Medina JH, Pozzo-Miller L (2004). ERK1/2 activation is necessary for BDNF to increase dendritic spine density in hippocampal CA1 pyramidal neurons. *Learn Mem* **11**(2): 172-8.

Alonso M, Vianna MR, Depino AM, Mello e Souza T, Pereira P, Szapiro G, Viola H, Pitossi F, Izquierdo I, Medina JH (2002a). BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. *Hippocampus* **12**(4): 551-60.

Alonso M, Vianna MR, Izquierdo I, Medina JH (2002b). Signaling mechanisms mediating BDNF modulation of memory formation in vivo in the hippocampus. *Cell Mol Neurobiol* **22**(5-6): 663-74.

Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ (1997). Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* **389**(6653): 856-60.

Altar CA, Siuciak JA, Wright P, Ip NY, Lindsay RM, Wiegand SJ (1994). In situ hybridization of trkB and trkC receptor mRNA in rat forebrain and association with high-affinity binding of [125I]BDNF, [125I]NT-4/5 and [125I]NT-3. *Eur J Neurosci* **6**(9): 1389-405.

Altshuler HL (1981). Animal models for alcohol research. *Curr Alcohol* **8**: 343-57.

Atwal JK, Massie B, Miller FD, Kaplan DR (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. *Neuron* **27**(2): 265-77.

Aubert I, Ghorayeb I, Normand E, Bloch B (2000). Phenotypical characterization of the neurons expressing the D1 and D2 dopamine receptors in the monkey striatum. *J Comp Neurol* **418**(1): 22-32.

Baek JK, Heaton MB, Walker DW (1996). Up-regulation of high-affinity neurotrophin receptor, trk B-like protein on western blots of rat cortex after chronic ethanol treatment. *Brain Res Mol Brain Res* **40**(1): 161-4.

Baquet ZC, Gorski JA, Jones KR (2004). Early striatal dendrite deficits followed by neuron loss with advanced age in the absence of anterograde cortical brain-derived neurotrophic factor. *J Neurosci* **24**(17): 4250-8.

Barbacid M, Lamballe F, Pulido D, Klein R (1991). The trk family of tyrosine protein kinase receptors. *Biochim Biophys Acta* **1072**(2-3): 115-27.

Barde YA, Edgar D, Thoenen H (1982). Purification of a new neurotrophic factor from mammalian brain. *Embo J* **1**(5): 549-53.

Barnea A, Roberts J (2001). Induction of functional and morphological expression of neuropeptide Y (NPY) in cortical cultures by brain-derived neurotrophic factor (BDNF): evidence for a requirement for extracellular-regulated kinase (ERK)-dependent and ERK-independent mechanisms. *Brain Res* **919**(1): 57-69.

Barrett SP, Boileau I, Okker J, Pihl RO, Dagher A (2004). The hedonic response to cigarette smoking is proportional to dopamine release in the human striatum as measured by positron emission tomography and [11C]raclopride. *Synapse* **54**(2): 65-71.

Beadles-Bohling AS, Crabbe JC, Wiren KM (2000). Elevated prodynorphin expression associated with ethanol withdrawal convulsions. *Neurochem Int* **37**(5-6): 463-72.

Becker HC, Lopez MF (2004). Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. *Alcohol Clin Exp Res* **28**(12): 1829-38.

Beitner-Johnson D, Guitart X, Nestler EJ (1992). Common intracellular actions of chronic morphine and cocaine in dopaminergic brain reward regions. *Ann N Y Acad Sci* **654**: 70-87.

Berhow MT, Hiroi N, Nestler EJ (1996). Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. *J Neurosci* **16**(15): 4707-15.

Berhow MT, Russell DS, Terwilliger RZ, Beitner-Johnson D, Self DW, Lindsay RM, Nestler EJ (1995). Influence of neurotrophic factors on morphine- and cocaine-induced biochemical changes in the mesolimbic dopamine system. *Neuroscience* **68**(4): 969-79.

Berke JD, Hyman SE (2000). Addiction, dopamine, and the molecular mechanisms of memory. *Neuron* **25**(3): 515-32.

Blednov YA, Walker D, Martinez M, Harris RA (2006). Reduced alcohol consumption in mice lacking preprodynorphin. *Alcohol* **40**(2): 73-86.

Bliss TV, Collingridge GL (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**(6407): 31-9.

Blizard DA (2007). Sweet and bitter taste of ethanol in C57BL/6J and DBA2/J mouse strains. *Behav Genet* **37**(1): 146-59.

Bolanos CA, Nestler EJ (2004). Neurotrophic mechanisms in drug addiction. *Neuromolecular Med* **5**(1): 69-83.

Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990). In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. *J Neurosci* **10**(6): 1788-98.

Cai YQ, Cai GQ, Liu GX, Cai Q, Shi JH, Shi J, Ma SK, Sun X, Sheng ZJ, Mei ZT, Cui D, Guo L, Wang Z, Fei J (2006). Mice with genetically altered GABA transporter subtype I (GAT1) expression show altered behavioral responses to ethanol. *J Neurosci Res* **84**(2): 255-67.

Carlezon WA, Jr., Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998). Regulation of cocaine reward by CREB. *Science* **282**(5397): 2272-5.

Centonze D, Picconi B, Gubellini P, Bernardi G, Calabresi P (2001). Dopaminergic control of synaptic plasticity in the dorsal striatum. *Eur J Neurosci* **13**(6): 1071-7.

Chao MV, Bothwell M (2002). Neurotrophins: to cleave or not to cleave. *Neuron* **33**(1): 9-12.

Chao MV, Hempstead BL (1995). p75 and Trk: a two-receptor system. *Trends Neurosci* **18**(7): 321-6.

Chavkin C, James IF, Goldstein A (1982). Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* **215**(4531): 413-5.

Chefer VI, Shippenberg TS (2006). Paradoxical effects of prodynorphin gene deletion on basal and cocaine-evoked dopaminergic neurotransmission in the nucleus accumbens. *Eur J Neurosci* **23**(1): 229-38.

Civelli O, Douglass J, Goldstein A, Herbert E (1985). Sequence and expression of the rat prodynorphin gene. *Proc Natl Acad Sci U S A* **82**(12): 4291-5.

Cole RL, Konradi C, Douglass J, Hyman SE (1995). Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron* **14**(4): 813-23.

Constantinescu A, Diamond I, Gordon AS (1999). Ethanol-induced translocation of cAMP-dependent protein kinase to the nucleus. Mechanism and functional consequences. *J Biol Chem* **274**(38): 26985-91.

Cowen MS, Lawrence AJ (2001). Alterations in central preproenkephalin mRNA expression after chronic free-choice ethanol consumption by fawn-hooded rats. *Alcohol Clin Exp Res* **25**(8): 1126-33.

Crabbe JC, Kosobud A, Young ER, Janowsky JS (1983). Polygenic and single-gene determination of responses to ethanol in BXD/Ty recombinant inbred mouse strains. *Neurobehav Toxicol Teratol* **5**(2): 181-7.

Croll SD, Wiegand SJ, Anderson KD, Lindsay RM, Nawa H (1994). Regulation of neuropeptides in adult rat forebrain by the neurotrophins BDNF and NGF. *Eur J Neurosci* **6**(8): 1343-53.

Curran EJ, Watson SJ, Jr. (1995). Dopamine receptor mRNA expression patterns by opioid peptide cells in the nucleus accumbens of the rat: a double in situ hybridization study. *J Comp Neurol* **361**(1): 57-76.

D'Addario C, Di Benedetto M, Izenwasser S, Candeletti S, Romualdi P (2007). Role of serotonin in the regulation of the dynorphinergic system by a kappa-opioid agonist and cocaine treatment in rat CNS. *Neuroscience* **144**(1): 157-64.

Davis RJ (1995). Transcriptional regulation by MAP kinases. *Mol Reprod Dev* **42**(4): 459-67.

Del Boca FK (1994). Sex, gender, and alcoholic typologies. *Ann N Y Acad Sci* **708**: 34-48.

Deng YP, Lei WL, Reiner A (2006). Differential perikaryal localization in rats of D1 and D2 dopamine receptors on striatal projection neuron types identified by retrograde labeling. *J Chem Neuroanat* **32**(2-4): 101-16.

DePaoli AM, Hurley KM, Yasada K, Reisine T, Bell G (1994). Distribution of kappa opioid receptor mRNA in adult mouse brain: an in situ hybridization histochemistry study. *Mol Cell Neurosci* **5**(4): 327-35.

Di Chiara G, Imperato A (1985). Ethanol preferentially stimulates dopamine release in the nucleus accumbens of freely moving rats. *Eur J Pharmacol* **115**(1): 131-2.

Di Chiara G, Imperato A (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci U S A* **85**(14): 5274-8.

Dohrman DP, Diamond I, Gordon AS (1996). Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. *Proc Natl Acad Sci U S A* **93**(19): 10217-21.

Dole VP, Gentry RT (1984). Toward an analogue of alcoholism in mice: scale factors in the model. *Proc Natl Acad Sci U S A* **81**(11): 3543-6.

Dole VP, Ho A, Gentry RT (1985). Toward an analogue of alcoholism in mice: criteria for recognition of pharmacologically motivated drinking. *Proc Natl Acad Sci U S A* **82**(10): 3469-71.

Douglass J, McKinzie AA, Pollock KM (1994). Identification of multiple DNA elements regulating basal and protein kinase A-induced transcriptional expression of the rat prodynorphin gene. *Mol Endocrinol* **8**(3): 333-44.

Doyon WM, Howard EC, Shippenberg TS, Gonzales RA (2006). Kappa-opioid receptor modulation of accumbal dopamine concentration during operant ethanol self-administration. *Neuropharmacology* **51**(3): 487-96.

Drake CT, Terman GW, Simmons ML, Milner TA, Kunkel DD, Schwartzkroin PA, Chavkin C (1994). Dynorphin opioids present in dentate granule cells may function as retrograde inhibitory neurotransmitters. *J Neurosci* **14**(6): 3736-50.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA (1993). Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363**(6424): 45-51.

Endoh T, Matsuura H, Tanaka C, Nagase H (1992). Nor-binaltorphimine: a potent and selective kappa-opioid receptor antagonist with long-lasting activity in vivo. *Arch Int Pharmacodyn Ther* **316**: 30-42.

English JD, Sweatt JD (1997). A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. *J Biol Chem* **272**(31): 19103-6.

Epstein EE, Ginsburg BE, Hesselbrock VM, Schwarz JC (1994). Alcohol and drug abusers subtyped by antisocial personality and primary or secondary depressive disorder. *Ann N Y Acad Sci* **708**: 187-201.

Ernfors P, Kucera J, Lee KF, Loring J, Jaenisch R (1995). Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. *Int J Dev Biol* **39**(5): 799-807.

Ernfors P, Lee KF, Jaenisch R (1994). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368**(6467): 147-50.

Everitt BJ, Robbins TW (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat Neurosci* **8**(11): 1481-9.

Fagergren P, Smith HR, Daunais JB, Nader MA, Porrino LJ, Hurd YL (2003). Temporal upregulation of prodynorphin mRNA in the primate striatum after cocaine self-administration. *Eur J Neurosci* **17**(10): 2212-8.

Falk JL, Samson HH, Winger G (1972). Behavioral maintenance of high concentrations of blood ethanol and physical dependence in the rat. *Science* **177**(51): 811-3.

Falk JL, Tang M (1988). What schedule-induced polydipsia can tell us about alcoholism. *Alcohol Clin Exp Res* **12**(5): 577-85.

Falk T, Zhang S, Erbe EL, Sherman SJ (2006). Neurochemical and electrophysiological characteristics of rat striatal neurons in primary culture. *J Comp Neurol* **494**(2): 275-89.

Fallon JH, Leslie FM (1986). Distribution of dynorphin and enkephalin peptides in the rat brain. *J Comp Neurol* **249**(3): 293-336.

Fattori V, Abe SI, Kobayashi K, Costa LG, Tsuji R (2007). Effects of postnatal ethanol exposure on neurotrophic factors and signal transduction pathways in rat brain. *J Appl Toxicol*.

Filip M, Faron-Gorecka A, Kusmider M, Golda A, Frankowska M, Dziedzicka-Wasylewska M (2006). Alterations in BDNF and trkB mRNAs following acute or sensitizing cocaine treatments and withdrawal. *Brain Res* **1071**(1): 218-25.

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME (1997). CREB: a major mediator of neuronal neurotrophin responses. *Neuron* **19**(5): 1031-47.

Finn DA, Belknap JK, Cronise K, Yoneyama N, Murillo A, Crabbe JC (2005). A procedure to produce high alcohol intake in mice. *Psychopharmacology (Berl)* **178**(4): 471-80.

Freeman AY, Pierce RC (2002). Neutralization of neutrophin-3 in the ventral tegmental area or nucleus accumbens differentially modulates cocaine-induced behavioral plasticity in rats. *Synapse* **46**(2): 57-65.

Fuchs RA, Branham RK, See RE (2006). Different neural substrates mediate cocaine seeking after abstinence versus extinction training: a critical role for the dorsolateral caudate-putamen. *J Neurosci* **26**(13): 3584-8.

Funada M, Suzuki T, Narita M, Misawa M, Nagase H (1993). Blockade of morphine reward through the activation of kappa-opioid receptors in mice. *Neuropharmacology* **32**(12): 1315-23.

Gabriel KI, Cunningham CL (2005). Effects of topiramate on ethanol and saccharin consumption and preferences in C57BL/6J mice. *Alcohol Clin Exp Res* **29**(1): 75-80.

Gaiddon C, Loeffler JP, Larmet Y (1996). Brain-derived neurotrophic factor stimulates AP-1 and cyclic AMP-responsive element dependent transcriptional activity in central nervous system neurons. *J Neurochem* **66**(6): 2279-86.

Gerdeman GL, Partridge JG, Lupica CR, Lovinger DM (2003). It could be habit forming: drugs of abuse and striatal synaptic plasticity. *Trends Neurosci* **26**(4): 184-92.

Gerfen CR (1988). Synaptic organization of the striatum. *J Electron Microsc Tech* **10**(3): 265-81.

Gooney M, Shaw K, Kelly A, O'Mara SM, Lynch MA (2002). Long-term potentiation and spatial learning are associated with increased phosphorylation of TrkB and extracellular signal-regulated kinase (ERK) in the dentate gyrus: evidence for a role for brain-derived neurotrophic factor. *Behav Neurosci* **116**(3): 455-63.

Gottschalk W, Pozzo-Miller LD, Figurov A, Lu B (1998). Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus. *J Neurosci* **18**(17): 6830-9.

Graham DL, Edwards S, Bachtell RK, Dileone RJ, Rios M, Self DW (2007). Dynamic BDNF activity in nucleus accumbens with cocaine use increases self-administration and relapse. *Nat Neurosci* **10**(8): 1029-37.

Grahame NJ, Cunningham CL (1997). Intravenous ethanol self-administration in C57BL/6J and DBA/2J mice. *Alcohol Clin Exp Res* **21**(1): 56-62.

Grahame NJ, Grose AM (2003). Blood alcohol concentrations after scheduled access in high-alcohol-preferring mice. *Alcohol* **31**(1-2): 99-104.

Grant BF, Dawson DA, Stinson FS, Chou SP, Dufour MC, Pickering RP (2004). The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991-1992 and 2001-2002. *Drug Alcohol Depend* **74**(3): 223-34.

Grant KA, Samson HH (1985). Induction and maintenance of ethanol self-administration without food deprivation in the rat. *Psychopharmacology (Berl)* **86**(4): 475-9.

Grewal SS, York RD, Stork PJ (1999). Extracellular-signal-regulated kinase signalling in neurons. *Curr Opin Neurobiol* **9**(5): 544-53.

Grimm JW, Lu L, Hayashi T, Hope BT, Su TP, Shaham Y (2003). Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. *J Neurosci* **23**(3): 742-7.

Guiton M, Gunn-Moore FJ, Stitt TN, Yancopoulos GD, Tavare JM (1994). Identification of in vivo brain-derived neurotrophic factor-stimulated autophosphorylation sites on the TrkB receptor tyrosine kinase by site-directed mutagenesis. *J Biol Chem* **269**(48): 30370-7.

Gulya K, Orpana AK, Sikela JM, Hoffman PL (1993). Prodynorphin and vasopressin mRNA levels are differentially affected by chronic ethanol ingestion in the mouse. *Brain Res Mol Brain Res* **20**(1-2): 1-8.

Haber SN, Kim KS, Mailly P, Calzavara R (2006). Reward-related cortical inputs define a large striatal region in primates that interface with associative cortical connections, providing a substrate for incentive-based learning. *J Neurosci* **26**(32): 8368-76.

Hall W, Zador D (1997). The alcohol withdrawal syndrome. *Lancet* **349**(9069): 1897-900.

Harwood HJ, Fountain D, Fountain G (1999). Economic cost of alcohol and drug abuse in the United States, 1992: a report. *Addiction* **94**(5): 631-5.

He DY, Vagts AJ, Yaka R, Ron D (2002). Ethanol induces gene expression via nuclear compartmentalization of receptor for activated C kinase 1. *Mol Pharmacol* **62**(2): 272-80.

Helton TE, Daunais JB, McGinty JF (1993). Convulsant doses of cocaine alter immediate early gene and opioid peptide expression in rat limbic forebrain. *Brain Res Mol Brain Res* **20**(3): 285-8.

Hensler JG, Ladenheim EE, Lyons WE (2003). Ethanol consumption and serotonin-1A (5-HT1A) receptor function in heterozygous BDNF (+/-) mice. *J Neurochem* **85**(5): 1139-47.

Herz A (1998). Opioid reward mechanisms: a key role in drug abuse? *Can J Physiol Pharmacol* **76**(3): 252-8.

Hjelmstad GO, Fields HL (2003). Kappa opioid receptor activation in the nucleus accumbens inhibits glutamate and GABA release through different mechanisms. *J Neurophysiol* **89**(5): 2389-95.

Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde YA (1990). Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *Embo J* **9**(8): 2459-64.

Holter SM, Henniger MS, Lipkowski AW, Spanagel R (2000). Kappa-opioid receptors and relapse-like drinking in long-term ethanol-experienced rats. *Psychopharmacology (Berl)* **153**(1): 93-102.

Horger BA, Iyasere CA, Berhow MT, Messer CJ, Nestler EJ, Taylor JR (1999). Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. *J Neurosci* **19**(10): 4110-22.

Huang EJ, Reichardt LF (2003). Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem* **72**: 609-42.

Hunt WA, Majchrowicz E, Dalton TK, Swartzwelder HS, Wixon H (1979). Alterations in neurotransmitter activity after acute and chronic ethanol treatment: studies of transmitter interactions. *Alcohol Clin Exp Res* **3**(4): 359-63.

Hurd YL, Herkenham M (1992). Influence of a single injection of cocaine, amphetamine or GBR 12909 on mRNA expression of striatal neuropeptides. *Brain Res Mol Brain Res* **16**(1-2): 97-104.

Hyman SE, Malenka RC, Nestler EJ (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci* **29**: 565-98.

Iwamoto ET (1985). Place-conditioning properties of mu, kappa, and sigma opioid agonists. *Alcohol Drug Res* **6**(5): 327-39.

Jamensky NT, Gianoulakis C (1997). Content of dynorphins and kappa-opioid receptors in distinct brain regions of C57BL/6 and DBA/2 mice. *Alcohol Clin Exp Res* **21**(8): 1455-64.

Jeanblanc J, He DY, McGough NN, Logrip ML, Phamluong K, Janak PH, Ron D (2006). The dopamine D3 receptor is part of a homeostatic pathway regulating ethanol consumption. *J Neurosci* **26**(5): 1457-64.

Jezierski MK, Sohrabji F (2000). Region- and peptide-specific regulation of the neurotrophins by estrogen. *Brain Res Mol Brain Res* **85**(1-2): 77-84.

Jezierski MK, Sohrabji F (2003). Estrogen enhances retrograde transport of brain-derived neurotrophic factor in the rodent forebrain. *Endocrinology* **144**(11): 5022-9.

Joel D, Weiner I (1994). The organization of the basal ganglia-thalamocortical circuits: open interconnected rather than closed segregated. *Neuroscience* **63**(2): 363-79.

Kang H, Welcher AA, Shelton D, Schuman EM (1997). Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. *Neuron* **19**(3): 653-64.

Kaplan DR, Miller FD (1997). Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol* **9**(2): 213-21.

Kaplan DR, Miller FD (2000). Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* **10**(3): 381-91.

Klein R (1994). Role of neurotrophins in mouse neuronal development. *Faseb J* **8**(10): 738-44.

Klein R, Conway D, Parada LF, Barbacid M (1990). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* **61**(4): 647-56.

Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991). The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**(2): 395-403.

Kokaia Z, Andsberg G, Yan Q, Lindvall O (1998). Rapid alterations of BDNF protein levels in the rat brain after focal ischemia: evidence for increased synthesis and anterograde axonal transport. *Exp Neurol* **154**(2): 289-301.

Kokaia Z, Bengzon J, Metsis M, Kokaia M, Persson H, Lindvall O (1993). Coexpression of neurotrophins and their receptors in neurons of the central nervous system. *Proc Natl Acad Sci U S A* **90**(14): 6711-5.

Kolbeck R, Bartke I, Eberle W, Barde YA (1999). Brain-derived neurotrophic factor levels in the nervous system of wild-type and neurotrophin gene mutant mice. *J Neurochem* **72**(5): 1930-8.

Koros E, Piasecki J, Kostowski W, Bienkowski P (1999). Development of alcohol deprivation effect in rats: lack of correlation with saccharin drinking and locomotor activity. *Alcohol Alcohol* **34**(4): 542-50.

Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc Natl Acad Sci U S A* **92**(19): 8856-60.

Korte M, Kang H, Bonhoeffer T, Schuman E (1998). A role for BDNF in the late-phase of hippocampal long-term potentiation. *Neuropharmacology* **37**(4-5): 553-9.

Kovacs KM, Szakall I, O'Brien D, Wang R, Vinod KY, Saito M, Simonin F, Kieffer BL, Vadasz C (2005). Decreased oral self-administration of alcohol in kappa-opioid receptor knock-out mice. *Alcohol Clin Exp Res* **29**(5): 730-8.

Kovalchuk Y, Hanse E, Kafitz KW, Konnerth A (2002). Postsynaptic Induction of BDNF-Mediated Long-Term Potentiation. *Science* **295**(5560): 1729-34.

Kuzmin AV, Semenova S, Gerrits MA, Zvartau EE, Van Ree JM (1997). Kappa-opioid receptor agonist U50,488H modulates cocaine and morphine self-administration in drug-naive rats and mice. *Eur J Pharmacol* **321**(3): 265-71.

Lack AK, Floyd DW, McCool BA (2005). Chronic ethanol ingestion modulates proanxiety factors expressed in rat central amygdala. *Alcohol* **36**(2): 83-90.

Le Bourhis B, Aufrere G (1983). Pattern of alcohol administration and physical dependence. *Alcohol Clin Exp Res* **7**(4): 378-81.

Le Foll B, Diaz J, Sokoloff P (2005). A single cocaine exposure increases BDNF and D3 receptor expression: implications for drug-conditioning. *Neuroreport* **16**(2): 175-8.

Le Moine C, Bloch B (1995). D1 and D2 dopamine receptor gene expression in the rat striatum: sensitive cRNA probes demonstrate prominent segregation of D1 and D2 mRNAs in distinct neuronal populations of the dorsal and ventral striatum. *J Comp Neurol* **355**(3): 418-26.

Legault G, Smith CT, Beninger RJ (2006). Post-training intra-striatal scopolamine or flupenthixol impairs radial maze learning in rats. *Behav Brain Res* **170**(1): 148-55.

Lester J, Fink S, Aronin N, DiFiglia M (1993). Colocalization of D1 and D2 dopamine receptor mRNAs in striatal neurons. *Brain Res* **621**(1): 106-10.

Lieber CS, DeCarli LM, Sorrell MF (1989). Experimental methods of ethanol administration. *Hepatology* **10**(4): 501-10.

Lindholm S, Ploj K, Franck J, Nylander I (2000). Repeated ethanol administration induces short- and long-term changes in enkephalin and dynorphin tissue concentrations in rat brain. *Alcohol* **22**(3): 165-71.

Lindholm S, Rosin A, Dahlin I, Georgieva J, Franck J (2007). Ethanol alters the effect of kappa receptor ligands on dopamine release in the nucleus accumbens. *Physiol Behav*.

Lindholm S, Werme M, Brene S, Franck J (2001). The selective kappa-opioid receptor agonist U50,488H attenuates voluntary ethanol intake in the rat. *Behav Brain Res* **120**(2): 137-46.

Liu QR, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR (2006). Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. *Brain Res* **1067**(1): 1-12.

Lopez MF, Becker HC (2005). Effect of pattern and number of chronic ethanol exposures on subsequent voluntary ethanol intake in C57BL/6J mice. *Psychopharmacology (Berl)* **181**(4): 688-96.

Lu B (2003). Pro-region of neurotrophins: role in synaptic modulation. *Neuron* **39**(5): 735-8.

Lu L, Dempsey J, Liu SY, Bossert JM, Shaham Y (2004). A single infusion of brain-derived neurotrophic factor into the ventral tegmental area induces long-lasting potentiation of cocaine seeking after withdrawal. *J Neurosci* **24**(7): 1604-11.

Lu L, Hope BT, Dempsey J, Liu SY, Bossert JM, Shaham Y (2005). Central amygdala ERK signaling pathway is critical to incubation of cocaine craving. *Nat Neurosci* **8**(2): 212-9.

Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, Watson SJ (1994). Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. *J Comp Neurol* **350**(3): 412-38.

Marchant NJ, Densmore VS, Osborne PB (2007). Coexpression of prodynorphin and corticotrophin-releasing hormone in the rat central amygdala: Evidence of two distinct endogenous opioid systems in the lateral division. *J Comp Neurol* **504**(6): 702-15.

Marinelli PW, Lam M, Bai L, Quirion R, Gianoulakis C (2006). A microdialysis profile of dynorphin A(1-8) release in the rat nucleus accumbens following alcohol administration. *Alcohol Clin Exp Res* **30**(6): 982-90.

Marsh HN, Scholz WK, Lamballe F, Klein R, Nanduri V, Barbacid M, Palfrey HC (1993). Signal transduction events mediated by the BDNF receptor gp 145trkB in primary hippocampal pyramidal cell culture. *J Neurosci* **13**(10): 4281-92.

Martin-Iverson MT, Todd KG, Altar CA (1994). Brain-derived neurotrophic factor and neurotrophin-3 activate striatal dopamine and serotonin metabolism and related behaviors: interactions with amphetamine. *J Neurosci* **14**(3 Pt 1): 1262-70.

Mathieu-Kia AM, Besson MJ (1998). Repeated administration of cocaine, nicotine and ethanol: effects on preprodynorphin, preprotachykinin A and preproenkephalin mRNA expression in the dorsal and the ventral striatum of the rat. *Brain Res Mol Brain Res* **54**(1): 141-51.

Matsushita S, Kimura M, Miyakawa T, Yoshino A, Murayama M, Masaki T, Higuchi S (2004). Association study of brain-derived neurotrophic factor gene polymorphism and alcoholism. *Alcohol Clin Exp Res* **28**(11): 1609-12.

McCarty JH, Feinstein SC (1998). Activation loop tyrosines contribute varying roles to TrkB autophosphorylation and signal transduction. *Oncogene* **16**(13): 1691-700.

McDaniel KL, Mundy WR, Tilson HA (1990). Microinjection of dynorphin into the hippocampus impairs spatial learning in rats. *Pharmacol Biochem Behav* **35**(2): 429-35.

McGeorge AJ, Faull RL (1989). The organization of the projection from the cerebral cortex to the striatum in the rat. *Neuroscience* **29**(3): 503-37.

McGough NN, He DY, Logrip ML, Jeanblanc J, Phamluong K, Luong K, Kharazia V, Janak PH, Ron D (2004). RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. *J Neurosci* **24**(46): 10542-52.

McKinzie DL, Nowak KL, Yorger L, McBride WJ, Murphy JM, Lumeng L, Li TK (1998). The alcohol deprivation effect in the alcohol-preferring P rat under free-drinking and operant access conditions. *Alcohol Clin Exp Res* **22**(5): 1170-6.

McLaughlin JP, Land BB, Li S, Pintar JE, Chavkin C (2006). Prior activation of kappa opioid receptors by U50,488 mimics repeated forced swim stress to potentiate cocaine place preference conditioning. *Neuropsychopharmacology* **31**(4): 787-94.

McLaughlin JP, Marton-Popovici M, Chavkin C (2003). Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci* **23**(13): 5674-83.

Meredith GE, Callen S, Scheuer DA (2002). Brain-derived neurotrophic factor expression is increased in the rat amygdala, piriform cortex and hypothalamus following repeated amphetamine administration. *Brain Res* **949**(1-2): 218-27.

Meshul CK, McGinty JF (2000). Kappa opioid receptor immunoreactivity in the nucleus accumbens and caudate-putamen is primarily associated with synaptic vesicles in axons. *Neuroscience* **96**(1): 91-9.

Messersmith DJ, Gu J, Dubner R, Douglass J, Iadarola MJ (1994). Basal and inducible transcriptional activity of an upstream AP-1/CRE element (DYNCRE3) in the prodynorphin promoter. *Mol Cell Neurosci* **5**(3): 238-45.

Messersmith DJ, Kim DJ, Gu J, Dubner R, Iadarola MJ (1996). c-Jun activation of the DYNCRE3 site in the prodynorphin promoter. *Brain Res Mol Brain Res* **40**(1): 15-21.

Meyer RE (1994). Toward a comprehensive theory of alcoholism. *Ann N Y Acad Sci* **708**: 238-50.

Middlemas DS, Lindberg RA, Hunter T (1991). trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. *Mol Cell Biol* **11**(1): 143-53.

Middlemas DS, Meisenhelder J, Hunter T (1994). Identification of TrkB autophosphorylation sites and evidence that phospholipase C-gamma 1 is a substrate of the TrkB receptor. *J Biol Chem* **269**(7): 5458-66.

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M (2002). Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron* **36**(1): 121-37.

Mitchell JM, Liang MT, Fields HL (2005). A single injection of the kappa opioid antagonist norbinaltorphimine increases ethanol consumption in rats. *Psychopharmacology (Berl)* **182**(3): 384-92.

Miyamoto E (2006). Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus. *J Pharmacol Sci* **100**(5): 433-42.

Moller C, Wiklund L, Sommer W, Thorsell A, Heilig M (1997). Decreased experimental anxiety and voluntary ethanol consumption in rats following central but not basolateral amygdala lesions. *Brain Res* **760**(1-2): 94-101.

Mooney SM, Miller MW (2003). Ethanol-induced neuronal death in organotypic cultures of rat cerebral cortex. *Brain Res Dev Brain Res* **147**(1-2): 135-41.

Nagy A (2000). Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**(2): 99-109.

Naranjo JR, Mellstrom B, Achaval M, Sassone-Corsi P (1991). Molecular pathways of pain: Fos/Jun-mediated activation of a noncanonical AP-1 site in the prodynorphin gene. *Neuron* **6**(4): 607-17.

Narita M, Aoki K, Takagi M, Yajima Y, Suzuki T (2003). Implication of brain-derived neurotrophic factor in the release of dopamine and dopamine-related behaviors induced by methamphetamine. *Neuroscience* **119**(3): 767-75.

Nawa H, Carnahan J, Gall C (1995). BDNF protein measured by a novel enzyme immunoassay in normal brain and after seizure: partial disagreement with mRNA levels. *Eur J Neurosci* **7**(7): 1527-35.

Negus SS, Mello NK, Portoghese PS, Lin CE (1997). Effects of kappa opioids on cocaine self-administration by rhesus monkeys. *J Pharmacol Exp Ther* **282**(1): 44-55.

Numan S, Lane-Ladd SB, Zhang L, Lundgren KH, Russell DS, Seroogy KB, Nestler EJ (1998). Differential regulation of neurotrophin and trk receptor mRNAs in catecholaminergic nuclei during chronic opiate treatment and withdrawal. *J Neurosci* **18**(24): 10700-8.

O'Brien CP (1994). Treatment of alcoholism as a chronic disorder. *Alcohol* **11**(6): 433-7.

Oroszi G, Goldman D (2004). Alcoholism: genes and mechanisms. *Pharmacogenomics* **5**(8): 1037-48.

Ortiz J, Fitzgerald LW, Charlton M, Lane S, Trevisan L, Guitart X, Shoemaker W, Duman RS, Nestler EJ (1995). Biochemical actions of chronic ethanol exposure in the mesolimbic dopamine system. *Synapse* **21**(4): 289-98.

Ou LC, Gean PW (2006). Regulation of amygdala-dependent learning by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol-3-kinase. *Neuropsychopharmacology* **31**(2): 287-96.

Pandey SC, Roy A, Zhang H, Xu T (2004). Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. *J Neurosci* **24**(21): 5022-30.

Pandey SC, Zhang D, Mittal N, Nayyar D (1999). Potential role of the gene transcription factor cyclic AMP-responsive element binding protein in ethanol withdrawal-related anxiety. *J Pharmacol Exp Ther* **288**(2): 866-78.

Pandey SC, Zhang H, Roy A, Misra K (2006). Central and medial amygdaloid brain-derived neurotrophic factor signaling plays a critical role in alcohol-drinking and anxiety-like behaviors. *J Neurosci* **26**(32): 8320-31.

Panula P, Wu JY, Emson P (1981). Ultrastructure of GABA-neurons in cultures of rat neostriatum. *Brain Res* **219**(1): 202-7.

Partridge JG, Tang KC, Lovinger DM (2000). Regional and postnatal heterogeneity of activity-dependent long-term changes in synaptic efficacy in the dorsal striatum. *J Neurophysiol* **84**(3): 1422-9.

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* **16**(6): 1137-45.

Patterson SL, Grover LM, Schwartzkroin PA, Bothwell M (1992). Neurotrophin expression in rat hippocampal slices: a stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. *Neuron* **9**(6): 1081-8.

Pierce RC, Bari AA (2001). The role of neurotrophic factors in psychostimulant-induced behavioral and neuronal plasticity. *Rev Neurosci* **12**(2): 95-110.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA, Jr. (2001). Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. *J Neurosci* **21**(18): 7397-403.

Ploj K, Roman E, Gustavsson L, Nylander I (2000). Basal levels and alcohol-induced changes in nociceptin/orphanin FQ, dynorphin, and enkephalin levels in C57BL/6J mice. *Brain Res Bull* **53**(2): 219-26.

Pohorecky LA (1981). The interaction of alcohol and stress. A review. *Neurosci Biobehav Rev* **5**(2): 209-29.

Porrino LJ, Lyons D, Smith HR, Daunais JB, Nader MA (2004). Cocaine self-administration produces a progressive involvement of limbic, association, and sensorimotor striatal domains. *J Neurosci* **24**(14): 3554-62.

Przewlocka B, Turchan J, Lason W, Przewlocki R (1997). Ethanol withdrawal enhances the prodynorphin system activity in the rat nucleus accumbens. *Neurosci Lett* **238**(1-2): 13-6.

Pu L, Liu QS, Poo MM (2006). BDNF-dependent synaptic sensitization in midbrain dopamine neurons after cocaine withdrawal. *Nat Neurosci* **9**(5): 605-7.

Quilliam LA, Rebhun JF, Castro AF (2002). A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. *Prog Nucleic Acid Res Mol Biol* **71**: 391-444.

Rasmussen DD, Mitton DR, Green J, Puchalski S (2001). Chronic daily ethanol and withdrawal: 2. Behavioral changes during prolonged abstinence. *Alcohol Clin Exp Res* **25**(7): 999-1005.

Rassnick S, Heinrichs SC, Britton KT, Koob GF (1993). Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. *Brain Res* **605**(1): 25-32.

Reid M, Herrera-Marschitz M, Hokfelt T, Terenius L, Ungerstedt U (1988). Differential modulation of striatal dopamine release by intranigral injection of gamma-aminobutyric acid (GABA), dynorphin A and substance P. *Eur J Pharmacol* **147**(3): 411-20.

Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiol Behav* **84**(1): 53-63.

Rhodes JS, Ford MM, Yu CH, Brown LL, Finn DA, Garland T, Jr., Crabbe JC (2007). Mouse inbred strain differences in ethanol drinking to intoxication. *Genes Brain Behav* **6**(1): 1-18.

Rios M, Fan G, Fekete C, Kelly J, Bates B, Kuehn R, Lechan RM, Jaenisch R (2001). Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol Endocrinol* **15**(10): 1748-57.

Robbins TW, Everitt BJ (2002). Limbic-striatal memory systems and drug addiction. *Neurobiol Learn Mem* **78**(3): 625-36.

Roberts AJ, Heyser CJ, Koob GF (1999). Operant self-administration of sweetened versus unsweetened ethanol: effects on blood alcohol levels. *Alcohol Clin Exp Res* **23**(7): 1151-7.

Rodd-Henricks ZA, McKinzie DL, Murphy JM, McBride WJ, Lumeng L, Li TK (2000). The expression of an alcohol deprivation effect in the high-alcohol-drinking replicate rat lines is dependent on repeated deprivations. *Alcohol Clin Exp Res* **24**(6): 747-53.

Ron D, Vagts AJ, Dohrman DP, Yaka R, Jiang Z, Yao L, Crabbe J, Grisel JE, Diamond I (2000). Uncoupling of betaIIPKC from its targeting protein RACK1 in response to ethanol in cultured cells and mouse brain. *Faseb J* **14**(14): 2303-14.

Samson H, Falk JL (1975). Pattern of daily blood ethanol elevation and the development of physical dependence. *Pharmacol Biochem Behav* **3**(6): 1119-23.

Samson HH (1986). Initiation of ethanol reinforcement using a sucrose-substitution procedure in food- and water-sated rats. *Alcohol Clin Exp Res* **10**(4): 436-42.

Samson HH, Czachowski CL (2003). Behavioral measures of alcohol self-administration and intake control: rodent models. *Int Rev Neurobiol* **54**: 107-43.

Sanchis-Segura C, Spanagel R (2006). Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. *Addict Biol* **11**(1): 2-38.

Sandin J, Nylander I, Georgieva J, Schott PA, Ogren SO, Terenius L (1998). Hippocampal dynorphin B injections impair spatial learning in rats: a kappa-opioid receptor-mediated effect. *Neuroscience* **85**(2): 375-82.

Sanna PP, Simpson C, Lutjens R, Koob G (2002). ERK regulation in chronic ethanol exposure and withdrawal. *Brain Res* **948**(1-2): 186-91.

Sato M, Suzuki K, Nakanishi S (2001). NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. *J Neurosci* **21**(11): 3797-805.

Sauer B, Henderson N (1988). Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* **85**(14): 5166-70.

Saylor AJ, Meredith GE, Vercillo MS, Zahm DS, McGinty JF (2006). BDNF heterozygous mice demonstrate age-related changes in striatal and nigral gene expression. *Exp Neurol* **199**(2): 362-72.

Schenk S, Partridge B, Shippenberg TS (1999). U69593, a kappa-opioid agonist, decreases cocaine self-administration and decreases cocaine-produced drug-seeking. *Psychopharmacology (Berl)* **144**(4): 339-46.

Schenk S, Partridge B, Shippenberg TS (2001). Effects of the kappa-opioid receptor agonist, U69593, on the development of sensitization and on the maintenance of cocaine self-administration. *Neuropsychopharmacology* **24**(4): 441-50.

Schlussman SD, Zhou Y, Bailey A, Ho A, Kreek MJ (2005). Steady-dose and escalating-dose "binge" administration of cocaine alter expression of behavioral stereotypy and striatal preprodynorphin mRNA levels in rats. *Brain Res Bull* **67**(3): 169-75.

Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **285**(5433): 1569-72.

Segal M, Greenberger V, Korkotian E (2003). Formation of dendritic spines in cultured striatal neurons depends on excitatory afferent activity. *Eur J Neurosci* **17**(12): 2573-85.

Segal RA (2003). Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci* **26**: 299-330.

Seizinger BR, Bovermann K, Maysinger D, Hollt V, Herz A (1983). Differential effects of acute and chronic ethanol treatment on particular opioid peptide systems in discrete regions of rat brain and pituitary. *Pharmacol Biochem Behav* **18 Suppl 1**: 361-9.

Self DW (2004). Regulation of drug-taking and -seeking behaviors by neuroadaptations in the mesolimbic dopamine system. *Neuropharmacology* **47 Suppl 1**: 242-55.

Self DW, McClenahan AW, Beitner-Johnson D, Terwilliger RZ, Nestler EJ (1995). Biochemical adaptations in the mesolimbic dopamine system in response to heroin self-administration. *Synapse* **21**(4): 312-8.

Sharpe AL, Tsivkovskaia NO, Ryabinin AE (2005). Ataxia and c-Fos expression in mice drinking ethanol in a limited access session. *Alcohol Clin Exp Res* **29**(8): 1419-26.

Shaywitz AJ, Greenberg ME (1999). CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* **68**: 821-61.

Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, Phillips HS, Carroll K, Spencer SD, Levinson AD (1995). Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. *J Neurosci* **15**(1 Pt 2): 477-91.

Shi X, McGinty JF (2006). Extracellular signal-regulated mitogen-activated protein kinase inhibitors decrease amphetamine-induced behavior and neuropeptide gene expression in the striatum. *Neuroscience* **138**(4): 1289-98.

Shippenberg TS, LeFevour A, Heidbreder C (1996). kappa-Opioid receptor agonists prevent sensitization to the conditioned rewarding effects of cocaine. *J Pharmacol Exp Ther* **276**(2): 545-54.

Signs SA, Yamamoto BK, Schechter MD (1987). In vivo electrochemical determination of extracellular dopamine in the caudate of freely-moving rats after a low dose of ethanol. *Neuropharmacology* **26**(11): 1653-6.

Simmons ML, Chavkin C (1996). Endogenous opioid regulation of hippocampal function. *Int Rev Neurobiol* **39**: 145-96.

Simpson CS, Morris BJ (1995). Induction of c-fos and zif/268 gene expression in rat striatal neurons, following stimulation of D1-like dopamine receptors, involves protein kinase A and protein kinase C. *Neuroscience* **68**(1): 97-106.

Smith AJ, McGinty JF (1994). Acute amphetamine or methamphetamine alters opioid peptide mRNA expression in rat striatum. *Brain Res Mol Brain Res* **21**(3-4): 359-62.

Sohrabji F, Miranda RC, Toran-Allerand CD (1995). Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. *Proc Natl Acad Sci U S A* **92**(24): 11110-4.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, et al. (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell* **65**(5): 895-903.

Spanagel R (1996). The influence of opioid antagonists on the discriminative stimulus effects of ethanol. *Pharmacol Biochem Behav* **54**(4): 645-9.

Spanagel R, Herz A, Shippenberg TS (1990). The effects of opioid peptides on dopamine release in the nucleus accumbens: an in vivo microdialysis study. *J Neurochem* **55**(5): 1734-40.

Spanagel R, Herz A, Shippenberg TS (1992). Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. *Proc Natl Acad Sci U S A* **89**(6): 2046-50.

Spangler R, Unterwald EM, Kreek MJ (1993). 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudate-putamen. *Brain Res Mol Brain Res* **19**(4): 323-7.

Steiner H, Gerfen CR (1998). Role of dynorphin and enkephalin in the regulation of striatal output pathways and behavior. *Exp Brain Res* **123**(1-2): 60-76.

Surmeier DJ, Ding J, Day M, Wang Z, Shen W (2007). D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. *Trends Neurosci* **30**(5): 228-35.

Surmeier DJ, Song WJ, Yan Z (1996). Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. *J Neurosci* **16**(20): 6579-91.

Suzuki T, Shiozaki Y, Masukawa Y, Misawa M, Nagase H (1992). The role of mu- and kappa-opioid receptors in cocaine-induced conditioned place preference. *Jpn J Pharmacol* **58**(4): 435-42.

Svingos AL, Chavkin C, Colago EE, Pickel VM (2001). Major coexpression of kappa-opioid receptors and the dopamine transporter in nucleus accumbens axonal profiles. *Synapse* **42**(3): 185-92.

Svingos AL, Colago EE, Pickel VM (1999). Cellular sites for dynorphin activation of kappa-opioid receptors in the rat nucleus accumbens shell. *J Neurosci* **19**(5): 1804-13.

Szumlinski KK, Diab ME, Friedman R, Henze LM, Lominac KD, Bowers MS (2007). Accumbens neurochemical adaptations produced by binge-like alcohol consumption. *Psychopharmacology (Berl)* **190**(4): 415-31.

Tang M, Falk JL (1983). Production of physical dependence on ethanol by a short drinking episode each day. *Pharmacol Biochem Behav* **19**(1): 53-5.

Tapia-Arancibia L, Rage F, Givalois L, Dingeon P, Arancibia S, Beauge F (2001). Effects of alcohol on brain-derived neurotrophic factor mRNA expression in discrete regions of the rat hippocampus and hypothalamus. *J Neurosci Res* **63**(2): 200-8.

Teyler TJ, Discenna P (1984). Long-term potentiation as a candidate mnemonic device. *Brain Res* **319**(1): 15-28.

The Gallup Organization (2007). The Gallup Poll Annual Surveys on Alcohol and Drinking, 1939-2007. As found at http://www.galluppoll.com/content/default.aspx?ci=1582.

Thompson AC, Zapata A, Justice JB, Jr., Vaughan RA, Sharpe LG, Shippenberg TS (2000). Kappa-opioid receptor activation modifies dopamine uptake in the nucleus accumbens and opposes the effects of cocaine. *J Neurosci* **20**(24): 9333-40.

Todtenkopf MS, Marcus JF, Portoghese PS, Carlezon WA, Jr. (2004). Effects of kappa-opioid receptor ligands on intracranial self-stimulation in rats. *Psychopharmacology (Berl)* **172**(4): 463-70.

Tsankova N, Renthal W, Kumar A, Nestler EJ (2007). Epigenetic regulation in psychiatric disorders. *Nat Rev Neurosci* **8**(5): 355-67.

Turchan J, Lason W, Budziszewska B, Przewlocka B (1997). Effects of single and repeated morphine administration on the prodynorphin, proenkephalin and dopamine D2 receptor gene expression in the mouse brain. *Neuropeptides* **31**(1): 24-8.

Turchan J, Przewlocka B, Lason W, Przewlocki R (1998). Effects of repeated psychostimulant administration on the prodynorphin system activity and kappa opioid receptor density in the rat brain. *Neuroscience* **85**(4): 1051-9.

Turrigiano G (2007). Homeostatic signaling: the positive side of negative feedback. *Curr Opin Neurobiol* **17**(3): 318-24.

Tzschentke TM (1998). Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog Neurobiol* **56**(6): 613-72.

Uhl GR, Liu QR, Walther D, Hess J, Naiman D (2001). Polysubstance abuse-vulnerability genes: genome scans for association, using 1,004 subjects and 1,494 single-nucleotide polymorphisms. *Am J Hum Genet* **69**(6): 1290-300.

Vagts AJ, He DY, Yaka R, Ron D (2003). Cellular adaptation to chronic ethanol results in altered compartmentalization and function of the scaffolding protein RACK1. *Alcohol Clin Exp Res* **27**(10): 1599-605.

Valjent E, Corvol JC, Pages C, Besson MJ, Maldonado R, Caboche J (2000). Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *J Neurosci* **20**(23): 8701-9.

Valjent E, Pages C, Herve D, Girault JA, Caboche J (2004). Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. *Eur J Neurosci* **19**(7): 1826-36.

Volkow ND, Wang GJ, Telang F, Fowler JS, Logan J, Childress AR, Jayne M, Ma Y, Wong C (2006). Cocaine cues and dopamine in dorsal striatum: mechanism of craving in cocaine addiction. *J Neurosci* **26**(24): 6583-8.

Voorn P, Vanderschuren LJ, Groenewegen HJ, Robbins TW, Pennartz CM (2004). Putting a spin on the dorsal-ventral divide of the striatum. *Trends Neurosci* **27**(8): 468-74.

Vrublevsky AG (1994). Variants of alcoholism: patterns in development, course, and prognosis. *Ann N Y Acad Sci* **708**: 86-96.

Wagner JJ, Terman GW, Chavkin C (1993). Endogenous dynorphins inhibit excitatory neurotransmission and block LTP induction in the hippocampus. *Nature* **363**(6428): 451-4.

Walker BM, Koob GF (2007). Pharmacological Evidence for a Motivational Role of kappa-Opioid Systems in Ethanol Dependence. *Neuropsychopharmacology*.

Wang JQ, Smith AJ, McGinty JF (1995). A single injection of amphetamine or methamphetamine induces dynamic alterations in c-fos, zif/268 and preprodynorphin messenger RNA expression in rat forebrain. *Neuroscience* **68**(1): 83-95.

Weiss F, Porrino LJ (2002). Behavioral neurobiology of alcohol addiction: recent advances and challenges. *J Neurosci* **22**(9): 3332-7.

Williams KL, Woods JH (1998). Oral ethanol-reinforced responding in rhesus monkeys: effects of opioid antagonists selective for the mu-, kappa-, or delta-receptor. *Alcohol Clin Exp Res* **22**(8): 1634-9.

Williams TJ, Laforge KS, Gordon D, Bart G, Kellogg S, Ott J, Kreek MJ (2007). Prodynorphin gene promoter repeat associated with cocaine/alcohol codependence. *Addict Biol*.

Winkler A, Spanagel R (1998). Differences in the kappa opioid receptor mRNA content in distinct brain regions of two inbred mice strains. *Neuroreport* **9**(7): 1459-64.

Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF (2000). The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. *J Neurosci* **20**(18): 6888-97.

Xuei X, Dick D, Flury-Wetherill L, Tian HJ, Agrawal A, Bierut L, Goate A, Bucholz K, Schuckit M, Nurnberger J, Jr., Tischfield J, Kuperman S, Porjesz B, Begleiter H, Foroud T, Edenberg HJ (2006). Association of the kappa-opioid system with alcohol dependence. *Mol Psychiatry* **11**(11): 1016-24.

Yaka R, He DY, Phamluong K, Ron D (2003). Pituitary adenylate cyclase-activating polypeptide (PACAP(1-38)) enhances N-methyl-D-aspartate receptor function and brain-derived neurotrophic factor expression via RACK1. *J Biol Chem* **278**(11): 9630-8.

Yan QS, Feng MJ, Yan SE (2005). Different expression of brain-derived neurotrophic factor in the nucleus accumbens of alcohol-preferring (P) and -nonpreferring (NP) rats. *Brain Res* **1035**(2): 215-8.

Yin HH, Knowlton BJ (2006). The role of the basal ganglia in habit formation. *Nat Rev Neurosci* **7**(6): 464-76.

Yin HH, Knowlton BJ, Balleine BW (2004). Lesions of dorsolateral striatum preserve outcome expectancy but disrupt habit formation in instrumental learning. *Eur J Neurosci* **19**(1): 181-9.

Yin HH, Knowlton BJ, Balleine BW (2006). Inactivation of dorsolateral striatum enhances sensitivity to changes in the action-outcome contingency in instrumental conditioning. *Behav Brain Res* **166**(2): 189-96.

Yin HH, Ostlund SB, Knowlton BJ, Balleine BW (2005). The role of the dorsomedial striatum in instrumental conditioning. *Eur J Neurosci* **22**(2): 513-23.

Yin HH, Park BS, Adermark L, Lovinger DM (2007). Ethanol reverses the direction of long-term synaptic plasticity in the dorsomedial striatum. *Eur J Neurosci* **25**(11): 3226-32.

You ZB, Herrera-Marschitz M, Terenius L (1999). Modulation of neurotransmitter release in the basal ganglia of the rat brain by dynorphin peptides. *J Pharmacol Exp Ther* **290**(3): 1307-15.

Yuen EC, Mobley WC (1999). Early BDNF, NT-3, and NT-4 signaling events. *Exp Neurol* **159**(1): 297-308.

Zapata A, Gonzales RA, Shippenberg TS (2006). Repeated ethanol intoxication induces behavioral sensitization in the absence of a sensitized accumbens dopamine response in C57BL/6J and DBA/2J mice. *Neuropsychopharmacology* **31**(2): 396-405.

Zapata A, Shippenberg TS (2006). Endogenous kappa opioid receptor systems modulate the responsiveness of mesoaccumbal dopamine neurons to ethanol. *Alcohol Clin Exp Res* **30**(4): 592-7.

Zhang D, Zhang L, Lou DW, Nakabeppu Y, Zhang J, Xu M (2002). The dopamine D1 receptor is a critical mediator for cocaine-induced gene expression. *J Neurochem* **82**(6): 1453-64.

Zhang H, Pandey SC (2003). Effects of PKA modulation on the expression of neuropeptide Y in rat amygdaloid structures during ethanol withdrawal. *Peptides* **24**(9): 1397-402.

Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ (2004). Effect of the endogenous kappa opioid agonist dynorphin A(1-17) on cocaine-evoked increases in striatal dopamine levels and cocaine-induced place preference in C57BL/6J mice. *Psychopharmacology (Berl)* **172**(4): 422-9.

Zhou Y, Spangler R, Schlussman SD, Yuferov VP, Sora I, Ho A, Uhl GR, Kreek MJ (2002). Effects of acute "binge" cocaine on preprodynorphin, preproenkephalin, proopiomelanocortin, and corticotropin-releasing hormone receptor mRNA levels in the striatum and hypothalamic-pituitary-adrenal axis of mu-opioid receptor knockout mice. *Synapse* **45**(4): 220-9.

Zimprich A, Kraus J, Woltje M, Mayer P, Rauch E, Hollt V (2000). An allelic variation in the human prodynorphin gene promoter alters stimulus-induced expression. *J Neurochem* **74**(2): 472-7.

APPENDIX

APPENDIX A: LIST OF COMMON ABBREVIATIONS

Molecular Signaling Components

Abbreviation	Full Name
AP-1	activator protein 1
BDNF	brain-derived neurotrophic factor
CaMK	calcium and calmodulin-dependent protein kinase
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
ERK1/2	extracellular signal-regulated kinases 1&2
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor-bound protein 2
KOR	kappa opioid receptor
MAPK	mitogen-activated protein kinase
MEK1/2	MAPK and ERK kinases 1&2
Pdyn	*preprodynorphin*
PI3K	phosphoinositide 3-kinase
PKA	cyclic AMP-dependent protein kinase
PLC-γ	phospholipase C-γ
RACK1	receptor for activated C kinase 1
RSK	ribosomal S6 kinase
SOS	son of sevenless
TH	tyrosine hydroxylase
TrkB	tropomyocin-related kinase B

Key Brain Regions:

Abbreviation	Full Name
SN	substantia nigra
SNc	pars compacta
SNr	pars reticulata
VTA	ventral tegmental area
NAc	nucleus accumbens
DLS	dorsolateral striatum
DMS	dorsomedial striatum
PFC	prefrontal cortex
DIV	days *in vitro*
CPu	caudate/putamen (striatum in primates)

Miscellaneous

Abbreviation	Full Name
CaMKcre	mice expressing Cre driven by the CaMKIIα promoter
cKO	conditional knockout
CPA	conditioned place aversion
CPP	conditioned place preference
DAPI	diamidino-2-phenylindole, a nuclear marker
GAD-67	glutamic acid decarboxylase, 67kDa isoform
GAPDH	*glyceraldehyde 3-phosphate dehydrogenase*
i.p.	intraperitoneal route of injection
KO	knockout
LTD	long-term depression
LTP	long-term potentiation
MAP2	microtubule-associated protein 2, a neuronal marker
nor-BNI	nor-binaltorphimine, a kappa receptor antagonist
U50	U50,488H,a kappa receptor agonist
WT	wild-type

APPENDIX B: MOUSE BRAIN DISSECTION DIAGRAMS

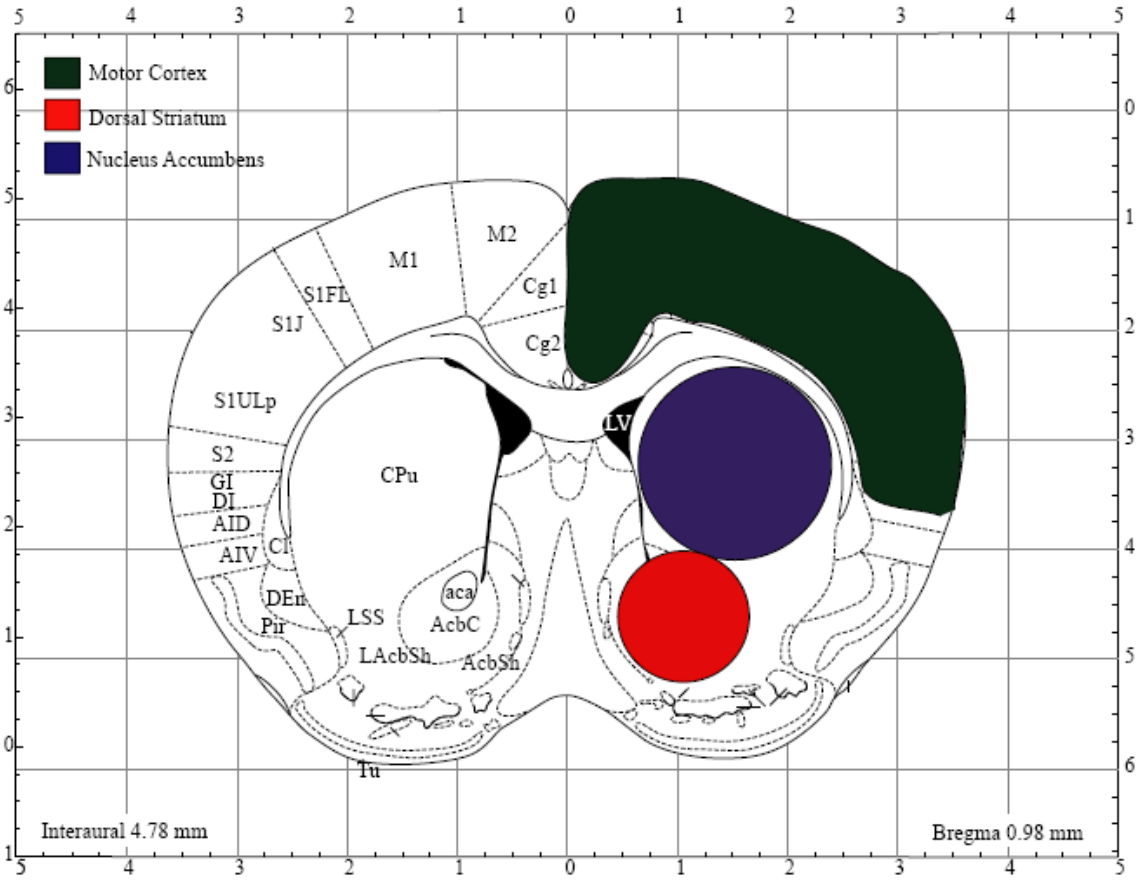


Figure A.1. Mouse forebrain regions microdissected for mRNA analysis.

Diagram depicts regions isolated for Motor Cortex (green), Dorsal Striatum (blue) and Nucleus Accumbens (red) mRNA determination. Dorsal striatum and nucleus accumbens were isolated via tissue punch of a frontal slice approximately 2 mm in width, beginning just posterior to the prefrontal cortical regions and extending to the region immediately anterior to the hypothalamus. The section shown here is the approximate center of the 2 mm slice used. The anterior commissure was used as a marker to select the NAc punch, and dorsal striatal punches were isolated from the region dorsal to the NAc and ventral to the corpus callosum. Following removal of the NAc and dorsal striatum, cortical regions were microdissected dorsal to the corpus callosum; the most ventral regions of cortex were generally removed to prevent inclusion of striatal regions remaining after tissue punch. Note that this resulted in the exclusion of most insular and piriform cortical regions. Abbreviations: aca: anterior commissure; AcbC: nucleus accumbens core; AcbSh: nucleus accumbens shell; AID: dorsal agranular insular cortex; AIV: ventral agranular insular cortex; Cg1/2: cingulated cortex areas 1 & 2; CPu: caudate/putamen; DEn: dorsal endopiriform nucleus; DI: dysgranular insular cortex; GI: granular insular cortex; LAcbSh: lateral nucleus accumbens shell; LSS: lateral stripe of the striatum; LV: lateral ventricle; M1: primary motor cortex; M2: secondary motor cortex; Pir: piriform cortex; S1: primary somatosensory cortical regions (FL = forelimb, J = jaw; ULp = upper lip); S2 = secondary somatosensory cortex.

Figures are reproduced with permission from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2004).

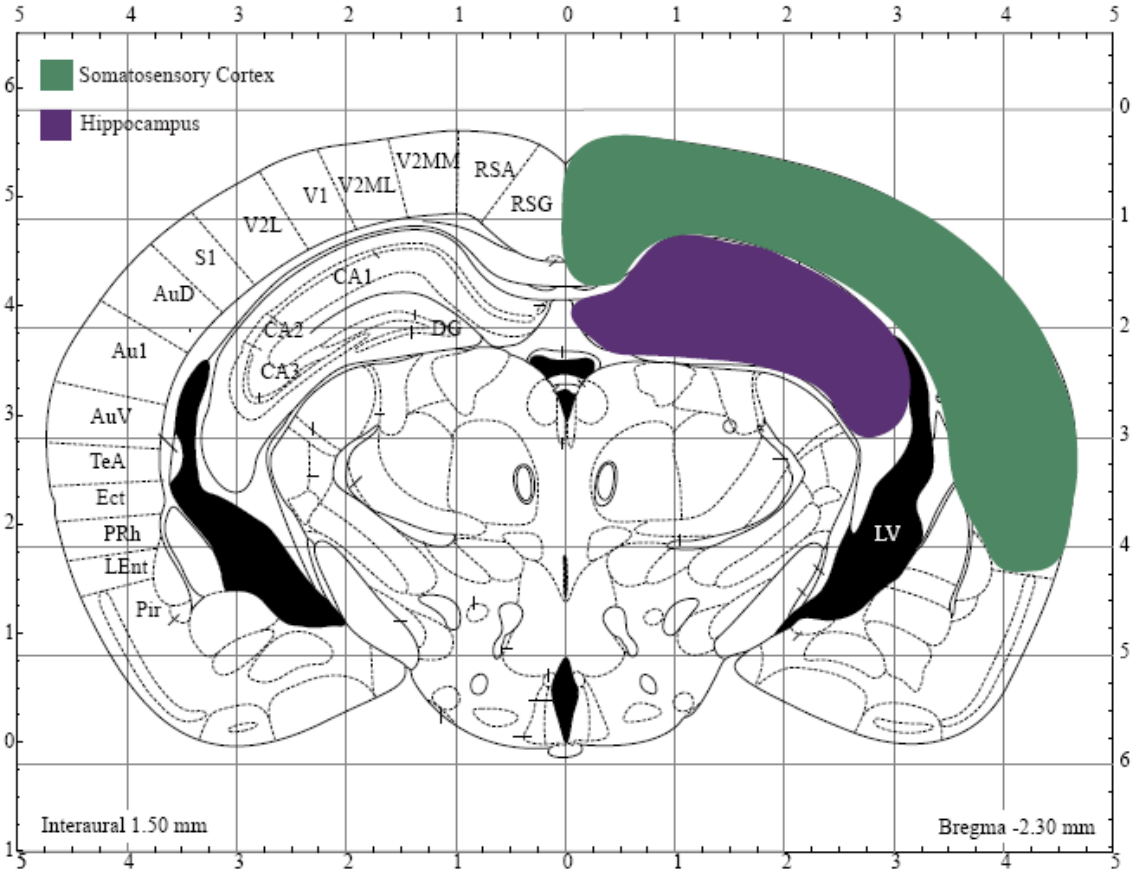


Figure A.2. Mouse hindbrain regions microdissected for mRNA analysis.

Diagram depicts regions isolated for Somatosensory Cortex (green) and Hippocampus (purple) mRNA determination. Hindbrain regions were isolated from the posterior half of the brain following transsection just anterior to the hypothalamus. A small incision was performed in line with the hemispheric division such that cortex and hippocampus could be folded outward and separated from the central portion of the brain and cerebellum. The hippocampal section was removed from the internal face of the section, and the remainder kept as Somatosensory Cortex, as shown by the shaded region. Abbreviations: Au1: primary auditory cortex; AuD: dorsal region of secondary auditory cortex; AuV: ventral area of secondary auditory cortex; CA1, CA2, CA3: fields CA1 – CA3 of the hippocampus; DG: dentate gyrus; Ect: ectorhinal cortex; LEnt: lateral entorhinal cortex; LV: lateral ventricle; Pir: piriform cortex; PRh: perirhinal cortex; RSA: retrosplenial agranular cortex; RSG: retrosplenial granular cortex; S1: primary somatosensory cortex; TeA: temporal association cortex; V1: primary visual cortex; V2L: lateral area of secondary visual cortex; V2ML: mediolateral area of secondary visual cortex; V2MM: medial area of secondary visual cortex.

Figures are reproduced with permission from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2004).

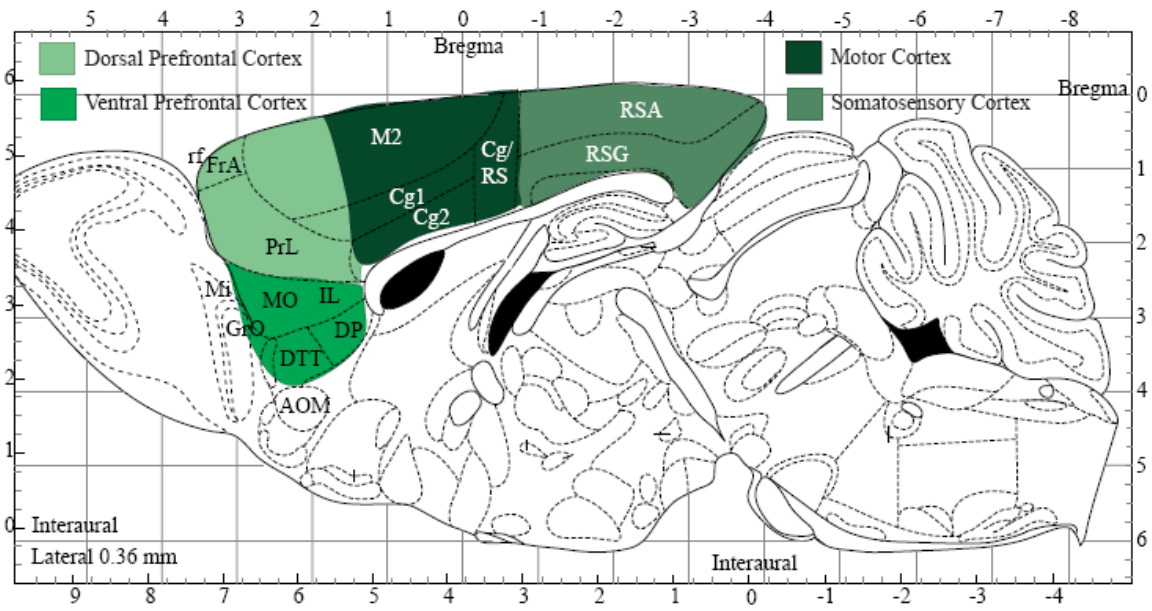


Figure A.3. Delineation of cortical regions included in the four primary subdivisions separated for mRNA analysis.

Diagram depicts regions isolated for Dorsal Prefrontal Cortex, Ventral Prefrontal Cortex, Motor Cortex and Somatosensory Cortex mRNA determination, as indicated. Motor and somatosensory cortices were isolated as described in figures S1 and S2. To obtain prefrontal regions, brains were dissected as described in A.1 to remove prefrontal regions from the remainder of the forebrain. A diagonal cut was made to remove the olfactory bulbs and a coronal transection made approximately in the middle of the section to separate the dorsal and ventral subdivisions of the prefrontal cortex. The cortical subdivisions contain the following regions, as shown on the diagram: Dorsal Prefrontal Cortex: frontal association cortex (FrA), prelimbic cortex (PrL), and portions of secondary motor cortex (M2) and area 1 of cingulate cortex; Ventral Prefrontal Cortex: orbital cortex (MO), infralimbic cortex (IL), dorsal peduncular cortex (DP), dorsal tenia tecta (DTT) and possibly portions of the anterior olfactory nucleus (AOM), depending on the angle of slice performed to remove the olfactory bulbs; Motor Cortex: primary and secondary motor cortex (M1 and M2), cingulate cortex areas 1 and 2 (Cg1 and 2) as well as the cingulate/retrosplenial cortex (Cg/RS) and additional regions detailed in Figure A.1; Somatosensory Cortex: retrosplenial agranular (RSA) and granular (RSG) cortices, as well as the more lateral sensory regions detailed in Figure A.2. Note that the figure depicts the medial aspect of these sections; thus in description of contents, the labels “medial” have been removed as the brain sections also included the more lateral aspects not shown in this diagram.

Figures are reproduced with permission from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2004).

APPENDIX C: REGRESSION ANALYSIS OF ETHANOL INTAKE AND CORTICAL *BDNF* EXPRESSION AFTER BREAKDOWN OF HOMEOSTASIS

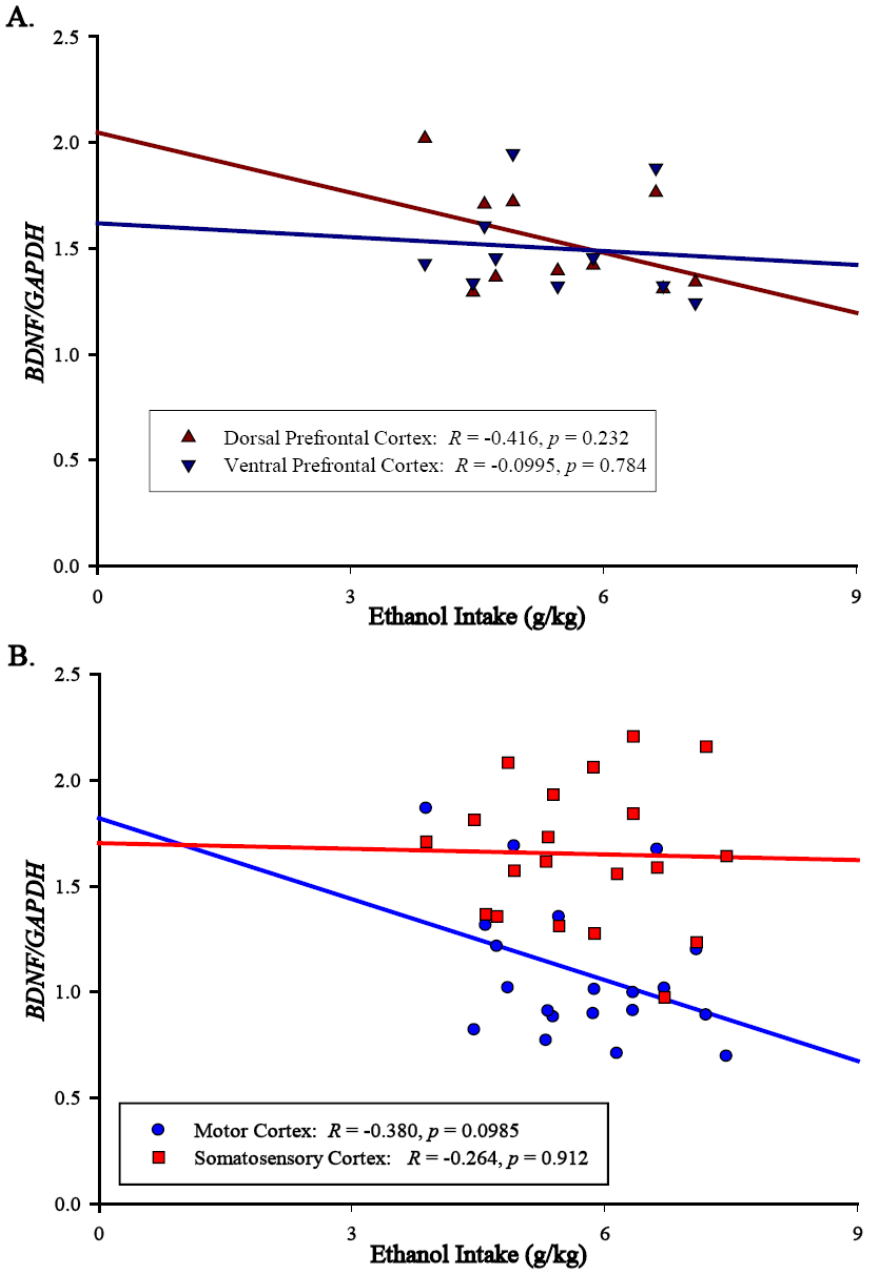


Figure A.4. Regression plots of ethanol intake and cortical *BDNF* expression following 6 weeks' limited access to ethanol.

C57BL/6J mice were provided with two-bottle choice access to 10% ethanol and water for 4 h per day over a six week period. Mice were sacrificed and brains microdissected immediately after the final ethanol access period. Regression plots show *BDNF* expression, calculated as a ratio to *GAPDH* levels, as a function of ethanol consumed, expressed as grams ethanol per kg body weight over 4 h. (A) *BDNF* in the dorsal PFC shows a greater inverse correlation with quantity of ethanol consumed during the final 4 h period than that in the ventral PFC. n = 10 per brain region. (B) *BDNF* in the motor cortex shows very high negative correlation with ethanol intake, while *BDNF* in the somatosensory cortex shows no direct correspondence to quantity of ethanol consumed. n = 20 per brain region. Note that *BDNF* expression in dorsal PFC and motor cortex are very highly correlated with one another ($R = 0.890$, $p < 0.001$, n = 10).

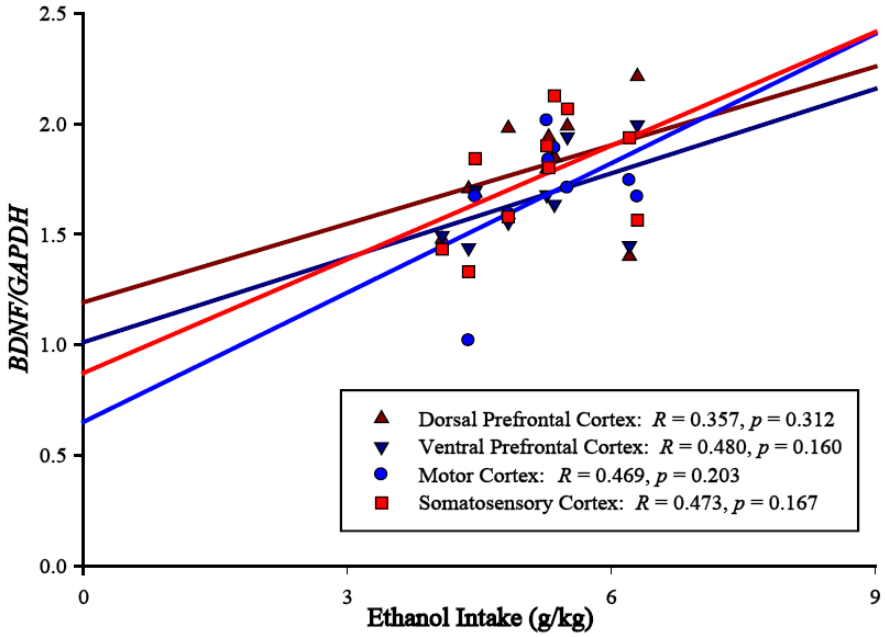


Figure A.5. Regression plots of ethanol intake and cortical *BDNF* expression after 24-hour withdrawal from 6 weeks' limited access to ethanol.

C57BL/6J mice were provided with two-bottle choice access to 10% ethanol and water for 4 h per day over a six week period. Mice were sacrificed and brains microdissected 24 h after the final ethanol access period. Regression plots show *BDNF* expression, calculated as a ratio to *GAPDH* levels, as a function of ethanol consumed, expressed as grams ethanol per kg body weight over 4 h. All cortical regions show positive correlations to quantity consumed 24 h prior to sacrifice; however, none are significant.. n = 10 per brain region. Note that 24 h into withdrawal, *BDNF* expression in dorsal and ventral PFC are highly correlated with one another ($R = 0.795$, $p < 0.01$, n = 10).

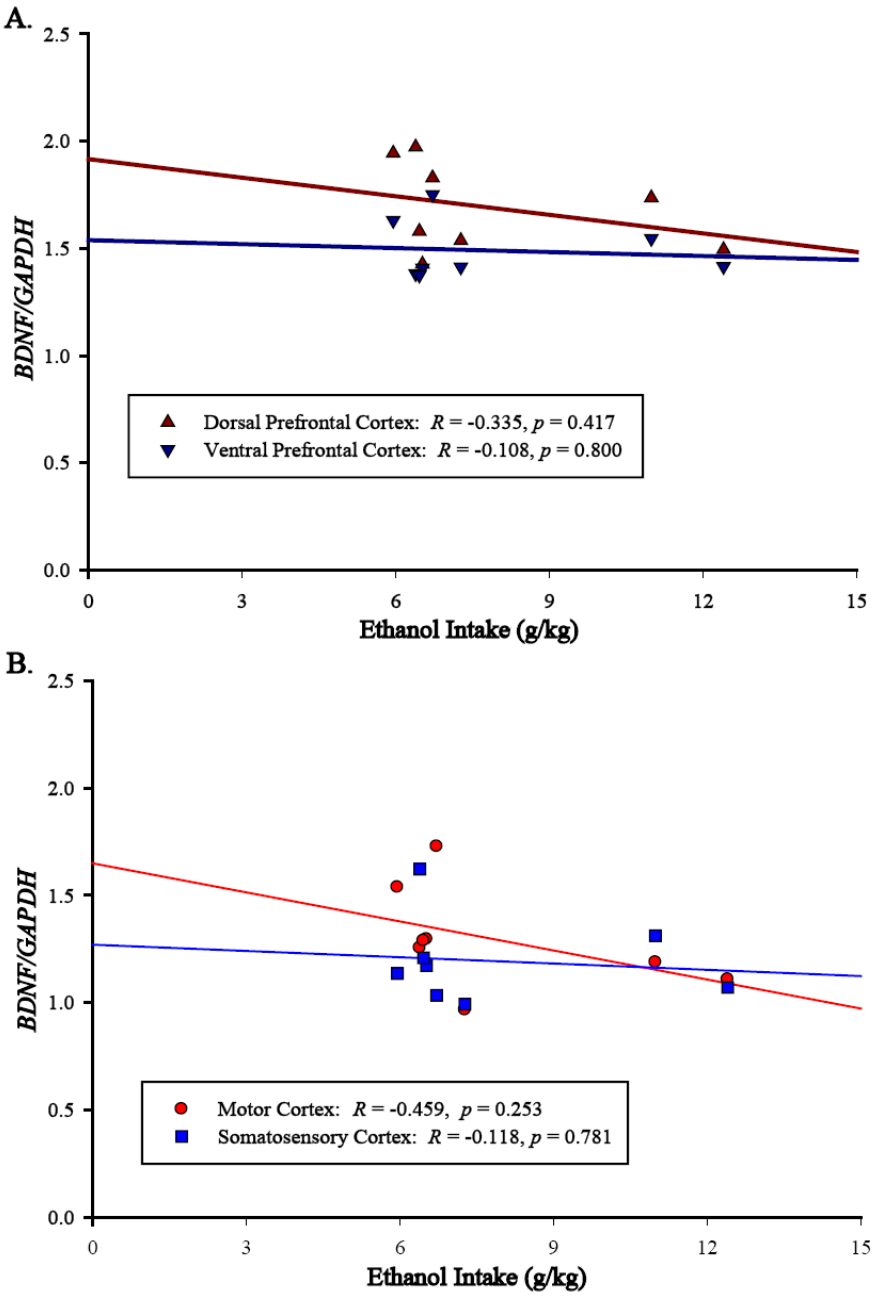


Figure A.6. Regression plots of ethanol intake and cortical *BDNF* expression after a single renewal session subsequent to 2 weeks' ethanol deprivation.

C57BL/6J mice were provided with two-bottle choice access to 10% ethanol and water for 4 h per day over an eight week period, then deprived of ethanol for 2 weeks. On the final day the mice were given a single 4 h renewal session, immediately after which they were sacrificed and brains microdissected. Regression plots show *BDNF* expression, calculated as a ratio to *GAPDH* levels, as a function of ethanol consumed, expressed as grams ethanol per kg body weight over 4 h. (A) *BDNF* in the dorsal PFC shows a slight negative correlation with ethanol consumed, while the ventral PFC shows no correlation. n = 8 per brain region. (B) *BDNF* in the motor cortex shows a slight inverse correlation with ethanol intake, while *BDNF* in the somatosensory cortex shows no correlation to quantity of ethanol consumed. n = 8 per brain region. Note that here *BDNF* expression in ventral PFC and motor cortex are highly correlated with one another ($R = 0.798$, $p < 0.05$, n = 8).

REFERENCE

Paxinos G, Franklin KBJ (2004). *The mouse brain in stereotaxic coordinates*. Amsterdam; Boston, Elsevier Academic Press.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses and dissertations. Copies of all UCSF theses and dissertations will be routed to the library via the Graduate Division. The library will make all theses and dissertations accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis or dissertation to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature

12/11/07

Date