Title

Glial cell line-derived neurotrophic factor is a novel alcohol-responsive gene: Implications for the treatment of alcohol addiction

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Publication Date

2012

Peer reviewed|Thesis/dissertation

Glial cell line-derived neurotrophic factor is a novel alcohol-responsive gene: Implications for the treatment of alcohol addiction

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

For my family.

Acknowledgements

I would be remiss if I didn't first thank my mentor, Dorit Ron. I am indebted to her for shaping my scientific view of the world, and for always encouraging me to ask (and to try to answer) the important questions. Dorit, as you always say, "It was worth it." I know I wouldn't be here if you hadn't pushed me to believe in myself. For this, I am, and will be, eternally grateful.

I also thank the faculty members on both my thesis and qualifying exam committees who dedicated much of their precious time critiquing and advising my research and future career options, as well as (sometimes) pondering life's many existential quandries: Robert Messing, Mark Von Zastrow, Benjamin Cheyette, Su Guo, and Ying-Hui Fu. Your combined knowledge humbles me, and I deeply appreciate having had the chance to pick your brains.

To my high school teachers and University of Nevada professors and mentors who inspired me (or rather, didn't discourage me) from pursuing a career in science, to Don Moses, Ellen Baker, David Schooley, Kathy Schegg, and Carol Condit: a sincere Thank You. I am also profoundly grateful to my labmates, past and present, for their patience and their willingness to share their cloning tricks, buffer recipes, research opinions, and editorial suggestions. My GDNF project partners, Sebastien Carnicella, Segev Barak, Jun Wang, and Quinn Yowell; and my go-to biochemistry and molecular biology gurus, Stuart Gibb, Dao-Yao He, Khanky Phamluong, and Jeremie Neasta have all contributed invaluable benchside knowledge to my training, without which I would have been continually spinning my wheels and going nowhere. My classmates, Stacy Musone, Jason Fernandes, Howard Horng, Melissa Calton, Michael Hicks, and Jennifer

Yokoyama are irreplaceable fixtures of my graduate school experience, and their input, companionship, and encouragement are among the best things I could have ever gotten out of this journey.

Many heartfelt thanks to my friends: April and Matthew Haas, Elissa Pope, Nicole and Chris Franklin, Laura and Paul Karayan, Rowena Suriben, and Andrew Lee. Special recognition is due to Marian Logrip. As Dorit's only other student, as well as my lab- bench- school- *and* room-mate, she and I share a very special bond. Marian, you are the older sister I never had, and navigating the murky waters of grad school would have been impossible without you. And that's a fact; $***p < 0.001$.

I must recognize the contribution of my family to this crazy adventure. I almost gave up. A lot. My parents pulled me back from the brink, every time. I love you both. Thank you for bringing me into this world, instilling the importance of an education into the core of my very being, and continuing to pick up the check every once in a while. My little sister, my best friend #1, the other (cuter, funnier!) half of me, I can never thank you enough for commiserating with me. I love you, silly girl.

And, to my best friend #2, my husband, my partner, my love, my Ryan: we make a good team, you and I; a most powerful new force. Your unwavering support from the very beginning of my grad school journey, when you still had every opportunity to back out, no strings attached, means that this dissertation is as much yours as it is mine.

Somayeh Ahmadiantehrani
September 2011

Funding and Co-Author Acknowledgements

Support for this work and my graduate career was provided by grants from the National Institute of Alcohol Abuse and Alcoholism: R01 AA014366 (D.R.) and F31 AA017801 (S.A.), and the National Institutes of Health T32 GM07175 (PSPG program); the Merck Research Fellow Award (S.A.); and funds from the state of California for medical research on alcohol and substance abuse through UCSF (D.R.).

The work presented in **Chapter 2** represents a collaborative study with co-author Segev Barak (Ahmadiantehrani, S.; S. Barak; and D. Ron. *In preparation*.). The RT- and qRT-PCR analyses, adenovirus preparation, and alcohol consumption training were conducted by Somayeh Ahmadiantehrani. Co-author Segev Barak performed the adenovirus infusions and associated alcohol drinking measurements. The manuscript is written by Somayeh Ahmadiantehrani. Dorit Ron supervised the study.

The pShuttle2-mCherry construct and the [^{3}H]-dopamine release assay protocol described in **Chapter 4** were developed and optimized by Stuart L. Gibb.

The data and figures presented in **Chapter 5** are reproduced here from a published study (Carnicella, S.; S. Ahmadiantehrani; D.Y. He; C.K. Nielsen; S.E. Bartlett; P.H. Janak; and D. Ron (2009). Cabergoline decreases alcohol drinking and seekingbehaviors via glial cell line-derived neurotrophic factor. Biological Psychiatry 66:146-153.) First author Sebastien Carnicella performed the behavioral experiments and wrote the manuscript. The *in vitro* RT-PCR, Western blot, and ELISA analyses were conducted by Somayeh Ahmadiantehrani. Co-authors Dao-Yao He and Carsten K. Nielsen performed the *in vivo* RT-PCR analysis and the [^{35}S]GTPγS binding assay, respectively. Selena A. Bartlett, Patricia H. Janak, and Dorit Ron supervised the study. The text of this chapter, while based on the published work, has been rewritten for this dissertation.

GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR IS A NOVEL ALCOHOL-RESPONSIVE GENE: IMPLICATIONS FOR THE TREATMENT OF ALCOHOL ADDICTION

by
Somayeh Ahmadiantehrani

Abstract

Alcohol abuse affects millions of people worldwide, and there is an alarming lack of efficient treatments for this disease. We previously identified a growth factor, glial cell line-derived neurotrophic factor (GDNF), that acts in the ventral tegmental area (VTA) to reduce alcohol drinking, seeking, and relapse in rodents (Carnicella et al., 2008), suggesting that targeting GDNF and/or its signaling pathway is a potential treatment for alcoholism. I set out to characterize the relationship between alcohol and GDNF, as well as its signaling pathway and its implications for the treatment of alcohol addiction.

Using rodent models, I identify *GDNF* as a novel alcohol-responsive gene in the VTA. I show that early alcohol drinking induces *GDNF* expression, which regulates the escalation of excessive drinking. I also demonstrate that after a long history of excessive drinking, *GDNF* levels are abnormally decreased, possibly contributing to the motivation to continue drinking. I also detail two putative molecular mechanisms involving the Zif268 and Pitx3 transcription factors, which may underlie the alcohol-mediated changes in endogenous *GDNF* expression in the VTA.

I next outline the identification of protein targets of the GDNF signaling pathway that may mitigate GDNF's anti-alcohol actions. I identify the synaptic vesicle protein,

Synapsin I as a novel target of GDNF signaling, which may play a role in dopamine release, consequently affecting alcohol drinking.

Finally, I include the results of a collaborative study in which we demonstrate that pharmacological activation of the GDNF signaling pathway by the FDA-approved drug, cabergoline, effectively lowers alcohol-drinking and -seeking behaviors in rodents. Importantly, cabergoline's actions are due to its ability to up-regulate *GDNF*, and thus activate its signaling pathway.

Together, these findings document a reciprocal relationship between alcohol and endogenous *GDNF* expression in the VTA, identify a novel target of GDNF signaling that may play a role in GDNF's anti-alcohol actions, and establish a model in which the targeted pharmacological up-regulation of *GDNF* can effectively reduce the risk of relapse.

Carnicella S, Kharazia V, Jeanblanc J, Janak PH, Ron D (2008). GDNF is a fast-acting potent inhibitor of alcohol consumption and relapse. PNAS 105:8114-8119.

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CHAPTER 1

INTRODUCTION

> O God, that men should put an enemy in their mouths to steal away their brains! that we should, with joy, pleasance, revel, and applause, transform ourselves into beasts!
>
> William Shakespeare, Othello II.i.289-293

Alcohol Addiction

The drinking of alcoholic beverages has long been a mainstay in human society. Archaeologists have detected remnants of fermented beverages in early Neolithic drinking vessels that date back to ca. 7000 B.C. (McGovern et al., 2004). The brewing and distilling of alcoholic drinks, and the customs and ceremony surrounding the dedication of such to deities are prominent features in the written history of early human civilizations, including the Ancient Egyptians, Babylonians, and Chinese (Potter, 2008). Sadly, for as long as alcohol has been made and offered in veneration, it has also been consumed to excess. While historical depictions of the disorderly drunk appear as early as Ancient Greece, it was only in 1852 that physician Magnus Huss coined the term "alcoholism," classifying addiction to alcohol as a disease with characteristic behavioral and physiological symptoms (Huss and Busch, 1852). Even so, it was still commonly regarded as little more than a social "evil," blamed primarily for causing family violence, ineptitude, and disorderly public behavior (Potter, 2008). Far less attention was paid to the adverse effects of alcohol on the physical health of the excessive drinker. Scholarly reports on its effects on the nervous system dating from as early as mid-1600's were, for the most part, ignored (Potter, 2008).

Today, we know that chronic, excessive drinking has detrimental physiological and psychological health effects. Alcohol abuse and addiction result in the deaths of 2.5

million people worldwide each year, making them the world's third largest contributor to disease burden, according to the World Health Organization (February 2011 Alcohol Fact Sheet No. 349). In the United States alone, alcoholism and alcohol dependence are estimated to cost upwards of $220 billion a year (Potter, 2008). Some estimate that this is more than the U.S. spends on either cancer or obesity-related diseases alone. Clearly, alcohol abuse and addiction constitute a large humanitarian and financial burden, and it is in the best interest of society to address and treat this devastating disease.

The Mesolimbic Reward Pathway & Dopamine

Integral to the study of addictive processes in the brain is the identification of the brain regions that are affected by drugs of abuse. In 1954, work by Olds and Milner demonstrated that electrical stimulation in certain areas of the rat brain was positively reinforcing, promoting continued and frequent voluntary self-stimulation on the part of the rat for as long as the stimulation was available (Olds and Milner, 1954). Later studies established that the midbrain and, in particular, the ventral tegmental area (VTA) of the midbrain, was especially sensitive to activation by this type of electrical stimulation (Corbett and Wise, 1980; Wise and Rompre, 1989). It has since been observed that natural rewards, as well as drugs of abuse activate dopaminergic neurons in the VTA, increasing dopamine release into the brain regions to which VTA neurons project, thus positively reinforcing rewarding stimuli (Di Chiara and Imperato, 1988; Wise and Rompre, 1989). Furthermore, administration of dopamine antagonists into these same regions, blocking the effect of drug- or alcohol-induced dopamine release, was found to suppress drug and alcohol consummatory behaviors (De Wit and Wise, 1977; Hyman et

al., 2006; Koob et al., 1998). As a result, the VTA has come to be regarded as a central locus of the brain's reward pathway, and plays a key role in the regulation of drug- and alcohol-related behaviors.

The VTA is comprised mostly of dopaminergic cell bodies, and, to a lesser extent, glutamatergic and γ-aminobutyric acid (GABA)-ergic neurons (Fields et al., 2007). Neurons in the VTA project to the nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala, hippocampus, and ventral pallidum (Fields et al., 2007). The dopaminergic compliment of each of these projections varies, depending on the target. Most of the projections to the NAc are dopaminergic, while very few to the hippocampus are dopaminergic in identity (Fields et al., 2007). Importantly, the VTA receives feedback from each of the regions to which it projects. For instance, the transmission of dopamine from the VTA to the NAc is subject to GABAergic feedback regulation via afferents originating from the NAc (Fields et al., 2007; Geisler and Zahm, 2005; Kalivas, 1993; Phillipson, 1979). More recent reports indicate that GABAergic interneurons *within* the VTA can also act to negatively regulate dopaminergic activity (Fields et al., 2007; Korotkova et al., 2004). Activation of dopaminergic neurons in the VTA in response to rewarding stimuli, such as alcohol, results in an enhancement of dopamine release into the NAc of both rodents (Di Chiara and Imperato, 1988; Pontieri et al., 1995) and humans (Volkow et al., 2007), as well as an increase in somatodentritic dopamine release within the VTA (Kohl et al., 1998). This acute, alcohol-mediated release of dopamine from the VTA into the NAc is due to active release, and not simply reuptake inhibition (Yim and Gonzales, 2000). Moreover, several independent studies verified that alcohol-mediated dopamine release is the result of an increase in GABAergic feedback from the NAc to the

VTA, inhibiting local GABAergic tone in the VTA, and causing a disinhibition of the dopaminergic neurons in the VTA (Kohl et al., 1998; Lof et al., 2007; Spanagel, 2009). Although GABAergic feedback clearly plays a major role in the regulation of midbrain dopaminergic activity, it does not preclude the possibility that VTA dopaminergic neurons are also subject to other sources of regulation.

Glutamatergic neurons within the VTA have recently been identified (Yamaguchi et al., 2007). Additionally, glutamatergic inputs from the PFC, amygdala, hippocampus, or lateral hypothalamus into the VTA (Omelchenko and Sesack, 2007) can also influence alcohol-induced dopaminergic activity (Xiao et al., 2009). Similarly, local activation of serotonin type 3 (5-HT_3) receptors in the VTA is known to increase dopaminergic activity in that brain region (Liu et al., 2006). Correspondingly, the alcohol-mediated dopamine release in the NAc (Carboni et al., 1989) and somatodendritic release of dopamine in the VTA (Campbell et al., 1996) are attenuated upon blockade of 5-HT_3 receptors in the VTA. There is also evidence that activation of nicotinic acetylcholine (nACh) receptors is required for the alcohol-mediated dopamine release in the VTA (Blomqvist et al., 1993). In sum, while it is apparent that multiple systems, via several different neurotransmitters, act in concert to manipulate midbrain dopaminergic activity in the presence of alcohol, it is clear that they converge on a common effect: an increase of dopaminergic overflow into the NAc. An additional confound is that, although alcohol initially induces an increase in dopamine activity, long-term exposure to alcohol has been shown to result in a decrease in the basal dopaminergic tone in both the ventral midbrain (Lanca, 1994) and the limbic system, including the NAc (Zhou et al., 1995). Thus, the function of these fluctuations in mesolimbic dopamine in the context of alcohol is of great interest. Not

surprisingly, the exact role of dopamine with regard to addiction behaviors is highly debated.

Dopamine, in addition to being essential for motor activity (Graeff, 1966), has been proposed to encode separate, distinct aspects of addiction: liking of a drug, wanting of a drug, and learning to predict where/when a drug is available (Wise, 2004). Efforts to attribute the role of dopamine to just one of these so-called "dopamine hypotheses" of addiction are further complicated by the fact the evidence supporting each has been gathered from highly varied experimental paradigms, ranging from cue-induced and/or operant self-administration in rodents, to subjective self-reporting by human subjects (Wise, 2004). It may very well be that the precise role of dopamine with regard to a drug, such as alcohol varies from individual to individual, whether it is one articulated by one (or more) of these hypotheses, or one that has yet to be postulated. Nonetheless, it is well-established that dopamine plays an integral role in the perception of, and response to, drugs of abuse and alcohol, and contributes to the development of addiction. Thus, factors that modulate dopaminergic activity are of keen interest as potential pharmacological targets to combat addictive processes. In the last twenty years, a potent molecular regulator of dopaminergic function has emerged: glial cell line-derived neurotrophic factor (GDNF) (Hudson et al., 1995; Lin et al., 1993).

GDNF: Glial cell line-Derived Neurotrophic Factor

GDNF was first isolated from a rat glioma cell line, and was at that time characterized as an essential growth and potent maturation factor for midbrain dopaminergic neurons *in vitro* (Lin et al., 1993). Extensive studies since its discovery

have revealed that while GDNF's prominent role is as a support factor for dopaminergic, as well as motor, neurons, it is also important for kidney development (Pichel et al., 1996b) and spermatogenesis (Meng et al., 2000). Indeed, the homozygous *null* mutation of GDNF ($GDNF^{-/-}$) in mice is lethal, due to incomplete and aberrant development of the kidneys (Pichel et al., 1996a). GDNF was also found to dramatically improve the survival of midbrain dopaminergic neurons after injury (Beck et al., 1995), making it an attractive therapeutic possibility for the treatment of Parkinson's disease (Rangasamy et al., 2010), which is characterized by the loss of dopaminergic neurons in the substantia nigra (SN) region of the midbrain. Currently, GDNF is also the focus of research aimed at developing therapies for amyotrophic lateral sclerosis (McGeer and McGeer, 2005), epilepsy (Kanter-Schlifke et al., 2009), pain management (Boucher et al., 2000), and stroke (Harvey et al., 2003). Unfortunately, treatment of these conditions with GDNF itself has proven to be less than fruitful, mainly due to GDNF's inability to cross the blood-brain barrier (Boado and Pardridge, 2009; Kastin et al., 2003). As a result, there is a great deal of interest in the targets of the GDNF signaling pathway, as well as how *GDNF* expression is regulated.

GDNF is one member of the GDNF-family of ligands (GFL), along with persephin, artemin, and neurturin (Airaksinen and Saarma, 2002). The GFLs are part of the transforming growth factor-β (TGFβ) superfamily, a structurally-defined group consisting of cystine-knot proteins that function as secreted homodimers. There are four high-affinity co-receptors, one for each of the four GFLs: GDNF family receptor-α 1-4 (GFRα1-4). GDNF's cognate co-receptor is GFRα1 (Jing et al., 1996; Treanor et al., 1996), although it has been shown to interact, albeit weakly, with GFRα2 (Figure 1.1A;

(Sariola and Saarma, 2003; Wang et al., 2000)). GFRα1 exists in two different forms: a glycosyl phosphatidylinositol (GPI)-anchored form, which localizes it to the outer leaflet of the plasma membrane (and likely to specific microdomains therein (Paratcha et al., 2001)) and a secreted form (Paratcha and Ledda, 2008). As a result, GDNF can signal either *in cis* or *in trans* (Figure 1.1B). In both conditions, the GDNF homodimer binds to GFRα1, promoting dimerization of the receptor (Jing et al., 1996). The GDNF-GFRα1 complex then recruits and promotes the dimerization of the Ret (rearranged during transfection) receptor tyrosine kinase, a membrane-spanning receptor (Robertson and Mason, 1997; Treanor et al., 1996; Trupp et al., 1998). This induces the autophosphorylation, and thus activation of the Ret receptor. Phosphorylated tyrosine residues on the Ret receptor serve as docking sites for a number of adaptor and scaffolding proteins, ultimately activating multiple intracellular pathways, including the extracellular signal-regulated kinase 1/2 (ERK1/2), phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinase (JNK), and phospholipase C-γ (PLCγ) signaling pathways (Figure 1.1C; (Airaksinen and Saarma, 2002; Hayashi et al., 2000; Murakami et al., 2002; Takahashi, 2001)). Of particular importance, autophosphorylation of the Tyr1062 residue in the Ret receptor is correlated with the activation of the ERK1/2 signaling pathway (Hayashi et al., 2000), and this is faithfully modeled in the human dopaminergic-like SH-SY5Y cell line (Figure 1.1D). Although Ret is the canonical GDNF receptor, the fact that GDNF treatment results in ERK1/2 activation in cells lacking the Ret receptor suggests that other signaling receptors exist for GDNF (Poteryaev et al., 1999; Trupp et al., 1999). In 2003, it was reported that GDNF binds and signals through the neuronal cell adhesion molecule (NCAM), activating the ERK1/2 pathway, and promoting synaptic activity

(Paratcha et al., 2003; Zhou et al., 2003). Additionally, the GDNF-GFRα1 complex has also been shown, in the absence of Ret, to act as a ligand-induced cell adhesion molecule

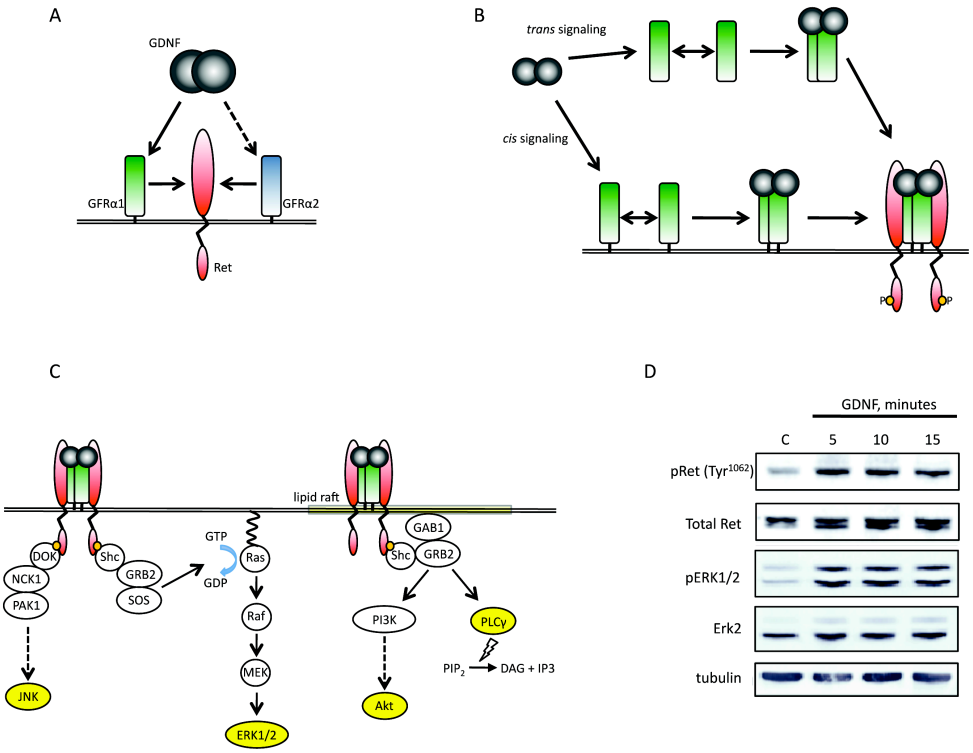

Figure 1-1: GDNF and its signaling pathway. A, GDNF, like all GFLs, signals through the Ret receptor tyrosine kinase via a high-affinity bond with the GFRα1 co-receptor. GDNF may also bind Ret through a weak association with GFRα2. **B,** GFRα1 is expressed in membrane-bound and soluble forms, enabling *cis* or *trans* signaling, respectively. **C,** Phosphorylation, and thus activation, of the Ret receptor provides binding sites for several scaffolding proteins and activation of several downstream pathways, including the JNK, ERK1/2, PI3K/Akt, and PLCγ pathways. There is evidence that membrane microdomains, such as lipid rafts, confer a level of specificity as to which pathways will be activated. **D,** Treatment of the human dopaminergic-like SH-SY5Y cell line with GDNF results in a rapid phosphorylation of the Ret receptor (pRet) at the Tyr^{1062} residue, as well as a robust phosphorylation, and activation, of the ERKs 1/2. Tubulin was detected as a loading control.

(LICAM), promoting synapse formation between hippocampal neurons via trans-homophilic interactions (Ledda et al., 2007). The GDNF signaling pathway is also susceptible to negative regulation. Ledda *et al*. identified a transmembrane protein, Lrig1 (leucine-rich repeat and Ig-like domain protein), which interacts with the Ret receptor

inhibiting its association with GDNF and thus inhibiting activation of downsteam kinases, such as ERK1/2 (Ledda et al., 2008). A more recent study showed that Rap1GAP (a GTPase-activating protein for Rap1) binds to the Ret receptor soon after Ret is activated by GDNF, thus down-regulating ERK1/2 activation and attenuating neurite outgrowth (Jiao et al., 2011).

In the adult mesolimbic pathway, GDNF is highly expressed in the striatum (including the NAc) (Barroso-Chinea et al., 2005; Trupp et al., 1997), while its receptors, Ret and GFRα1, are concentrated in the VTA and SN midbrain regions (Treanor et al., 1996; Trupp et al., 1997). Accordingly, Tomac and colleagues confirmed that striatal GDNF is a target-derived neurotrophic factor, and is retrogradely transported from the dorsal striatum to the SN by dopamine neurons (Tomac et al., 1995b). In a similar fashion, we have demonstrated that the GDNF that is expressed in the NAc is retrogradely transported to the VTA (Wang et al., 2010). Once in the VTA, GDNF is secreted, binds to its receptors on dopaminergic neurons, and activates the ERK1/2 signaling pathway (Wang et al., 2010). What follows is an ERK1/2-dependent increase in the spontaneous firing of these neurons, and a consequent increase in dopamine release into the NAc (Wang et al., 2010). Interestingly, GDNF also has the ability to up-regulate its own expression, both *in vitro* in the human dopaminergic-like SH-SY5Y cell line (He and Ron, 2006) and *in vivo* in the VTA of rats (Barak et al., 2011), resulting in a self-perpetuating persistence of GDNF signaling. This indicates that the long-lasting effects of GDNF may be due, in part, to a continued elevation of *GDNF* levels by its own signaling pathway.

Remarkably, while the long-term effects of GDNF on dopaminergic neuron survival (Beck et al., 1995; Borgal et al., 2007), differentiation (Widmer et al., 2000), function (Hudson et al., 1995; Pothos et al., 1998), repair following damage (Beck et al., 1995; Tomac et al., 1995a), and the expression of genes essential to dopaminergic function (such as tyrosine hydroxylase [TH] and the dopamine transporter [DAT]) (Consales et al., 2007) have been extensively documented, very little is known regarding the regulation of its own expression and secretion, or the immediate downstream targets of its signaling pathway and their acute effects on dopaminergic function. There have been, however, significant advances in our understanding of GDNF's involvement in addiction to drugs of abuse, including alcohol.

GDNF & Drugs of Abuse

Over the last decade, a number of studies on the effect of neurotrophic factors on drug-related behaviors and molecular neuroadaptations have been conducted (Ghitza et al., 2010). Consequently, GDNF has emerged as a key inhibitor of some of these drug- and alcohol-induced effects (Carnicella and Ron, 2009; Leggio et al., 2010).

Psychostimulants: cocaine & methamphetamine **–** Messer and colleagues demonstrated that infusion of GDNF directly into the rat VTA attenuated the increases in TH (the enzyme that catalyzes the rate-limiting step of dopamine biosynthesis) and the NR1 subunit of the *N*-methyl *D*-aspartate (NMDA) receptor that occur after repeated exposures to cocaine (Messer et al., 2000). They also showed that GDNF blocked the rewarding attributes of cocaine, as conditioned place preference (CPP) for cocaine was inhibited when GDNF was infused into the VTA of mice (Messer et al., 2000). Moreover,

when GDNF levels were low, either as a result of the infusion of GDNF inhibitory antibodies into the rat VTA, or in GDNF heterozygous knockout ($GDNF^{+/-}$) mice, CPP for cocaine and induction of TH by cocaine were both enhanced (Messer et al., 2000). Interestingly, transfusion of a cell line that expresses and secretes GDNF (Green-Sadan et al., 2003), as well as delivery of GDNF-conjugated nanoparticles (Green-Sadan et al., 2005) into the NAc lowered cocaine self-administration. These findings are in line with the determination that NAc-derived GDNF is retrogradely transported to the VTA (Wang et al., 2010). Not only did this body of work demonstrate that exogenous GDNF has therapeutic potential for the treatment of drug (specifically, cocaine) addiction, it also posited that the endogenous GDNF system likely plays a role in regulating the molecular and behavioral effects of drugs of abuse, such as cocaine. Additional experiments utilizing the $GDNF^{+/-}$ mice have also demonstrated that GDNF regulates the rewarding effects, self-administration, and seeking of methamphetamine (Niwa et al., 2007; Yan et al., 2007).

Opiates – Subsequent studies demonstrated that GDNF has the potential to block the molecular and behavioral effects of drugs other than those classified as psychostimulants (Ghitza et al., 2010). For example, in addition to showing that GDNF blocked cocaine-induced molecular neuroadaptations, Messer *et al*. also demonstrated that the same was true for morphine-induced increases in TH (Messer et al., 2000). In a separate study, increased CPP for morphine was also observed in $GDNF^{+/-}$ mice (Niwa et al., 2007). Taken together, these studies provide mounting evidence that GDNF is a general inhibitor of drug-induced reward.

Alcohol – Recent work conducted in our laboratory showed that GDNF is a robust regulator of alcohol consumption. These investigations were precipitated by the initial finding that the anti-addiction properties of a naturally-occurring alkaloid, ibogaine, were mediated by GDNF (He et al., 2005). Anecdotal reports, as well as animal studies, indicated that ibogaine had the potential to treat addiction to multiple drugs of abuse, including alcohol (Glick and Maisonneuve, 2000; Mash et al., 1998; Rezvani et al., 1995). Importantly, ibogaine's ability to attenuate drug- and alcohol-related behaviors had a very long effect period, with a single administration lasting up to 6 months (Popik et al., 1995). Ibogaine, due to its psychoactive properties, has been relegated to the Schedule I Controlled Substance grouping in the United States, making all non-research use of this compound illegal. Our group found that systemic administration of ibogaine increased GDNF expression in the midbrain of rats, and also lowered the operant self-administration of alcohol (He et al., 2005). Treatment of the human dopaminergic-like SH-SY5Y cell line with ibogaine resulted in the increased expression of GDNF, as well as activation of its signaling pathway (He et al., 2005). Moreover, when GDNF inhibitory antibodies were infused into the VTA, the ability of ibogaine to lower alcohol operant self-administration was attenuated (He et al., 2005). This seminal study supported further investigation into the effect of GDNF on alcohol drinking and seeking behaviors.

Since then, our lab has established that GDNF itself reverses alcohol-induced biochemical neuroadaptations, and is a potent inhibitor of alcohol consumption and reward. GDNF, via altering the activity of the molecular chaperone protein, heat shock protein 90 (HSP90), reversed the alcohol-mediated increase in TH levels (He and Ron, 2008), which is a characteristic biochemical adaptation resulting from chronic alcohol

exposure (Ortiz et al., 1995). Infusion of the recombinant protein directly into the VTA significantly, and ERK1/2-dependently, lowered alcohol consumption, seeking, and relapse in rats that had been trained to lever-press for an alcohol reward (Carnicella et al., 2008). Remarkably, this effect had a very short onset, occurring within 10 minutes of the infusion, suggesting a rapid, non-genomic mechanism (Carnicella et al., 2008). In addition to attenuating operant responding for alcohol, infusion of GDNF into the VTA of rats also blocked excessive voluntary alcohol drinking in the intermittent-access two-bottle choice drinking model (Carnicella et al., 2009c). In line with these findings, we also found that GDNF heterozygous knockout mice displayed increased CPP to alcohol and consumed more alcohol after a period of abstinence than their wild-type littermates (Carnicella et al., 2009b). It is, however, unlikely that GDNF itself can be used to treat alcohol dependence, as it cannot pass through the blood-brain barrier (Kastin et al., 2003). Therefore, selectively targeting the GDNF signaling pathway, or inducing its activation, may prove to be an effective pharmacotherapy for the treatment of alcohol dependence (Carnicella and Ron, 2009; Leggio et al., 2010). Discerning the molecular actions of GDNF, and the mechanisms by which they may lower alcohol drinking are of critical importance for discovering pharmacological targets.

Significance & Summary

As with other drug addictions, alcohol dependence is widely recognized as a medically treatable disease, necessitating the development of efficacious drug therapies for the alleviation of craving and withdrawal symptoms. Currently, there are only a handful of FDA-approved medications for the treatment of alcohol addiction: disulfuram,

acamprosate, and naltrexone. Unfortunately, these medications have had limited success in the treatment of alcoholism, mainly due to a low level of patient compliance as a result of unpleasant adverse side effects (Assanangkornchai and Srisurapanont, 2007; Bouza et al., 2004; Johnson, 2008). Thus, the development of more efficacious pharmacological targets is of great importance.

Overall, our previous findings indicate that alcohol and GDNF share an inverse relationship. When GDNF levels are increased, alcohol consumption and reward are reduced. Conversely, if GDNF levels are abnormally low, there is an enhancement of alcohol consumption and reward. The purpose of this dissertation was to further characterize GDNF and its signaling pathway with special regard to alcohol. Specifically, we set out to determine whether there exists a causal relationship between alcohol drinking and the endogenous GDNF system, and the precise molecular mechanism of the GDNF-mediated decrease in alcohol consumption.

The work put forth in this dissertation represents a multi-pronged approach to gain new understanding of GDNF and its signaling pathway, and its implications for alcohol-related behaviors. In Chapter 2, the effect of voluntary alcohol consumption on the levels of endogenous *GDNF* is characterized. Two putative molecular mechanisms driving alcohol-mediated alterations in endogenous *GDNF* expression are identified and described in Chapter 3. Chapter 4 details efforts to identify downstream targets of GDNF-activated ERK1/2 that may be responsible for the rapid, GDNF-mediated decrease in alcohol self-administration. Finally, Chapter 5 represents a collaborative, published study in which we demonstrated that pharmacological activation of the GDNF signaling pathway can precipitate a reduction in alcohol-consummatory and -seeking behaviors

(Carnicella et al., 2009a). Together, the findings presented here add an important molecular insight into the contribution of GDNF to the regulation of alcohol intake.

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CHAPTER 2

GDNF IS A NOVEL ALCOHOL-RESPONSIVE GENE

Abstract

Glial cell line-derived neurotrophic factor (GDNF) is an endogenous negative regulator of alcohol consumption in rodents (Carnicella et al., 2009b). We set out to determine the effect of alcohol on the expression of *GDNF* in the nucleus accumbens (NAc) and the ventral tegmental area (VTA), the source and site of action of GDNF, respectively (Wang et al., 2010). We found that *GDNF* expression in the VTA is up-regulated after either a single, systemic administration of alcohol or a short period of voluntary alcohol consumption. A long history of excessive alcohol intake induced a deprivation-associated decrease in basal *GDNF* levels in the VTA. An acute, 30 minute "binge-like" drinking period following deprivation caused a significant up-regulation of *GDNF*, which normalized to control basal levels at the end of a 24-hour drinking session. Importantly, we show that specific attenuation of the alcohol-induced *GDNF* expression, via RNA interference, facilitated the escalation of alcohol drinking. To our knowledge, this is the first report identifying *GDNF* as an alcohol-responsive gene in the VTA. We propose that the alcohol-mediated increase in *GDNF* expression in the VTA is a feedback signal that protects against the progression of excessive alcohol intake. This protective mechanism is dysregulated after a long history of excessive alcohol consumption, possibly incentivizing continued alcohol drinking in order to replace the deprivation-induced deficit in basal *GDNF* levels.

Introduction

Drugs of abuse, including alcohol, target the mesolimbic reward pathway in the brain, a major component of which is the dopaminergic projection from the VTA to the NAc (Berke and Hyman, 2000; Hyman et al., 2006). Cellular and molecular adaptations in response to alcohol, such as changes in gene expression, in these brain regions are thought to underlie the development and persistence of alcoholism (Hyman and Malenka, 2001). One gene product crucial for proper dopaminergic function in the VTA and NAc is GDNF (Airaksinen and Saarma, 2002).

GDNF is a secreted growth factor, initially identified for its ability to promote the survival of midbrain dopaminergic neurons in culture (Lin et al., 1993). It has since been found to be essential for the development, differentiation, proliferation, and function of midbrain dopaminergic neurons (Airaksinen and Saarma, 2002). The GDNF signaling pathway is initiated upon the binding of GDNF to its co-receptor, GDNF family receptor-α 1 (GFRα1). The GDNF-GFRα1 complex then recruits and promotes the dimerization of the rearranged during transfection (Ret) receptor tyrosine kinase (Airaksinen and Saarma, 2002). Dimerization of Ret leads to its autophosphorylation, which, in turn, results in the activation of a number of intracellular signaling cascades, ultimately affecting neuronal survival, maintenance, and function (Airaksinen and Saarma, 2002; Hayashi et al., 2000). In the mesolimbic circuit of the adult brain, GDNF is highly expressed in the NAc (Barroso-Chinea et al., 2005), while its receptors GFRα1 and Ret are largely restricted to the VTA (Treanor et al., 1996; Trupp et al., 1997). In order for GDNF to activate its signaling pathway, it is retrogradely transported to the VTA, where it can bind to its

receptors (Wang et al., 2010). Once in the VTA, GDNF acts as an enhancer of dopaminergic activity (Wang et al., 2010).

Interestingly, GDNF and its signaling pathway, potent contributors to dopaminergic activity, are susceptible to drug-induced alterations. For instance, Messer *et al.* reported that levels of phosphorylated, and thus activated, Ret was lowered after chronic exposure to cocaine or morphine (Messer et al., 2000). In addition, Green-Sadan and colleagues found that endogenous *GDNF* was decreased in the dorsal striatum, but not in the ventral striatum (NAc), 24 hours after the end of a 12-day-long cocaine self-administration procedure (Green-Sadan et al., 2003). In contrast, *GDNF* mRNA levels in the VTA and the substantia nigra (SN) were up-regulated following subchronic (5 days) systemic administration of phencyclidine (PCP) (Semba et al., 2004). Although precedent for drug-induced changes in *GDNF* levels, as well as its signaling pathway, has been set, the effect of alcohol on this system has yet to be established. The effect of GDNF and GDNF-mediated signaling on alcohol consumption, however, has been characterized in detail.

We showed that infusion of GDNF into the rat VTA rapidly and ERK1/2-dependently lowered alcohol operant self-administration (Carnicella et al., 2008), attenuated alcohol-seeking behaviors and relapse (Carnicella et al., 2008), and blocked excessive alcohol intake in the intermittent access two-bottle choice paradigm (Carnicella et al., 2009c). Administration of pharmacological compounds, such as cabergoline (Carnicella et al., 2009a) or ibogaine (He et al., 2005), lowered alcohol intake by up-regulating *GDNF* in the VTA, and subsequently activating its signaling pathway. Taken together, these findings indicate that when GDNF levels are elevated, either by infusion

of the GDNF protein itself or by pharmacological up-regulation of its expression, alcohol intake is decreased. Additionally, we found that GDNF heterozygote knockout mice display an increased conditioned place preference for alcohol, and consume more alcohol than their wild-type littermates following a period of abstinence (Carnicella et al., 2009b), suggesting that reduced endogenous levels of GDNF result in an increased behavioral response to alcohol. As a result, we hypothesized that endogenous *GDNF* is a part of a homeostatic mechanism that negatively regulates alcohol intake. Thus, we tested whether endogenous *GDNF* expression in the mesolimbic pathway is altered in the presence of alcohol, and what role it plays in the regulation of alcohol consumption.

Materials & Methods

Reagents **–** TRIzol reagent was purchased from Invitrogen. Deoxyribonuclease (DNAse) and ethidium bromide (EtBr) were both purchased from Sigma. The Reverse Transcription System and 2X PCR master mix were purchased from Promega. The Taqman gene expression 2X master mix and the primer/probe kits were purchased from Applied Biosystems, Inc. The pRNAT-H1.1/Shuttle vector was obtained from the GenScript Corporation. The Adeno-X vector, Expression System, and Purification and Rapid Titer kits were purchased from Clontech.

Animals **–** Adult male Long-Evans rats (250-300g at the beginning of experiments) were purchased from Harlan. Animals were individually housed with food and water available *ad libitum*, constant temperature (23°C) and humidity (50%), and a 12-hour light-dark cycle (lights on at 7:00AM). All experimental protocols were approved by the Ernest Gallo Research Center Institutional Animal Care and Use Committee

(IACUC), and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Intermittent-access 20% alcohol two-bottle choice **–** Following a one-week habituation, rats were started on the intermittent-access two-bottle choice drinking paradigm as previously described (Carnicella et al., 2009c; Simms et al., 2008). Briefly, rats were given a bottle containing an alcohol solution (20% v/v in tap water), in addition to a bottle of tap water, every other day for a total of three, 24-hour alcohol drinking sessions per week (i.e.: Monday, Wednesday, and Friday). Control subjects were given an additional bottle of water in place of the alcohol solution. Rats were sacrificed, and the NAc and VTA collected, 30 minutes after the beginning (binge drinking), immediately following, or 24 hours after (deprivation) the end of the last alcohol drinking session, as noted.

Reverse Transcription (RT) and quantitative Real-Time (qRT) Polymerase Chain Reaction (PCR) **–** Following dissection, tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. Frozen tissues were mechanically homogenized in Trizol reagent, and total RNA was isolated from each sample according to the manufacturer's recommended protocol. Total RNA was treated with DNAse. Messenger RNA (mRNA) was selectively reverse transcribed into cDNA using the Reverse Transcription System and with oligo(dT) primers. For the RT-PCR, amplification of *GDNF* and the housekeeping gene, *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH),* was conducted with the resulting cDNA using the following primers: rat *GDNF* upstream: 5'- GAC GTC ATG GAT TTT ATT CAA GCC ACC -3'; rat *GDNF* downstream: 5'- CTG GCC TAC TTT GTC ACT TGT TAG CCT -3'; rat

GAPDH upstream: 5'- TGA AGG TCG GTG TCA ACG GAT TTG GC -3'; rat *GAPDH* downstream: 5'- CAT GTA GGC CAT GAG GTC CAC CAC -3'. For *GDNF,* 33-35 amplification cycles were used, while 27 cycles were used for *GAPDH*. PCR products were resolved on a 1.8% agarose gel supplemented with 0.05% EtBr for visualization under UV light. Images were captured using Eagle Eye 2 software. Band intensities were quantified using NIH ImageJ software. For the qPCR, cDNA samples were analyzed in triplicate on a 384-well plate using the Taqman gene expression 2X master mix, and the following gene expression primer/probe kits: rat *GDNF,* Rn00569510_m1, and rat *GAPDH*, Rn99999916_s1. Relative *GDNF* and *GAPDH* mRNA concentrations were determined based on a standard curve constructed using triplicate Ct readings of serial dilutions of whole striatal cDNA, which was prepared in parallel with the samples to be tested.

Adenoviral-mediated shRNA down-regulation of GDNF – We have previously described the construction and preparation of the GDNF small hairpin RNA (shGDNF) and the scrambled (SCR) control recombinant adenoviruses (Wang et al., 2010). Briefly, double-stranded oligonucleotides containing a 20-nucleotide small interfering RNA sequence against the coding region of GDNF mRNA was subcloned into the pRNAT-H1.1/Shuttle vector containing a green fluorescent protein (GFP) marker. The resulting shGDNF expression cassette was subcloned into the Adeno-X viral genome. Adenoviral particles were packaged and amplified in HEK293 cells according to the Adeno-X Expression System 1 protocol. Virus was purified with the Adeno-X Maxi Purification kit, and titered using the Adeno-X Rapid Titer kit. Preparation of the SCR virus was done in parallel. Verification of *in vivo* adenoviral-mediated knock-down of *GDNF*, as well as

the timecourse, is described in Appendix B. Virus (1.3 x 10^8 TU/mL), containing the shGDNF (AdV-shGDNF) or SCR (AdV-SCR) construct, was bilaterally infused into the VTA of naïve rats. Infusion of the viruses was conducted similarly to our previous report (Wang et al., 2010). Briefly, rats were continuously anesthetized with isolfurane. Injectors (33 ga) connected to 25 µl Hamilton syringes driven by an osmotic pump (Harvard Apparatus, Holliston, MA) were placed bilaterally into the VTA (coordinates, in mm: -5.6 AP to bregma, ± 0.75 ML, -8.4 DV to the skull surface). Virus (1.2 µl/side) was infused over a 3-minute period. The injectors were left in place for an additional 10 minutes. After allowing 11-13 days for surgical recovery and expression of shGDNF, the intermittent-access 20% alcohol two-bottle choice paradigm was started. This timepoint was chosen based on experiments demonstrating significant shRNA-mediated knockdown of *GDNF* in the VTA occurs by Day 14 post-viral infusion (Appendix B, Figure A-1).

Statistical Analysis – Statistical significance was determined by Student's t-test or one-way analysis of variance (ANOVA), with *post-hoc* Student-Newman-Keuls test. Alcohol consumption was analyzed in a mixed model two-way ANOVA with a between subjects factor of Virus infection and a repeated measurements factor of Day post-infection, followed by comparisons using method of contrasts.

Results

Acute, systemic administration and short-term voluntary consumption of alcohol increases GDNF expression in the VTA – We set out to determine whether alcohol affects endogenous *GDNF* expression levels. We first tested whether a single

exposure to alcohol would alter the expression of *GDNF* in the VTA or the NAc. Rats were administered 1.8 g/kg alcohol (i.p.), and the VTA and NAc were collected 0.5, 2, 4, 10, and 24 hours later. We observed a significant up-regulation of *GDNF* in the VTA 10 hours after the administration of alcohol, which appeared to decline 24 hours after the injection (Figure 2-1A). We did not observe any alteration in the expression of *GDNF* in the NAc at any of the timepoints tested (Figure 2-1B), suggesting that alcohol-mediated effects on *GDNF* expression in the mesolimbic VTA-NAc connection are specific to the VTA.

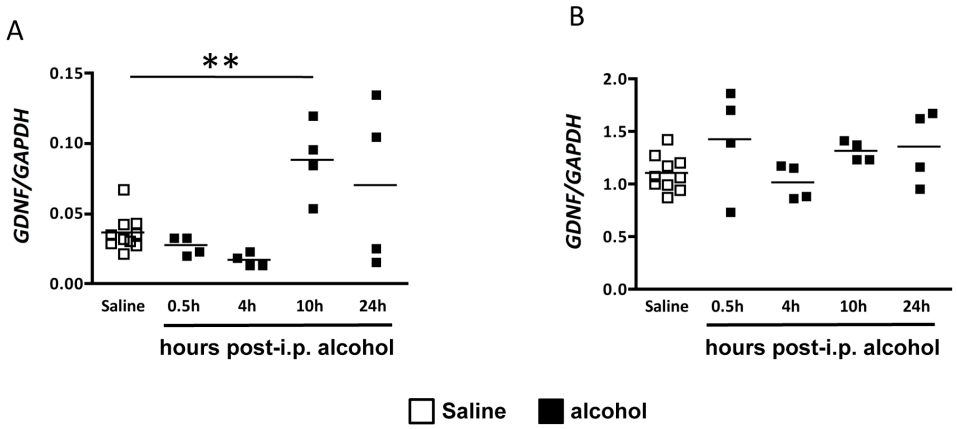

Figure 2-1. *GDNF* is increased in the VTA, but not the NAc, after a single, systemic administration of alcohol. Rats were injected i.p. with alcohol at a dose of 1.8 g/kg, with saline as a vehicle. Control rats were injected i.p. with a similar volume of saline only. The VTA and NAc were collected 0.5, 4, 10 and 24 hours after the injection. **A & B,** *GDNF* expression levels in the VTA **(A)** and the NAC **(B)** were measured by Taqman qRT-PCR. Points on the graph represent the *GDNF/GAPDH* ratio of the individual subjects, with the mean being depicted by a bar. Empty squares: control saline administration; filled squares: EtOH administration, n = 10 animals for the saline group and 4 for each timepoint. ** $p < 0.01$, compared to saline-injected control.

Since a single, acute administration of alcohol increased *GDNF* expression in the VTA, we next tested whether *GDNF* expression levels would be altered following a more physiologically relevant model of alcohol exposure: voluntary drinking. Rats were provided access to a 20% v/v alcohol in tap water in a two-bottle choice model on an

intermittent-access schedule for one week, for a total of four drinking sessions. The VTA and NAc tissues were collected immediately after the end of the last alcohol drinking session. The average alcohol consumption during the last drinking session was 4.03 ± 0.61 g/kg/24 hours (Table 2-1).

Table 2-1: Alcohol intake during the last drinking session:

Drinking History	Alcohol intake during last drinking session (Average ± SEM)
Short-term EtOH	4.03 ± 0.61 g/kg/24 hrs
Long-term EtOH	5.48 ± 0.88 g/kg/24 hrs
Long-term EtOH + "Binge"	1.28 ± 0.24 g/kg/30 mins

We found that *GDNF* levels in the VTA were significantly up-regulated after this short period of voluntary drinking (Figure 2-2A). As with the single alcohol i.p. administration, *GDNF* levels in the NAc were unaffected (Figure 2-2B). We next tested what effect long-term high levels of alcohol consumption would have on *GDNF* expression. Again, rats were trained to drink alcohol in the intermittent-access to 20% alcohol two-bottle choice paradigm. After a period of 6-8 weeks, when alcohol consumption had reached a stable baseline range averaging 5.48 ± 0.88 g/kg/24 hours (Table 2-1), the VTA and NAc were collected at the end of the last drinking session. We observed that, in both brain regions, *GDNF* levels in the alcohol-drinking subjects were the same as those in the water-only control subjects (Figure 2-2A, B), suggesting that the early effects of alcohol exposure on *GDNF* expression in the VTA had been normalized after a long history of repeated bouts of excessive alcohol drinking.

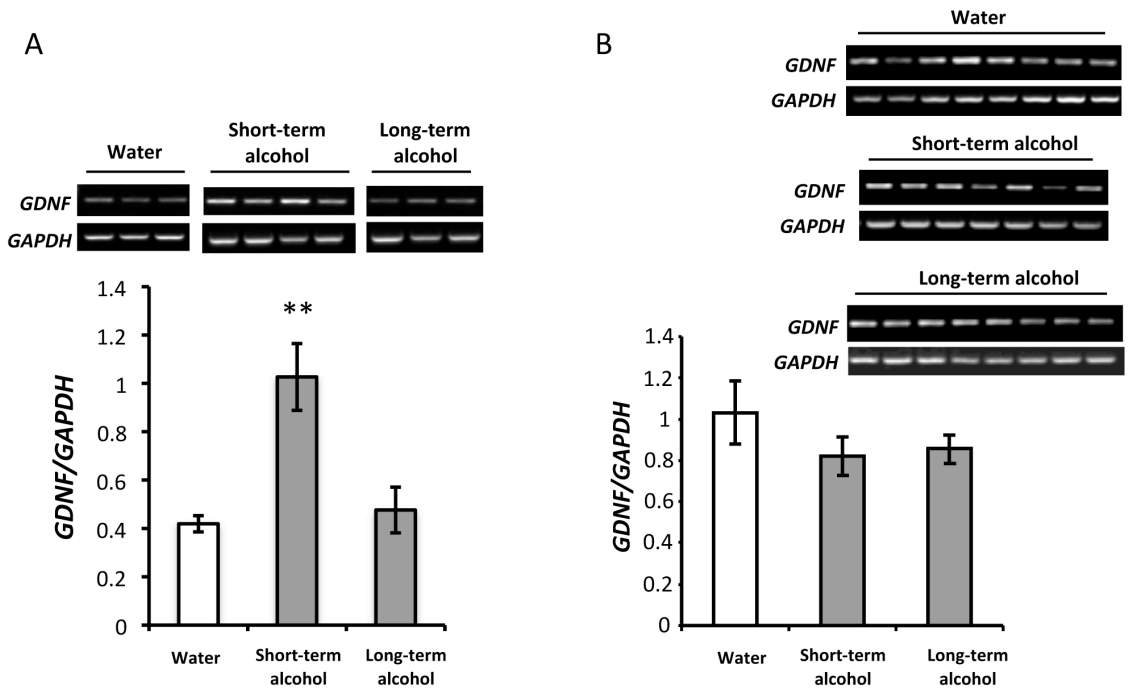

Figure 2-2. VTA *GDNF* is increased following a short history of alcohol intake and returns to baseline after a long history of alcohol consumption. Rats consumed alcohol in the intermittent-access, two-bottle choice paradigm for 1week or 6-8 weeks. The VTA and NAc were collected immediately following the end of the last drinking session. **A & B,** *GDNF* expression in the VTA **(A)** and the NAc **(B)** were determined by RT-PCR. Gel image in **(A)** is representative of two independent experiments. Bar graphs represent the mean *GDNF/GAPDH* ratio ± SEM, n = 7-8 per group. ** $p < 0.01$, compared to water control.

Deprivation following long-term consumption of high levels of alcohol results in a depressed level of GDNFexpression in the VTA – Although *GDNF* levels were normalized to basal levels in long-term drinkers following a drinking session (Figures 2-2A), it is possible that long-lasting, alcohol-induced changes in *GDNF* expression can only be discerned in the absence of alcohol. Thus, we set out to determine *GDNF* expression levels during a period of deprivation after long-term consumption of alcohol. To do so, we assessed the level of *GDNF* expression in the VTA and NAc of rats that consumed alcohol in the intermittent-access two-bottle choice paradigm for several weeks, as above, and were sacrificed 24 hours after the end of the last alcohol drinking

session. We found that, in the VTA, *GDNF* mRNA levels were significantly decreased after a 24-hour deprivation period following a long history of high levels of alcohol intake (Figure 2-3A). No difference was observed in the NAc (Figure 2-3B). This observation indicates that after a long history of excessive alcohol consumption, *GDNF* levels in the VTA are abnormally low during deprivation, when alcohol is no longer present for consumption, and has long since been metabolized.

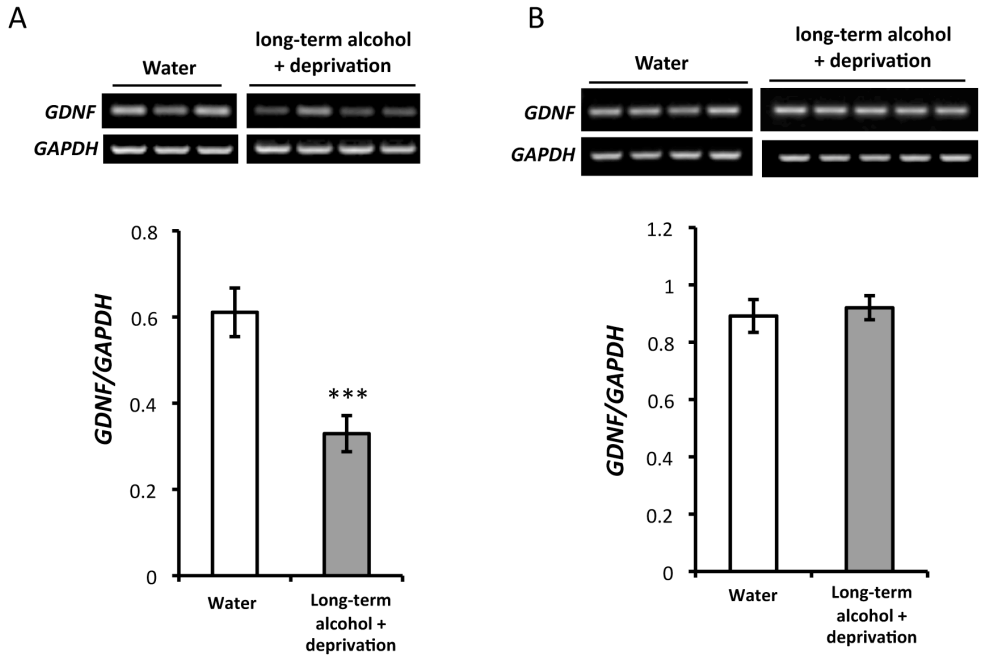

Figure 2-3. *GDNF* is down-regulated in the VTA after a period of deprivation following a long history of high levels of alcohol intake. Rats consumed 20% v/v alcohol in the intermittent-access two-bottle choice paradigm for at least six weeks. VTA and NAc tissues were dissected 24 hours after the end of the last alcohol drinking session. *GDNF* expression levels were determined by RT-PCR. **A,** *GDNF* expression is significantly decreased in the VTA. Gel image is representative of two independent experiments. Bar graph represents the mean *GDNF*/*GAPDH* ratio, ± SEM. *** $p < 0.001$, n = 6-8 per group. **B,** *GDNF* expression was unaltered in the NAc. Bar graph depicts the mean *GDNF*/*GAPDH* ratio ± SEM, n = 4-5.

GDNF expression in the VTA is significantly increased after a period of excessive, "binge-like" alcohol intake – We found that *GDNF* expression is decreased during a period of deprivation following a long history of excessive alcohol drinking

(Figure 2-3A), and returns to basal levels after a 24-hour drinking session (Figure 2-2A), suggesting that alcohol alters *GDNF* expression during the drinking period. We previously reported that during the first 30 minutes of the drinking session in the intermittent-access to 20% alcohol two-bottle choice procedure, rats consume an excessive amount of alcohol (1-1.5 g/kg/30 minutes; see also Table 2-1), resulting in a blood alcohol concentration (BAC) of 80.9 ± 7.2 mg% (17.5 ± 1.5 mM), which meets the National Institute on Alcohol Abuse and Alcoholism definition of binge drinking (BAC of 80 mg% or more) in humans. We therefore tested whether *GDNF* expression is affected by this period of excessive, "binge-like" alcohol drinking. We observed an increase in *GDNF* mRNA in the VTA 30 minutes after the beginning of the alcohol drinking session, as compared to water-only controls (Figure 2-4). These results indicate that alcohol drinking continues to induce an up-regulation of *GDNF,* even after a long

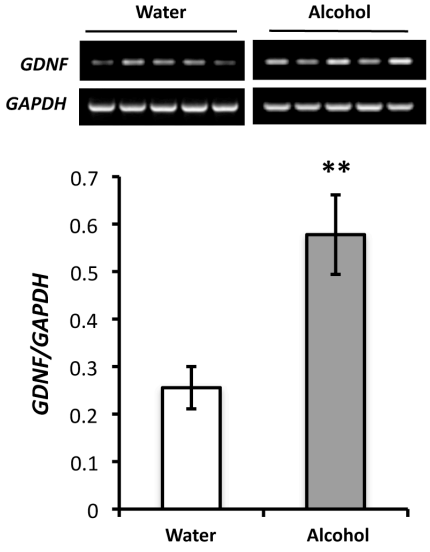

Figure 2-4. Excessive, "binge-like" alcohol consumption after a period of deprivation elevates *GDNF* levels in the VTA. Following a 24-hour deprivation period, rats consumed an excess amount of alcohol during a short, 30 minute episode. The VTA was collected at the end of this 30-minute "binge-like" drinking session. *GDNF* expression levels were measured by RT-PCR. Bar graph represents the average *GDNF*/*GAPDH* ratio ± SEM. ** $p < 0.01$, n = 10 per group.

history of high levels of alcohol consumption, but that this increase occurs very rapidly, and is normalized to basal levels by the end of the 24-hour drinking session.

The short-term, alcohol-mediated induction of GDNF expression in the VTA regulates the early stages of alcohol intake – We next set out to determine the precise function of the alcohol-mediated increase in *GDNF* expression during the early stages of alcohol drinking (Figure 2-2A). Previously, we demonstrated that GDNF in the VTA lowers the consumption of alcohol (Carnicella et al., 2009c; Carnicella et al., 2008; Carnicella and Ron, 2009), and that endogenous GDNF is a negative regulator of alcohol-mediated reward (Carnicella et al., 2009b). We therefore hypothesized that endogenous GDNF may be part of a homeostatic mechanism that is activated by alcohol to negatively regulate alcohol drinking. To test this, we delivered, via adenovirus, shRNA (short hairpin RNA) targeted against GDNF (shGDNF) into the VTA to block the alcohol-mediated increase in *GDNF* levels in this brain region. Following the infusion of the shGDNF adenovirus into the VTAs of alcohol-naïve rats, we assessed the levels of alcohol intake in the intermittent-access two-bottle choice paradigm. Over the course of the first four drinking sessions, we observed a significantly more rapid escalation in alcohol consumption in the subjects that received the shGDNF virus, as compared to those who had received the scrambled (SCR) control virus (Figure 2-5A). ANOVA revealed no effect of Virus infection [$F(1,16)=1.08$, $p = 0.31$], but a significant main effect of Day post-infection [$F(10, 160)=3.41$, $p < 0.001$] and a significant interaction [$F(10, 106)=2.55$, $p < 0.01$]. Further analysis using the method of contrasts showed a significant difference in EtOH intake between shGDNF- and SCR-treated animals during days 11-20 post-virus infusion [$F(1,16)=8.92$, $p < 0.01$]. Water consumption was

unaltered, regardless of viral treatment (Figure 2-5B). Importantly, drinking levels are equal between the shGDNF and SCR groups, indicating not only that this effect is not permanent, which is likely due to the transient nature of adenovirus-mediated expression of shRNA, but that the difference in alcohol intake is not simply due to an aversion to alcohol in the SCR group. These results suggest that the function of the alcohol-mediated increase in *GDNF* levels is to suppress the escalation of alcohol consumption during early alcohol drinking experiences.

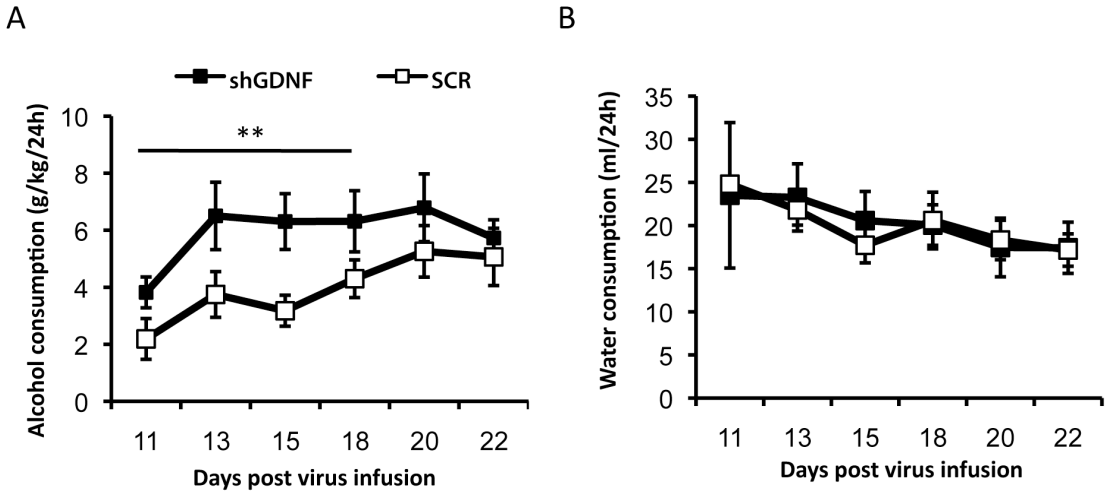

Figure 2-5. shRNA-mediated blockade of alcohol-induced *GDNF* in the VTA results in escalated drinking. Recombinant adenovirus containing shRNA against GDNF (shGDNF) or a scrambled control sequence (SCR) were bilaterally infused into the VTA of rats. 24-hour alcohol **(A)** and water **(B)** consumption in the intermittent-access two-bottle choice procedure was measured beginning 11 days after the viral infusion. Graph represents amount consumed ± SEM, n = 9 per group. Filled squares: shGDNF-infused rats; empty squares: SCR controls. $^{**}p < 0.01$.

Down-regulation of endogenous GDNF after a long history of excessive drinking does not affect alcohol consumption – After a long history of excessive drinking, we found that alcohol continues to up-regulate *GDNF* after a short, binge-like" drinking episode (Figure 2-4). Since we found that blocking the alcohol-induced increase

in *GDNF* during the early stages of drinking results in rapid escalation of alcohol consumption (Figure 2-5A and 2-6B), we tested whether attenuation of the up-regulation of *GDNF* after "binge-like" drinking also alters alcohol intake. Adenovirus encoding shGDNF, or SCR, was infused into the VTAs of rats that had been trained to drink and excessive amount of alcohol in the intermittent-access paradigm. After allowing 11 days for surgical recovery and expression of the shGDNF construct, rats were re-exposed to alcohol in the same two-bottle choice procedure, and alcohol intake was measured. We found that, after a long-term history of high levels of alcohol drinking, attenuation of the "binge"-mediated increase in *GDNF* levels did not affect the amount of alcohol consumed (Figure 2-6C).

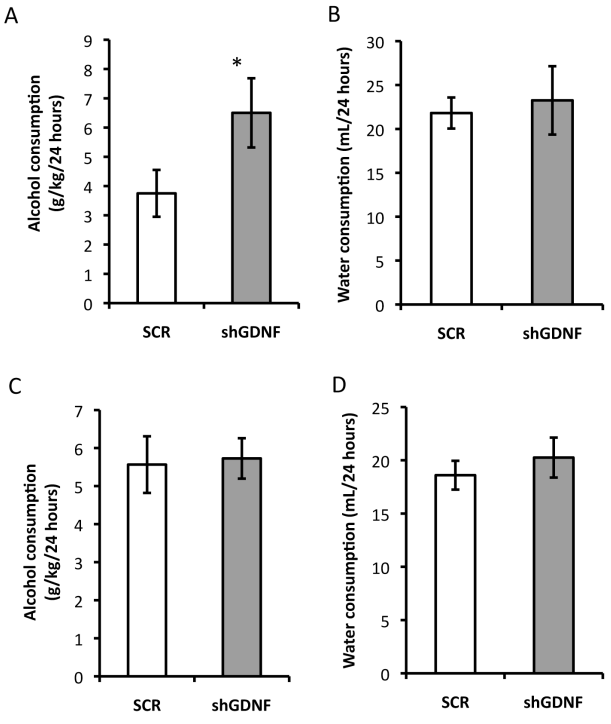

Figure 2-6. Down-regulation of *GDNF* enhances alcohol consumption in alcohol-naïve rats, but not rats trained to consume excessive amounts of alcohol. Adenovirus expressing shRNA against GDNF (shGDNF) or a scrambled control sequence (SCR) was infused into the VTA of rats. Alcohol consumption was measured in the intermittent-access to 20% alcohol two-bottle choice paradigm 11-13 days after the infusion of the virus. Knock-down of *GDNF* enhanced alcohol intake **(A)** in alcohol-naïve rats, but did not affect water consumption **(B).** Virus was infused into the VTAs of rats that had not been previously exposed to alcohol, n = 9 per group, $*p < 0.05$, compared to SCR controls. **C & D,** Down-regulation of *GDNF* does not alter alcohol **(C)** or water **(D)** consumption in rats trained to consume alcohol in the intermittent-access two-bottle choice procedure. Virus was infused into the VTA of rats that had been trained to drink excessive amounts of alcohol for at least 6 weeks, n = 7 per group.

Discussion

Here, we report the effects of alcohol on the expression levels of GDNF, a protein that we have previously demonstrated to exogenously lower the consumption of alcohol, as well as endogenously attenuate the rewarding properties of alcohol. We found that a single administration, as well as short-term voluntary drinking of alcohol results in an up-regulation of *GDNF* in the VTA (Figures 2-1A and 2-2A). We also found that shRNA-mediated blockade of this alcohol-mediated up-regulation of *GDNF* expression in the VTA facilitates the escalation of alcohol drinking in the intermittent-access two-bottle choice drinking model (Figure 2.5A). In addition, we observed that a period of deprivation after long-term, repeated bouts of excessive alcohol drinking results in a significant down-regulation of *GDNF* mRNA in the VTA (Figure 2-3A). However, after a short, "binge-like" drinking period following deprivation, *GDNF* levels are significantly increased (Figure 2-4), and normalized to control, basal levels by the end of the 24-hour drinking session (Figure 2-2A). Together, these results demonstrate that GDNF is an alcohol-responsive gene in the VTA, and its response is dependent on alcohol-drinking history. Expression of *GDNF* is up-regulated by initial exposures to alcohol to mediate the progression of excessive alcohol consumption. However, a long history of high levels of alcohol drinking results in the dysregulation of *GDNF* expression, possibly contributing to the maintenance of high levels of alcohol consumption (Figure 2-7).

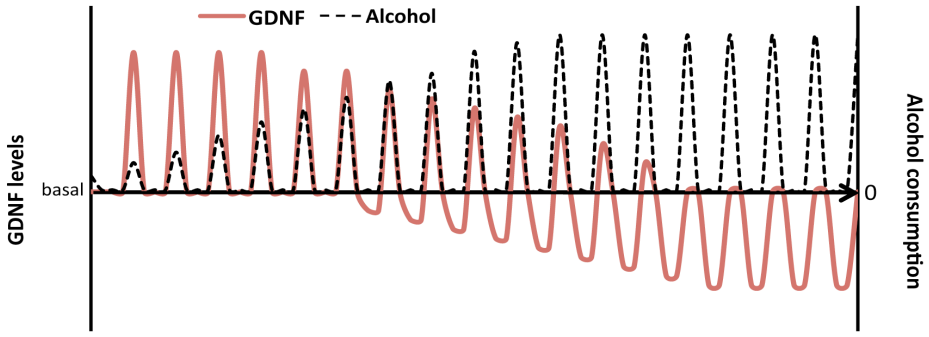

Figure 2-7. Model: *GDNF* levels are altered by alcohol consumption in a drinking history-dependent manner. During early alcohol (EtOH) drinking experiences, *GDNF* is up-regulated and acts to regulate the escalation of excessive drinking in the intermittent-access drinking model. Over time, as EtOH intake increases, endogenous control over basal *GDNF* expression becomes dysregulated, resulting in abnormally low levels of *GDNF* during alcohol deprivation. *GDNF* levels then normalize to former basal levels over the course of a drinking session. In this model, *GDNF* levels are again decreased during subsequent deprivation periods, contributing to the potentiation of drinking behaviors, resulting in lifetime-long, repeated cycles of withdrawal and relapse.

GDNF is an alcohol-responsive gene, and plays a protective role during the early, but not later, stages of drinking – Alcoholism manifests and progresses gradually over time. Furthermore, the development of alcohol dependence is not an end result of every incidence of alcohol drinking. For both of these reasons, it is highly likely that there are endogenous mechanisms, activated by alcohol intake, which inherently protect against the development of this disease. Indeed, we have previously identified such a role for brain-derived neurotrophic factor (BDNF). *BDNF* is induced in the presence of alcohol to reduce alcohol intake (Jeanblanc et al., 2009; McGough et al., 2004). Based on our current findings, we propose that GDNF is another such homeostatic mediator of alcohol intake. We observed an induction of *GDNF*, a known negative regulator of alcohol intake, after both a single systemic administration, and after a short period of voluntary intake of alcohol. Attenuation of this alcohol-mediated up-regulation of *GDNF* facilitated the escalation of alcohol intake during the same short period of voluntary

alcohol drinking. This strongly suggests that the GDNF signaling pathway is activated during the early stages of alcohol drinking to suppress excessive alcohol intake.

Interestingly, the alcohol-mediated up-regulation of *GDNF* during the later stages of drinking does not appear to play the same role in the regulation of alcohol consumption (Figure 2-6C). In fact, down-regulation of the alcohol-induced *GDNF* did not have any affect on alcohol intake. It is possible that, after a long history of excessive drinking, the physiological upper limit of voluntary alcohol consumption has been reached. Consequently, any increase in alcohol consumption would be occluded by this "ceiling effect." Another possibility is that other neuroadaptations resulting from a long history of excessive alcohol consumption intercept the effect of *GDNF* down-regulation on alcohol drinking. Indeed, such neuroadaptationa may well undelie a "ceiling effect" of voluntary consumption. Regardless, if alcohol-induced *GDNF* does indeed play a role in the regulation of the amount of alcohol consumed after a long history of excessive drinking, it is possible that it simply may not be discerned using this particular drinking model.

Deprivation-induced deficits in GDNF levels contribute to the maintenance of excessive alcohol drinking – Maintaining sobriety is a major hurdle for recovering alcoholics. In their allostatic model of addiction, Koob and LeMoal propose that chronic, repeated use of drugs of abuse, including alcohol, results in an allostasis of the hedonic affective state, which contributes to craving and the risk of relapse (Koob and Le Moal, 2001). This suggests that there are long-lasting, alcohol-induced neuroadaptations that alter the affective state and drive the motivation to further consume alcohol, even after the rewarding effect have dissipated, in order to regain psychosomatic normalcy (Koob

and Le Moal, 2001). We found that *GDNF* levels are abnormally low during a period of deprivation that follows long-term, repeated, excessive alcohol intake. Interestingly, a similar observation was recently made in humans. Heberlein *et al.* reported a long-lasting decrease in GDNF serum levels in human alcoholics during withdrawal from alcohol (Heberlein et al., 2010). A central function of GDNF is the maintenance of dopaminergic tone in the mesolimbic system (Wang et al., 2010). As such, deficits in GDNF levels during deprivation, such as we report here and those reported by Heberlein and colleagues (Heberlein et al., 2010), may contribute to the decrease in dopamine levels observed in the NAc of alcohol-dependent rats during withdrawal (Barak et al., 2011; Diana et al., 1993; Rossetti et al., 1992; Weiss et al., 1996). Because the neurotransmitter dopamine plays a key role in goal-directed behaviors and regulation of mood, it is possible that a down-regulation of *GDNF* levels underlies, in part, the alcohol-induced allostatic hedonic affective state. We propose that the abnormally low level of *GDNF* observed during deprivation is one example of a long-lasting neuroadaptation induced by alcohol, and is likely to contribute to the withdrawal-associated dopamine deficiency. The latter, in turn, may be associated with an abnormally depressed mood state and a susceptibility to relapse (Koob and Le Moal, 2001; Weiss et al., 1996). We also found that "binge-like" alcohol intake during the first 30 minutes of a drinking session that follows a deprivation period induces *GDNF* expression in the VTA, which then normalizes to basal levels at the end of the 24-hour drinking session. This suggests that although long-term, excessive consumption of alcohol has lowered the basal levels of *GDNF* in the absence of alcohol, *GDNF* continues to be up-regulated when alcohol is present. Therefore, one possible incentive for the continued consumption of alcohol is

that it functions to recover the deprivation-induced deficit in GDNF, and, by extension, dopaminergic tone and its associated elevated emotional affective state, to former baseline levels.

***Does exogenous intra-VTA GDNF reduce alcohol intake by reversing the deprivation-induced deficit in VTA GDNF levels?* –** Our finding that alcohol increases *GDNF* expression in the VTA, as opposed to the NAc, is in agreement with our previous studies localizing GDNF's ability to lower alcohol consumption to the VTA (Carnicella et al., 2009c; Carnicella et al., 2008). Specifically, infusion of the protein into the VTA of rats significantly lowered operant responding for alcohol, and blocked excessive intake and relapse. Importantly, in these studies, the GDNF protein was infused after a period of deprivation, during which, according to the observations made here, *GDNF* levels within the VTA are abnormally low. We therefore propose that the exogenous protein replaces the deficit in endogenous *GDNF*, negating the incentive to consume alcohol to up-regulate *GDNF*, and thus corresponding to a decrease in alcohol consumption.

***Molecular consequence of alcohol-induced GDNF* –** GDNF, via activation of its receptor Ret, leads to the activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, subsequently activating the transcription factor cAMP response element binding protein (CREB) in the nucleus (Hayashi et al., 2000). Thus, GDNF signaling plays a role in the alteration of gene expression (Hayashi et al., 2000; Jongen et al., 2005; Trupp et al., 1999). Notably, GDNF signaling was found to modulate the expression of genes involved in synaptic plasticity, including the calcineurin subunits ppp3R1 and pppC3B, and calcium-calmodulin-dependent protein kinase II-β (CaMKIIβ) (Consales et al., 2007), as well as the calcium binding proteins frequenin (Wang et al.,

2001) and calbindin (Wang et al., 2008), all of which function in synaptic transmission. Thus, a role for alcohol-induced *GDNF* in the up-regulation of these genes, in addition to a direct role influencing dopaminergic activity (Wang et al., 2010), cannot be ruled out. Thus, the molecular consequence(s) of the alcohol-mediated up-regulation of *GDNF* warrants further investigation.

Summary

This chapter identifies *GDNF* as an alcohol-responsive gene, and describes its role in the progression and maintenance of alcohol drinking. Our results indicate that the alcohol-mediated up-regulation of *GDNF* expression is part of a homeostatic pathway, activated by alcohol to endogenously regulate alcohol consumption in the early stages of drinking. Furthermore, long-term, repeated bouts of excessive alcohol intake results in a dysregulation of basal *GDNF* expression. Of further interest to us is the mechanism of both the alcohol-mediated up-regulation and subsequent aberrant regulation of *GDNF* levels. In the next chapter, possible molecular pathways that may underlie these alcohol-induced effects are identified.

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CHAPTER 3

DRIVING GDNF EXPRESSION: INVOLVEMENT OF THE DOPAMINE D2 RECEPTOR & THE TRANSCRIPTION FACTORS ZIF268 & PITX3

Abstract

We previously demonstrated that glial cell line-derived neurotrophic factor (GDNF) is an alcohol-responsive gene in the ventral tegmental area (VTA), with its expression levels increasing during early alcohol drinking experiences, and ultimately decreasing below basal levels during alcohol deprivation after a long history of excessive drinking (Chapter 2). We set out to determine the molecular mechanism underlying the alcohol-mediated alterations in *GDNF* expression. We found that voluntary alcohol consumption increased levels of the transcription factor Zif268, whose genomic targets include the *GDNF* promoter region (Shin et al., 2009), in the VTA. Additionally, a single systemic administration of alcohol was found to induce a rapid up-regulation of *Zif268* mRNA in the VTA, which was followed at a later timepoint by an increase in *GDNF* levels. We also observed that activation of the dopamine D2 receptor (D2R), via systemic administration of the D2R agonist quinpirole, rapidly up-regulated *Zif268* levels in the VTA. In line with these results, we found that treatment of the dopaminergic-like SH-SY5Y cell line with quinpirole led to the up-regulation of *Zif268*, which was followed by increases in *GDNF*. Moreover, the D2R-mediated increase in *Zif268* levels was due to a Gβγ-dependent activation of the ERK1/2 signaling pathway. Together, these results highlight a potential mechanism by which dopaminergic activity in response to alcohol consumption can regulate *GDNF* expression. In addition, we observed a deprivation-associated decrease in the midbrain-specific (Korotkova et al., 2005) transcription factor Pitx3, which is known to maintain *GDNF* expression in the midbrain, including the VTA (Peng et al., 2007). This suggests that dysregulation of *GDNF*

expression after a long history of alcohol consumption may be a result of a down-regulation of *Pitx3*.

Introduction

We found that *GDNF* expression levels in the rat VTA change throughout its drinking history (Chapter 2). First, *GDNF* levels are increased during early alcohol drinking episodes, and we demonstrated that this up-regulation of *GDNF* mediates, in part, the escalation of alcohol drinking (Chapter 2). Chronic, excessive alcohol intake subsequently causes a decrease in basal *GDNF* levels during deprivation, possibly incentivizing continued drinking, as *GDNF* expression was normalized back to control, alcohol-naïve levels following a drinking session (Chapter 2). We set out to elucidate the molecular mechanism of 1) the dysregulation of *GDNF* expression following chronic alcohol drinking; and 2) the up-regulation of *GDNF* during short-term alcohol drinking.

To date, while the regulation of *GDNF* expression in the developing kidney is very well understood and characterized, little is known about the postnatal control of *GDNF* expression in the brain (Saavedra et al., 2008). The expression of neurotrophic factors is regulated by neuronal activity (Hughes et al., 1999), suggesting that, in the mesolimbic system, dopamine may play a role in the expression of *GDNF*. In support of this, *GDNF* levels are 40-50% lower in the mesolimbic and mesostriatal regions of dopamine D2 receptor knockout ($D2R^{-/-}$) mice (Bozzi and Borrelli, 1999; Saavedra et al., 2008). Conversely, *in vitro* treatment of mesolimbic neuronal (Guo et al., 2002) or astrocytic (Ohta et al., 2004; Ohta et al., 2010; Ohta et al., 2003; Ohta et al., 2000) cultures with D2R agonists results in an up-regulation of *GDNF* (Saavedra et al., 2008).

Together, these findings indicate that dopamine signaling via the D2R regulates *GDNF* expression.

Alcohol, while also inducing dopamine release into the nucleus accumbens (NAc) from the VTA (Di Chiara and Imperato, 1988), has been shown to cause an increase in the somatodendritic release of dopamine locally, within the VTA (Campbell et al., 1996; Kohl et al., 1998; Xiao et al., 2009). This release of dopamine would consequently activate D2Rs in the VTA (Adell and Artigas, 2004; Mansour et al., 1990; Perra et al., 2011). Activation of the D2R, a G-protein coupled receptor (GPCR) leads to the inhibition of adenylate cyclase via the $G\alpha_i$ subunit, blocking the formation of cyclic adenosine monophosphate (cAMP) (Neve et al., 2004). In addition, activation of the D2R GPCR results in the activation of the ERK1/2 signaling pathway via a mechanism involving the $G\beta\gamma$ subunits (Beaulieu and Gainetdinov, 2011; Choi et al., 1999; Faure et al., 1994; Ghahremani et al., 2000). A well-known function of the ERK1/2 pathway is the activation of gene expression (Davis, 1995).

Gene expression may be controlled at several steps, including transcription initiation and elongation, and mRNA processing, stability and translation, however, most regulation is thought to occur at the level of transcription initiation (Maston et al., 2006; McKnight et al., 1982). Importantly, a prominent and well-known consequence of the ERK1/2 signaling pathway is the activation of numerous transcription factors (Davis, 1995), which bind to upstream transcriptional regulatory sequences in genomic DNA, either alone or in complex with others, to promote or block the recruitment of RNA polymerase II to a gene, and thus up- or down-regulating the expression of a

gene(Maston et al., 2006). Zif268 and Pitx3 are two such transcription factors that may influence the expression of *GDNF*.

Zif268 (zinc-finger protein 268), also known as early growth response protein-1 (Egr-1), belongs to the immediate-early response gene family of transcription factors (Knapska and Kaczmarek, 2004). As such, its expression is activated in the absence of *de novo* protein synthesis, and rises strikingly and transiently in response to stimuli. A recent study showed that the Zif268 transcription factor is up-regulated in response to stimulation with fibroblast growth factor-2 (FGF2), and enhances the expression of *GDNF* in cultured astrocytes (Shin et al., 2009). Moreover, Shin *et al.* demonstrated that FGF2-mediated activation of the ERK1/2 signaling pathway was required for the up-regulation of *Zif268* (Shin et al., 2009). Another recent report indicated that fibroblast growth factor-1 (FGF1) promoted neurite outgrowth in rat PC12 cells via an ERK1/2-*Zif268* mechanism, which presumably contributed to a persistence of GDNF-signaling (Lin et al., 2009). Interestingly, *Zif268* has been shown to be an alcohol-responsive gene in the central nervous system (CNS) (Canales, 2004; Depaz et al., 2000; Vilpoux et al., 2009), albeit not in the VTA, warranting further investigation into whether it plays a role in the alcohol-mediated up-regulation of *GDNF*. Because Zif268, a transcriptional activator of *GDNF*, is up-regulated downstream of ERK1/2 activation, which can be mediated by D2R signaling, we also tested whether activation of the D2R up-regulates *Zif268*, consequently promoting *GDNF* expression.

Pitx3 (paired-like homeodomain transcription factor-3 or pituitary homeobox-3) was identified by two independent groups in 1997 as the third member of the homeodomain-containing transcription factor family (Semina et al., 1997; Smidt et al.,

1997). Although *Pitx3* has a wide expression pattern during embryogenesis (it is found in skeletal muscle, as well as in the developing eye lens (Semina et al., 1997)), its postnatal expression is highly restricted to the VTA and substantia nigra (SN) regions of the midbrain (Korotkova et al., 2005; Smidt et al., 1997), where it is constitutively expressed and is important for the differentiation and maintenance of dopaminergic neurons. Notably, Pitx3 has been found to up-regulate *GDNF* expression and secretion (Li et al., 2009; Yang et al., 2008). Taken together with the fact that GDNF is a potent contributor to the maintenance of dopaminergic neurons, it is likely that Pitx3 promotes the health of these neurons via GDNF. While the effect of alcohol on *Pitx3* levels in the midbrain are unknown, genetic deletion of Pitx3 results in the selective degeneration of midbrain dopamine neurons, and a corresponding decrease in striatal dopamine levels (van den Munckhof et al., 2003). Together, this evidence provides an intriguing possibility for a Pitx3-mediated molecular mechanism that underlies the down-regulation of *GDNF* in the VTA after prolonged, repeated bouts of ethanol drinking and deprivation. We therefore investigated whether long-term, excessive alcohol drinking alters *Pitx3* expression levels in the VTA.

Materials & Methods

Reagents **–** TRIzol reagent, pre-cast SDS-PAGE gels, and running and transfer buffers were purchased from Invitrogen. Phosphatase inhibitor cocktails II and III, deoxyribonuclease (DNase) and ethidium bromide (EtBr) were purchased from Sigma. The protease inhibitor mini-tablets were purchased from Roche. The Reverse Transcription System and 2X PCR master mix were purchased from Promega.

Quinpirole, U0126, PD98059, and gallein were purchased from Tocris. The BCA Protein Assay Kit was purchased from Pierce Biotechnology. The GDNF antibody was obtained from R&D Systems. The Zif268 antibody was purchased from Cell Signaling Technology. Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-Goat and anti-Rabbit IgGs), anti-ERK2, anti-pERK1/2, and anti-GAPDH polyclonal antibodies were purchased from Santa Cruz Biotechnology. The enhanced chemiluminescence (ECL) detection reagents were purchased from GE Healthcare.

Cell culture – The human dopaminergic-like SH-SY5Y cell line was plated at a density of 2e5 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10% FBS), penicillin/streptomycin, and non-essential amino acids. Cells were differentiated into a neuronal phenotype by supplementing a 1% FBS DMEM solution containing 10 μM retinoic acid (RA) for three days, at which point the medium was changed again, to 1% FBS DMEM without RA for a further 18-24 hours. This was done to remove the RA, which can itself activate signaling pathways (such as ERK1/2) (Miloso et al., 2004), thus obscuring any effects mediated by GDNF-activated ERK1/2. At this point, cells were treated with 50 μM quinpirole in saline vehicle. For indicated pre-treatments, U0126 (10 μM), PD90859 (10 μM), or gallein (20 μM) were added to the cells 10 minutes before the addition of quinpriole (all inhibitors were dissolved in DMSO; final concentration of DMSO: 0.1%). At the end of treatments, cells were briefly washed in phospho-buffered saline (PBS), then lysed and collected in TRIzol reagent.

Animals – Adult male Long-Evans rats (250-300g at the beginning of experiments) were purchased from Harlan. Animals were individually housed with food

and water available *ad libitum*, constant temperature (23°C) and humidity (50%), and a 12-hour light-dark cycle (lights on at 7:00AM). All experimental protocols were approved by the Ernest Gallo Research Center Institutional Animal Care and Use Committee (IACUC), and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Intermittent-access 20% alcohol two-bottle choice – Following a one-week habituation to the animal housing facility, rats were started on the intermittent-access two-bottle choice drinking paradigm as previously described (Carnicella et al., 2009b; Simms et al., 2008). Briefly, rats were given a bottle containing an alcohol solution (20% v/v in tap water), in addition to a bottle of tap water, every other day for a total of three, 24-hour-long alcohol drinking sessions per week (i.e.: Monday, Wednesday, and Friday). Control subjects were given an additional bottle of water in place of the alcohol solution. Rats were sacrificed and the VTA collected immediately following, or 24 hours after (deprivation), the end of the last alcohol drinking session, as noted. Following dissection, tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. Frozen tissues were mechanically homogenized in TRIzol reagent.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) –Total RNA was isolated from cell lysates or tissue homogenates according to the manufacturer's recommended protocol. Following DNase treatment, messenger RNA (mRNA) was selectively reverse transcribed into cDNA using the Reverse Transcription System with oligo(dT) primers. For the PCR, amplifications of *GDNF, Zif268, Pitx3* and the housekeeping gene, *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH),* were conducted with the resulting cDNA using the following primers: rat *GDNF* upstream: 5'-

GAC GTC ATG GAT TTT ATT CAA GCC ACC -3'; rat *GDNF* downstream: 5'- CTG GCC TAC TTT GTC ACT TGT TAG CCT -3'; human *GDNF* upstream: 5'- TGC CAG AGG ATT ATC CTG ATC AGT TCG ATG -3'; human *GDNF* downstream: 5'- GAT ACA TCC ACA CCT TTT AGC GGA ATG CTT -3'; rat *Zif268* upstream: 5'- AGG TCT CCC TGT TGT TGT GG -3'; rat *Zif268* downstream: 5'- TGC ACC CAC CTT TCC TAC TC -3' ; human *Zif268* upstream: 5'- TGA CCG CAG AGT CTT TTC CT -3'; human *Zif268* downstream: 5'- TGG GTT GGT CAT GCT CAC TA -3'; rat *Pitx3* upstream: 5'- GAG CAC AGT GAC TCG GAG AAG G -3'; rat *Pitx3* downstream: 5'- AAG GCG AAC GGG AAG GTC -3'; rat/human *GAPDH* upstream: 5'- TGA AGG TCG GTG TCA ACG GAT TTG GC -3'; rat/human *GAPDH* downstream: 5'- CAT GTA GGC CAT GAG GTC CAC CAC -3'. For *GDNF,* 33-35 amplification cycles were used, while 27 cycles were used for *Zif268, Pitx3* and *GAPDH.* PCR products were resolved on 1.8% agarose gels supplemented with 0.05% EtBr for visualization under UV light. Images were captured using Eagle Eye 2 software. Band intensities were quantified using NIH ImageJ software.

Western blot analysis – Cells were lysed and collected in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 120 mM NaCl, 1% NP-40, 0.1% deoxycholate, and 0.5% SDS) containing protease and phosphatase inhibitors. Samples were briefly sonicated, and allowed to rest on ice for 30 minutes. Protein concentrations were determined using the BCA Protein Assay kit. Equal amounts of total protein from each sample were resolved on NuPAGE 10% Bis-Tris gels (for Zif268 and ERKs 1/2) or 16% Tris-Glycine gels (for GDNF), and transferred onto nitrocellulose membranes (Millipore). For phosphorylated ERK1/2 (pERK1/2) and ERK2

detection, membranes were first probed for pERK1/2, then stripped (25 mM glycine-HCl, 1% SDS, pH 3, for 30 minutes at room temperature) and reprobed for ERK2. The primary antibodies for protein detection used were as follows: anti-GDNF (1:250), anti-GAPDH (1:5000), anti-ERK2 (1:2000), anti-pERK1/2 (1:2000), and anti-Zif268 (1:500). HRP-conjugated secondary antibodies (1:1000) were used to detect immunoreactivity via an enhanced chemiluminescent reaction. Images were developed on Kodak film, and digitally scanned for densitometric quantification using NIH ImageJ software.

Statistical analysis – Statistical significance between two groups were determined by Student's t-test. One- or two-way analysis of variance (ANOVA) were used to compare multiple groups. Significant main effects or interactions of the ANOVAs were further investigated *post-hoc* using the Bonferroni or Student-Newman-Keuls tests.

Results

The levels of the transcription factor Zif268 are increased in response to alcohol exposure in vivo – In chapter 2, we found that either a single, systemic administration or short-term voluntary alcohol drinking induces the expression of *GDNF* in the VTA of rats. We set out to determine the molecular mechanism of this alcohol-mediated increase. We first tested the effect of an acute administration of alcohol on the mRNA levels of the inducible transcription factor, *Zif268*, in the VTA. Rats were injected i.p. with 1.8 g/kg of alcohol (or an equivalent volume of saline), and VTA tissue collected 0.5, 2, 4, and 10 hours later. We found a significant, rapid and transient induction of *Zif268* after a single acute exposure to alcohol (Figure 3-1A). Interestingly, the up-regulation of *Zif268* by alcohol occurred at a time point (0.5 hours) that preceded

the time point at which we observed the increase in *GDNF* expression (Figure 2-1A), providing temporal support of our hypothesis that the alcohol-induced *GDNF* increase occurs via a Zif268-mediated mechanism. Moreover, we observed the same up-regulation of *Zif268* in rats that voluntarily consumed alcohol in the intermittent-access two-bottle choice model for one week (Figure 3-1B), indicating that a short-term, physiologically-relevant exposure to alcohol activates Zif268, which, in turn, may cause the subsequent increase in *GDNF* expression that we observed previously (Figure 2-2A).

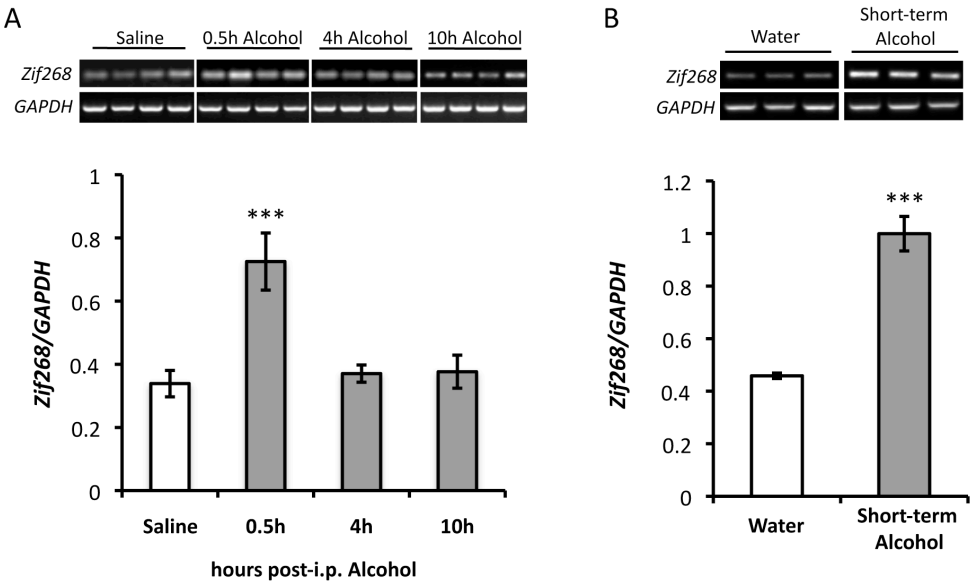

Figure 3-1. *In vivo* alcohol exposure up-regulates *Zif268* expression in the rat VTA. A, Rats were given a single, acute systemic administration of alcohol (1.8 g/kg), and the VTAs were collected 0.5, 4, and 10 hours later to assess *Zif268* levels. Bar graph represents the mean *Zif268*/*GAPDH* ± SEM, n = 4-6. **B,** Rats voluntarily drank from a 20% alcohol solution in the intermittent-access two-bottle choice model for one week. The VTAs were collected immediately following the end of the last alcohol-drinking session, and *Zif268* mRNA levels were measured by RT-PCR. Data are represented as the average *Zif268*/*GAPDH* ± SEM, n = 3 per group. ****p < 0.001*, compared to saline-injected or water-only controls.

Activation of the D2R results in Zif268 up-regulation in vitro and in vivo – Next, we set out to determine the means by which *Zif268* may be induced in the VTA. We hypothesized that activation of Zif268 is a consequence of D2R-mediated signaling.

We first tested whether D2R activation would increase *Zif268* expression and protein levels in the human dopaminergic-like SH-SY5Y cell line. Cells were treated with the D2R agonist, quinpriole (50μM), for 30, 60, and 240 minutes, and RT-PCR and Western blot analyses were used to determine Zif268 mRNA and protein levels, respectively. As shown in Figure 3-2A, there was a significant and transient increase in the expression levels of *Zif268*. The increase in *Zif268* at 30 minutes was followed by a corresponding increase in the amount of the protein at 60 minutes (Figure 3-2B), indicating that activation of D2R signaling results in the up-regulation of Zif268. To test whether D2R activation induces *Zif268* in the VTA, rats were systemically treated with quinpirole (1 mg/kg i.p., in saline vehicle), and the VTA tissue was dissected 30 minutes later for RT-PCR analysis. *Zif268* levels were significantly elevated in the VTA as a result of D2R activation (Figure 3-2C). Interestingly, this effect was localized to the VTA region of the mesolimbic system, as *Zif268* was not up-regulated in the NAc (Figure 3-2C), where D2Rs are also expressed (Mansour et al., 1990). These results indicate that D2R-mediated signaling *in vivo* plays a role in the up-regulation of *Zif268* in a region-specific manner. Furthermore, because D2R activation increases Zif268 levels in the SH-SY5Y cell line, it is a viable model for further characterizing this signaling pathway.

The D2R-mediated increase in Zif268 in vitro is followed by the up-regulation of GDNF expression and protein levels – In alcohol-treated rats, we observed an increase in *Zif268* (Figure 3-1A) that preceded the alcohol-mediated up-regulation of *GDNF* (Figure 2-1A). Because D2R activation up-regulated *Zif268,* we next tested

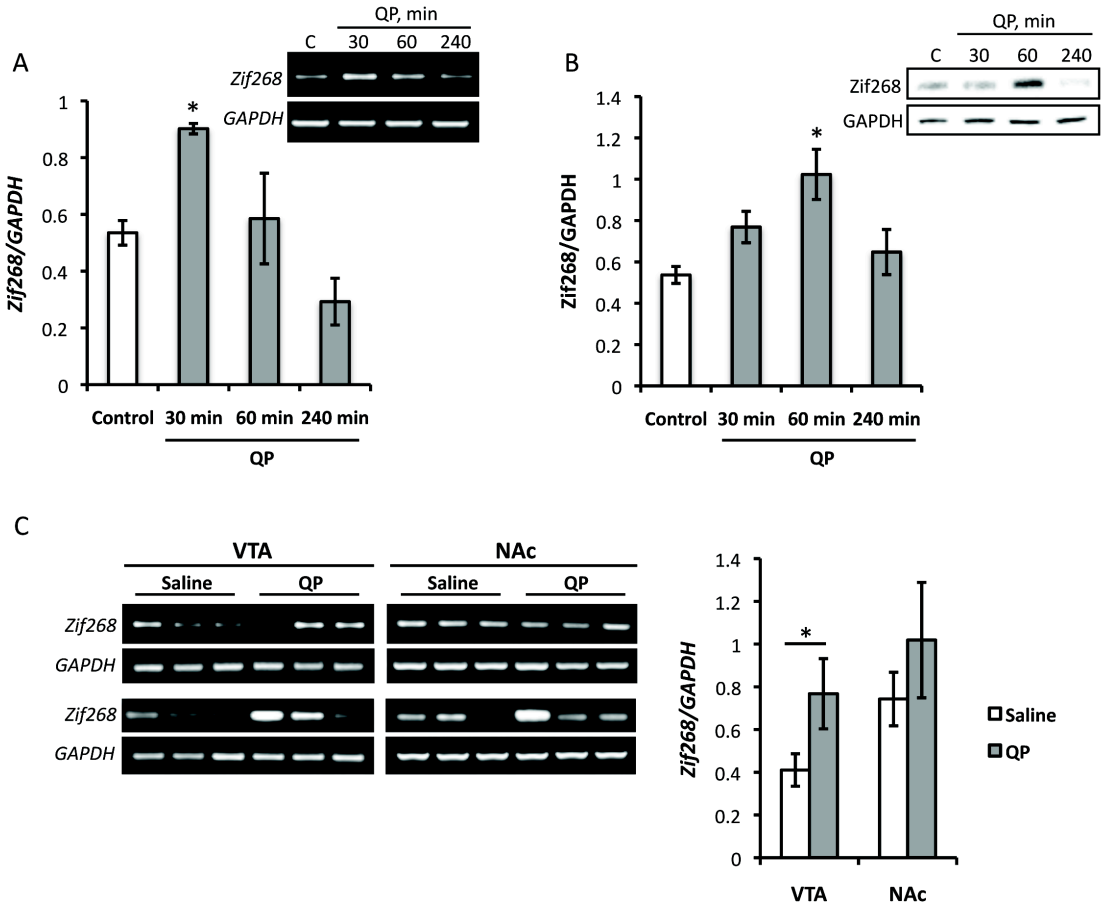

Figure 3-2. D2R activation up-regulates *Zif268* levels *in vitro* and *in vivo*. A & B, Treatment of SH-SY5Y cells with 50μM quinpirole results in an up-regulation of *Zif268* mRNA **(A)**, followed by an increase in Zif268 protein levels **(B)**. Data are represented as the average Zif268/GAPDH ± SEM, n = 4 independent experiments. **C,** Systemic quinpirole (1 mg/kg, i.p.) increased the level of *Zif268* in the VTA, but not the NAc, of rats. VTA and NAc tissues were collected 30 minutes after the quinpirole treatment for the evaluation of *Zif268* mRNA levels, n = 6 animals per treatment group. *$p < 0.05$, compared to saline-treated controls.

whether activation of the D2R would cause an increase in *GDNF*, and whether it occurred after the D2R-mediated increase in Zif268. SH-SY5Y cells were treated with quinpirole (50 μM) for 30, 60, and 240 minutes, and GDNF mRNA and protein levels were assessed. As shown in Figure 3-3A, after a 240-minute quinpirole treatment, there was a significant increase in the expression levels of *GDNF*. There was also a corresponding increase in GDNF protein levels (Figure 3-3B) indicating that GDNF

levels were increased after D2R activation. Importantly, the D2R-mediated up-regulation of GDNF occurred after the observed increases in Zif268 (Figure 3-2A and B). This suggests that D2R signaling first causes an increase in Zif268, which is followed by an up-regulation of GDNF, a genomic target of Zif268.

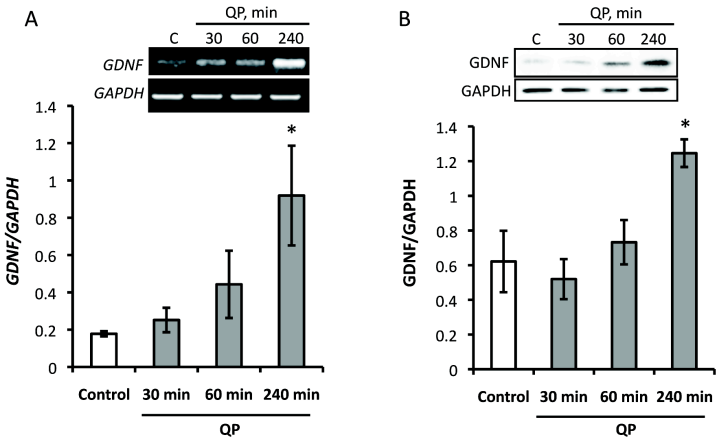

Figure 3-3. Activation of the D2R results in up-regulation of GDNF levels. Treatment of SH-SY5Y cells with quinpriole (50 μM) significantly increases *GDNF* mRNA **(A)**, and protein levels **(B)**, n = 4. Data are shown as the mean GDNF/GAPDH ± SEM. *$p < 0.05$, compared to saline-treated control.

D2R activation results in a rapid, Gβγ-dependent activation of the ERK1/2 signaling pathway in vitro – To further characterize the signaling pathway involved in the D2R-mediated up-regulation of *Zif268,* we tested whether ERKs 1/2 were activated in response to D2R signaling. We treated SH-SY5Y cells with 50 μM quinpriole for 5, 15, and 30 minutes, and assessed the levels of phosphorylated, and thus activated, ERK1/2. Activation of the D2R resulted in an increase in the levels of phosphorylated ERK1/2 after 15 minutes (Figure 3-4A). Because the activation of the D2R has been suggested to activate ERK1/2 via its Gβγ subunit complex (Beaulieu and Gainetdinov, 2011; Choi et al., 1999; Faure et al., 1994; Ghahremani et al., 2000), we hypothesized that the quinpirole-mediated activation of the ERK1/2 signaling

pathway occurs via the Gβγ subunits of this G-protein coupled receptor. To test this possibility, we pre-treated SH-SY5Y cells with the Gβγ inhibitor gallein (20μM in DMSO vehicle) for 10 minutes prior to treatment with quinpirole. As shown in Figure 3-4B, inhibition of Gβγ-mediated signaling blocked the activation of the ERK1/2 pathway by quinpriole. Thus, in the dopaminergic-like SH-SY5Y cells, activation of the D2R subsequently activates the ERK1/2 signaling pathway, in a Gβγ-dependent mechanism.

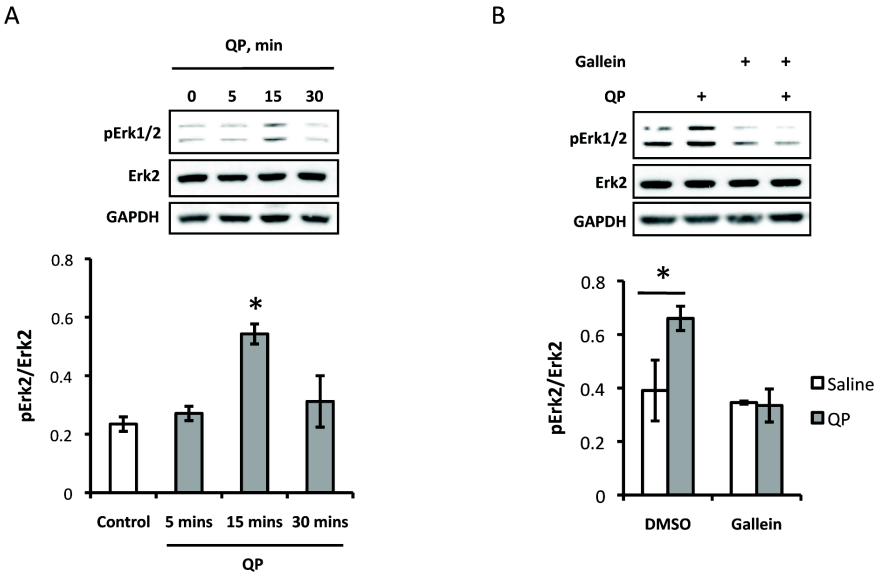

Figure 3-4. Activation of D2R signaling increases ERK pathway activity in a Gβγ-dependent manner. A, SH-SY5Y cells were treated with quinpirole (50 μM), and Western blot analysis was used to assess pERK2 levels. **B,** Cells were pre-treated with the Gβγ inhibitor gallein (20 μM) for 10 minutes prior to the addition of quinpirole. Data are represented as mean pERK2/ERK2 ± SEM, n = 4. *$p < 0.05$, compared to saline control.

The D2R-mediated up-regulation of Zif268 in vitro is Gβγ- and ERK1/2-dependent – Because *Zif268* expression has been shown to be a downstream consequence of ERK1/2 signaling, we hypothesized that the D2R-mediated activation of ERK1/2 (Figure 3-4) is required for up-regulation of *Zif268* in response to quinpirole treatment. We therefore pre-treated SH-SY5Y cells with the U0126 and PD98059 compounds, both of which specifically inhibit the mitogen-activated protein kinase (MAPK) kinase (MEK)

that phosphorylates, and thus activates, ERK1/2. Treatment of the SH-SY5Y cells with quinpirole in the presence of either U0126 or PD98059 attenuated the D2R-mediated induction of *Zif268* (Figure 3-5). We also tested whether Gβγ-mediated signaling was required for the up-regulation of *Zif268* in response to quinpriole treatment. As shown in Figure 3-5, in the presence of the Gβγ inhibitor, gallein, the D2R-mediated increase in *Zif268* was blocked. Together with the observation that D2R-mediated activation of ERK1/2 is Gβγ-dependent (Figure 3-4B), these results indicate that one consequence of the D2R-Gβγ-ERK1/2 signaling pathway is the up-regulation of *Zif268*.

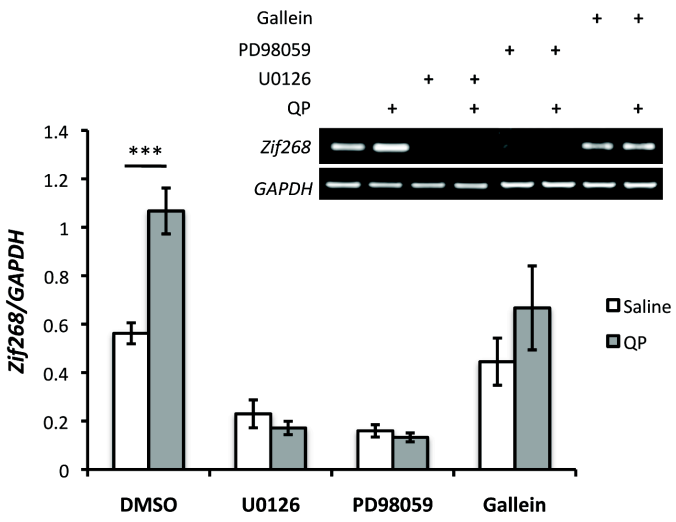

Figure 3-5. Gβγ- and ERK1/2-mediated signaling is required for D2R-induced up-regulation of *Zif268*. SH-SY5Y cells were given a 10-minute pre-treatment with the MEK inhibitors U0126 (10 μM) or PD98059 (10 μM), or the Gβγ inhibitor, gallein (20 μM), before treatment with quinpriole (50 μM for 30 minutes). RT-PCR was used to measure *Zif268* and *GAPDH* expression levels. Data is represented as the mean *Zif268*/*GAPDH* ± SEM, n = 4. ***$p < 0.001$, compared to saline control.

The expression of the midbrain-specific transcription factor Pitx3 is decreased during deprivation after a long history of voluntary alcohol intake – Previously, we noted that *GDNF* expression in the VTA is abnormally lowered during a deprivation period following a long history of excessive alcohol drinking (Figure 2.3A). We therefore set out to determine what factors might contribute to this disregulation of *GDNF*. We hypothesized that Pitx3, a constitutively-expressed, midbrain-specific transcription factor,

plays a role in the maintenance of *GDNF* expression in the VTA, and that a deprivation-associated decrease in *Pitx3* could result in the down-regulation of *GDNF*. As in chapter 2, rats were trained over the course of several weeks to voluntarily drink high levels of alcohol in the intermittent access two-bottle choice paradigm. *Pitx3* mRNA levels in the VTA were assessed after a 24-hour deprivation period; i.e., the same time point at which *GDNF* levels were abnormally decreased (Figure 2-3A). We observed a significant decrease in *Pitx3* levels in the subjects that had experienced a deprivation period following long-term excessive alcohol consumption, as compared to the water-only controls (Figure 3-6). This deprivation-associated decrease in *Pitx3* levels, taken together with data showing that Pitx3 is not only important for dopaminergic neuron health, but also contributes to *GDNF* expression, suggests that a down-regulation of *Pitx3* may underlie the deprivation-induced decrease in basal *GDNF* levels.

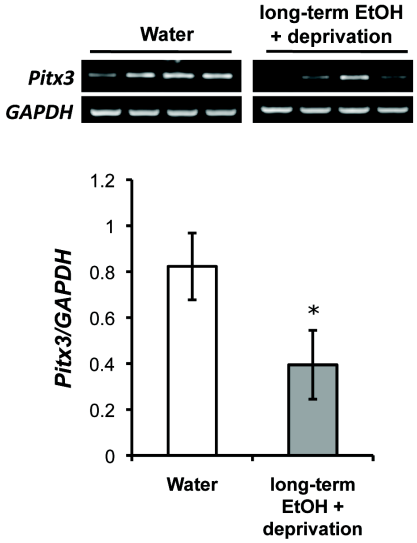

Figure 3-6. The transcription factor, *Pitx3,* is down-regulated during a period of deprivation following a long history of excessive voluntary alcohol consumption. Rats voluntarily consumed excessive amounts of alcohol in the intermittent access to 20% alcohol two-bottle choice model over a period of several weeks. The VTA was collected after a 24-hour deprivation period, and *Pitx3* mRNA levels were assessed by RT-PCR. Bar graph represents mean *Pitx3/GAPDH* ± SEM, n = 4 per group. *$p < 0.05$, compared to water control.

Discussion

Here, we identified two putative mechanisms that may underlie the alcohol-induced alterations in *GDNF* expression in the VTA that are detailed in Chapter 2. First, we observed a rapid, alcohol-mediated increase in *Zif268* expression (Figure 3-1A), which followed a single, acute systemic administration of alcohol and preceded the alcohol-induced up-regulation of *GDNF* (Figure 2-1A), as well as an increase in *Zif268* levels following short-term voluntary alcohol drinking (Figure 3-1B). Finally, the D2R agonist quinpirole up-regulated *Zif268 in vivo* in the VTA of rats, as well as *in vitro* in the human dopaminergic-like SH-SY5Y cell line (Figure 3-2). Importantly, the increase in *Zif268* levels *in vitro* was followed by a significant up-regulation of *GDNF* (Figure 3-3). Furthermore, the quinpriole-mediated increase in *Zif268* levels *in vitro* was found to be a consequence of D2R-Gβγ-ERK1/2 signaling (Figures 3-4 & 3-5). Together, these results put forward a putative mechanism for the alcohol-induced up-regulation of *GDNF,* in which alcohol-mediated somatodendritic release of dopamine in the VTA activates the D2Rs, increasing *Zif268* and its target, *GDNF* (Figure 3-7). We also found that the expression levels of *Pitx3* are reduced during deprivation (Figure 3-6), which may contribute to the dysregulation of *GDNF* that we observed during this period (Figure 2-3A).

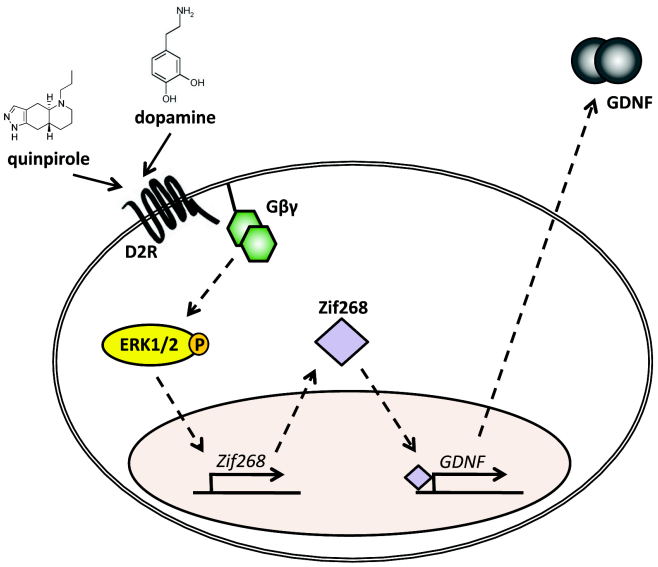

Figure 3-7. Proposed model: activation of the D2R induces *GDNF* expression in a Gβγ- and ERK1/2-dependent mechanism involving Zif268. Quinpriole or somatodendritic dopamine release in the VTA activates the D2R. The ERK1/2 pathway is then activated in a Gβγ-dependent manner. Phosphorylated ERK1/2 contributes to the up-regulation of *Zif268*. The Zif268 protein subsequently up-regulates *GDNF* expression, thus increasing GDNF protein levels.

The D2R-Gβγ-ERK1/2-Zif268-GDNF pathway: Implications for endogenous GDNF expression & alcohol consumption – The findings discussed in this chapter add to our as yet unclear understanding of the detailed transcriptional regulation of endogenous *GDNF* in the nervous system. Specifically, we propose that *GDNF* is up-regulated in a mechanism that involves a D2R-Gβγ-ERK1/2-Zif68 signaling pathway (Figure 3-7). Interestingly, this finding suggests that stimuli or behavior that causes a release of dopamine in the vicinity of D2Rs might induce the expression of endogenous *GDNF*. Alterations in endogenous *GDNF* expression have been shown to have various biochemical and behavioral effects. For example, adenoviral-mediated knock-down of endogenous *GDNF* in the nucleus accumbens (NAc) resulted in a decrease in the firing rate of midbrain dopaminergic neurons (Wang et al., 2010). Extensive studies have also demonstrated that GDNF heterozygous knock-out ($GDNF^{+/-}$) mice, which express a lower than normal level of *GDNF,* display greater reward responses (as measured by CPP) to alcohol (Carnicella et al., 2009a), cocaine (Messer et al., 2000), methamphetamine (Niwa

et al., 2007b), and morphine (Niwa et al., 2007a). Together, these reports suggest that a variable level of control over endogenous *GDNF* expression is required for behavioral responses to stimuli. In support of this, Uchida and colleagues recently observed an up-regulation of the *GDNF* transcript in a mouse strain that was able to adapt to chronic daily stress (B6 mice), as compared to another mouse strain that was not (BALB mice) (Uchida et al., 2011). Importantly, over-expression of *GDNF* in the BALB mice resulted in an adaptive response to the chronic daily stress (Uchida et al., 2011), suggesting that the up-regulation of *GDNF* as a result of stress constitutes an adaptive response. In light of these studies, our proposed model for the D2R-mediated regulation of *GDNF* expression strengthens the hypothesis that dopamine signaling contributes to the expression of endogenous *GDNF*, thereby influencing neuronal activity, adaptations to stress, and consumption of drugs of abuse.

Importantly, further work is needed to verify the causal link between the increase in the Zif268 transcription factor and the up-regulation of GDNF. While we have shown strong temporal evidence in support of this connection (Figures 3-2A & B, and 3-3A & B), whether D2R-mediated up-regulation of *GDNF* is, in fact, Zif268-dependent remains to be tested. To this end, future studies will utilize RNA interference against *Zif268* to determine the requirement of this transcription factor in the D2R-mediated increase of *GDNF*. We expect that blocking the D2R-mediated increase in Zif268 will also attenuate the subsequent increase in *GDNF* expression.

Zif268 has previously been shown to be an alcohol-responsive gene in various regions of the central nervous system (CNS) (Canales, 2004; Depaz et al., 2000; Vilpoux et al., 2009), although VTA-specific changes in *Zif268* expression in response to alcohol

had not been demonstrated. Here, we show that *in vivo* exposure to alcohol induces the expression of *Zif268* in the VTA (Figure 3-1A & B), and we have posited here, and in the previous chapter, that the alcohol-induced up-regulation of *GDNF* in the VTA, possibly via Zif268, acts to regulate alcohol consumption during early drinking experiences. Therefore, if the alcohol-mediated up-regulation of *GDNF* in the VTA requires *Zif268*, then blocking the alcohol-mediated induction of *Zif268* should result in a facilitated escalation of drinking, as in Figure 2-5. Additionally, it is of interest to demonstrate that the up-regulation of *Zif268* and *GDNF* by alcohol is via D2R-mediated signaling. Thus additional future experiments will address this via systemic pre-treatment with a D2R antagonist prior to the systemic administration of alcohol, as in Figure 3-2A, with the prediction that blockade of D2R-mediated signaling will attenuate alcohol-induced *Zif268* and *GDNF*.

Putative contribution of the down-regulation of the midbrain-specific Pitx3 transcription factor to the deprivation-associated deficit in basal GDNF expression – In addition to the abnormally low levels of *GDNF* expression found during deprivation (Chapter 2), we also observed a down-regulation of the midbrain-specific Pitx3 transcription factor (Figure 3-1), one of whose genomic targets is *GDNF*. While this association suggests the likelihood of a causal relationship between the transcription factor and its target gene, what remains to be demonstrated is whether and how the deprivation-associated decrease in *Pitx3* directly contributes to the down-regulation of *GDNF*. One possibility involves the participation of microRNAs (miRNAs), small, non-coding RNA species that mediate the targeted degradation of protein-coding messenger RNAs (mRNAs), effectively lowering the levels of specific proteins (Ambros, 2001;

Kosik, 2006). Very recently, miRNAs have emerged as key players in drug, as well as alcohol, addiction (Dreyer, 2010). For example, cocaine-mediated alterations in miRNA expression have been shown to not only affect the reward value of cocaine as measured by conditioned place preference (CPP) (Chandrasekar and Dreyer, 2011), but also to regulate cocaine-mediated changes in gene expression (Chandrasekar and Dreyer, 2009; Im et al., 2010), as well as cocaine intake (Hollander et al., 2010; Im et al., 2010). In the case of alcohol, an elegant study by Pietrzykowski and colleagues demonstrated that the alcohol-induced up-regulation of miR-9 targets the mRNA transcript of an alcohol-sensitive BK channel splice variant, thus contributing to tolerance to alcohol (Pietrzykowski et al., 2008; Treistman and Martin, 2009). An effort has since been made to identify which miRNAs display altered expression patterns in the brains of human alcoholics (Lewohl et al., 2011) but thus far, most functional studies linking miRNAs to the effects of alcohol have been relegated to the realm of alcohol-related liver toxicity (Miranda et al., 2010).

Interestingly, the discovery of a negative-feedback circuit that includes Pitx3 and the midbrain-specific miRNA miR-133b suggests a possible mechanism for the deprivation-associated decrease in *Pitx3* that we report here. Kim *et al*. not only determined that miR-133b is specifically expressed in midbrain dopaminergic neurons, but that it regulates the translation of *Pitx3* (Kim et al., 2007). Additionally, miR-133b was found to be under positive transcriptional control of Pitx3 (Kim et al., 2007). Therefore, a possible mechanism of the deprivation-associated down-regulation of *Pitx3* that we report here involves an alcohol-mediated up-regulation of miR-133b. This intriguing possibility clearly warrants further investigation.

Summary

In this chapter, we have outlined two putative mechanisms to explain the changes in endogenous *GDNF* expression in the VTA as a result of alcohol exposure. First, down-regulation of the midbrain-specific *Pitx3* transcription factor may contribute to the abnormally low levels of VTA *GDNF* during a deprivation period. Second, up-regulation of *GDNF* during acute and short-term exposures to alcohol may involve a D2R-mediated, Gβγ- and ERK1/2-dependent signaling mechanism that results in the up-regulation of the *Zif268* transcription factor, whose genomic targets include *GDNF*. Together, these findings shed light on the transcriptional control of endogenous *GDNF*, which then regulates alcohol consumption (Chapter 2). What remains to be determined is via what molecular mechanism(s) GDNF might regulate alcohol consumption. In the next chapter, two putative targets of the GDNF signaling pathway are investigated.

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CHAPTER 4

DOWNSTREAM TARGETS OF GDNF-ACTIVATED ERK1/2: IDENTIFICATION AND CHARACTERIZATION

Abstract

GDNF was previously found to rapidly and ERK1/2-dependently lower operant self-administration of alcohol in rodents (Carnicella et al., 2008). The very fast effect onset indicates that this GDNF-mediated change in behavior is the result of a non-genomic mechanism. Interestingly, GDNF was found to alleviate the alcohol-induced decrease in dopamine levels (Barak et al., 2011), an effect that could underlie the GDNF-mediated cessation of alcohol drinking. Using an *in vitro* [^{3}H]-dopamine ([^{3}H]-DA) release assay, we determined that GDNF enhances the potassium chloride (KCl)-evoked [^{3}H]-DA release in the human dopaminergic-like SH-SY5Y cell line, via an ERK1/2-dependent mechanism. In order to further characterize the mechanism of the GDNF-induced decrease in alcohol consumption, we next set out to identify downstream targets of GDNF-activated ERK1/2 that may play a role in dopaminergic transmission. Using a candidate-based approach, we tested whether GDNF-activated ERK1/2 phosphorylates the A-type potassium channel subunit, K_v4.3, and/or the synaptic vesicle protein, Synapsin I (SynI). We found that GDNF did not induce phosphorylation of K_v4.3 at the predicted ERK1/2 consensus sites. Conversely, GDNF-activated ERK1/2 phosphorylated SynI, both in SH-SY5Y cells over-expressing SynI, as well as in slices prepared from rat midbrain, suggesting that this protein target of GDNF signaling might play a role in the GDNF-mediated enhancement of evoked dopamine release.

Introduction

Intra-ventral tegmental area (VTA) infusion of GDNF lowered operant self-administration of alcohol in rats via an ERK1/2-dependent mechanism (Carnicella et al., 2008). Most interestingly, this effect had a very rapid onset, requiring only 10 minutes to alter alcohol consumption (Carnicella et al., 2008), indicating that a non-genomic mechanism underlies the GDNF-mediated decrease in alcohol self-administration. While a great deal is known regarding the long-term actions of GDNF signaling, very few of GDNF's acute actions, or molecular targets of its signaling pathway, have been described. Recent microdialysis studies conducted in our lab, however, have demonstrated that GDNF infused into the VTA very rapidly induces an ERK1/2-dependent (Wang et al., 2010) increase in dopamine overflow into the nucleus accumbens (NAc). Interestingly, intra-VTA GDNF also reversed the alcohol withdrawal-associated decrease in dopamine levels in the mesolimbic circuit (Barak et al., 2011). Because dopamine transmission is a major determinant of drug- and alcohol-mediated behaviors, it follows that the GDNF-induced enhancement of dopaminergic activity may underlie the decrease in alcohol consumption mediated by this neurotrophic factor. We therefore used a candidate-based approach to identify downstream targets of GDNF-activated ERK1/2 that could affect dopamine release.

One example of the acute effect of GDNF signaling on dopaminergic activity comes from the Lu laboratory. Yang *et al*. treated cultured midbrain neurons with GDNF, and observed a rapid enhancement in both the frequency and generation of dopaminergic neuron action potentials (Yang et al., 2001). Further testing indicated that GDNF selectively reduced the inhibitory A-current (I_A), and blockade of the I_A (by application of the A-type channel blocker 4-aminopyridine [4-AP]) occluded the excitatory effect of

GDNF on both action potential frequency, as well as the latency to the first spike (Yang et al., 2001). Moreover, application of U0126 or PD98059, inhibitors of the mitogen activated protein kinase kinase (MEK, the enzyme that directly phosphorylates and activates ERK1/2), not only attenuated the GDNF-mediated inhibition of I_A, it also blocked the enhancement of dopaminergic firing by GDNF (Yang et al., 2001). Together, these experiments provided strong evidence that GDNF enhances midbrain dopaminergic activity via an ERK1/2-dependent inhibition of the I_A.

The A-type potassium channel $K_v4.3$ belongs to a family of voltage-gated potassium channels that conduct the rapidly inactivating inhibitory I_A (Bourdeau et al., 2007; Dilks et al., 1999; Jerng et al., 2004; Rudy, 1988). When activated, $K_v4.3$ prolongs the inter-action potential interval, negatively regulating neuron firing (Liss et al., 2001; Shibata et al., 2000). Inactivation of this channel subsequently contributes to an increase in neuronal excitability via an increase in the rate of action potential firing. A-type potassium channels are found as homo- or heterotetramers, containing four α-subunits (of which $K_v4.3$ is one) that comprise the main pore-forming unit, along with a host of interacting β-subunits that function in the trafficking and localization of the channel (Lai and Jan, 2006; Serodio et al., 1994). *In situ* hybridization data revealed that $K_v4.3$ mRNA is highly expressed in the ventral midbrain, including the VTA (Serodio and Rudy, 1998), and additional immunohistochemical analysis indicated that $K_v4.3$ is localized to the somatodendritic regions of midbrain dopaminergic neurons (Liss et al., 2001). Thus, it is highly likely that the GDNF-mediated increase in midbrain dopaminergic activity reported by Yang *et al*. may be due to an ERK1/2-mediated inhibition of $K_v4.3$-containing A-type potassium channels.

Remarkably, the regulation of $K_v4.3$-containing channels by ERK1/2 has not been investigated. There is, however, evidence that A-type potassium channels containing the closely-related $K_v4.2$ (which is not expressed in the VTA (Serodio and Rudy, 1998)) subunit are susceptible to direct ERK1/2-mediated phosphorylation and inhibition. Several reports from the Johnston laboratory first established that activation of protein kinase A (PKA) and protein kinase C (PKC) caused a decrease in I_A via ERK1/2 (Hoffman and Johnston, 1998, 1999; Watanabe et al., 2002; Yuan et al., 2006). Following this, Adams *et al*. reported that ERK1/2 directly phosphorylates the $K_v4.2$ subunit at amino acid residues Thr^{602}, Thr^{607}, and Ser^{616} (Adams et al., 2000). Site-directed mutagenesis, exchanging the threonine residue at position 607 with aspartate (thus mimicking phosphorylation at this position) resulted in the same electrophysiological changes elicited by ERK1/2 activation, namely, a reduction of the I_A (Schrader et al., 2005). Thus, ERK1/2 phosphroylation of $K_v4.2$ at Thr^{607} inhibits A-type potassium channel function, and enhancing neuronal activity. $K_v4.2$ and $K_v4.3$ share a high degree of amino acid sequence identity (75.8% identical overall), and are 80% identical in the 20-amino acid sequence surrounding the $K_v4.2$ Thr^{607} residue. We therefore tested whether $K_v4.3$ is a substrate of GDNF-activated ERK1/2 at this residue.

Another possible target of GDNF-activated ERK1/2 is Synapsin I (SynI), a nerve terminal protein that exists in two splice isoforms, Ia and Ib (De Camilli et al., 1983a). SynI is a synaptic vesicle-associated phosphoprotein, localized on the outer surface of synaptic vesicles (De Camilli et al., 1983b). Knock-out studies in mice implicated SynI in the modulation of presynaptic plasticity (Kushner et al., 2005) and in the mediation of synaptic vesicle exocytosis (Ryan et al., 1996). More specifically, it functions in the

regulation of the migration of synaptic vesicles from the reserve pool, distal to the synaptic terminal, to the more proximal, "readily-releasable" pool (Hilfiker et al., 1999; Turner et al., 1999). This function is due to the ability of SynI to bind to the actin cytoskeleton, an interaction that is disrupted upon phosphorylation of SynI (Bahler and Greengard, 1987; Hilfiker et al., 1999). During a depolarization event, a number of kinases, including calcium calmodulin kinase II (CaMKII) (Lin et al., 1990), cyclic adenosine monophosphate (cAMP) -dependent protein kinase A (PKA) (Bonanomi et al., 2005; Hosaka et al., 1999; Menegon et al., 2006), and the ERKs 1/2 (Jovanovic et al., 1996; Jovanovic et al., 2000; Matsubara et al., 1996), phosphorylate serine and threonine residues on SynI, releasing the synaptic vesicles containing transmitter from the actin cytoskeleton and emabling their moblization to the proximity of the synaptic terminal. ERK1/2-dependent phosphorylation of SynI was specifically shown to enhance synaptic activity, namely by increasing the number of vesicles docked at the synaptic terminal (Kushner et al., 2005). In 1996, Jovanovic *et al*. reported that two neurotrophic factors, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), induced ERK1/2 phosphorylation of SynI, increasing glutamatergic release (Jovanovic et al., 1996). Because GDNF signaling enhances both neurotransmitter release and ERK1/2 activity, we set out to test whether SynI is a substrate of GDNF-activated ERK1/2, and what effect it may have on dopamine release.

Materials & Methods

Reagents – TRIzol reagent, pre-cast SDS-PAGE gels, and running and transfer buffers were purchased from Invitrogen. Phosphatase inhibitor cocktails III and III,

deoxyribonuclease (DNase), dimethyl sulfoxide (DMSO), and ethidium bromide (EtBr) were purchased from Sigma. The protease inhibitor mini-tablets were purchased from Roche. The Reverse Transcription System and 2X PCR master mix were purchased from Promega. Wortmannin and U0126 were purchased from Tocris. The BCA Protein Assay Kit was purchased from Pierce Biotechnology. The ERK1/2-phosphorylated-SynIa/b (pSynIa/b; detects phosphorylation of sites 4 and 5, $Ser^{62,67}$) antibody was obtained from Antibodies, Incorporated. Anti-K_v4.3 antibodies were purchased from Neuromab at the University of California, Davis. Horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-Goat, anti-Mouse, and anti-Rabbit IgGs), phospho-Ret (pRet), Ret, ERK2, phosphoERK1/2 (pERK1/2), phospho-K_v (pK_v, Thr^{607}), SynIa/b, green fluorescent protein (GFP), tyrosine hydroxylase (TH), microtubule-associated protein 2 (MAP2), tubulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibodies were purchased from Santa Cruz Biotechnology. The [^{3}H]-labeled dopamine ([^{3}H]-DA), and the enhanced chemiluminescence (ECL) detection reagents were purchased from GE Healthcare. The ScintiSafe 30% cocktail was purchased from Fisher Cemical.

Cell culture – The dopaminergic-like SH-SY5Y cell line was plated at a density of 2e5 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10% FBS), penicillin/streptomycin, and non-essential amino acids. Cells were differentiated into a neuronal phenotype by supplementing a 1% FBS DMEM solution with 10 μM retinoic acid (RA) for three days, at which point the medium was changed again to 1% FBS DMEM without RA for a further 18-24 hours. This was done to remove the RA, which can itself activate signaling pathways (such as ERK1/2)

(Miloso et al., 2004), thus obscuring any effects mediated by GDNF-activated ERK1/2. At this point, cells were utilized for the [^{3}H]dopamine release assay or viral-mediated over-expression studies.

Animals **–** Male Sprague Dawley rats (23-30 days old at the time of experiments) were purchased from Harlan. Rats were housed in pairs with food and water available *ad libitum* under 12-hour light-dark cycle conditions (lights on at 7:00AM). Male C57BL/6J (C57) mice were obtained at 6-8 weeks of age from Jackson Laboratories. Mice were singly housed in the 12-hour reverse light-dark cycle room with lights on at 10:00PM with food and water available *ad lib*. All experimental protocols were approved by the Ernest Gallo Research Center Institutional Animal Care and Use Committee (IACUC), and were conducted in agreement with the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

KCl-evoked [^{3}H]-dopamine ([^{3}H]-DA) Release Assay **–** [^{3}H] radio-labeled dopamine ([^{3}H]-DA) was loaded into differentiated SH-SY5Y cells during a 30-minute incubation period with [^{3}H]-DA (0.8 μCi per 2 mL well of a 6-well plate) in a low-KCl "load" solution (135 mM NaCl, 5 mM KCl, 1.3 mM Na2HPO4, 6 mM glucose, 10 mM HEPES, 0.2 mM sodium ascorbate, 0.6 mM MgCl2, 2.5 mM CaCl2, and 0.1% bovine serum albumin [BSA]). Excess [^{3}H]-DA was eliminated over three, 15-minute washes in "load" solution. [^{3}H]-DA release was evoked over 100 seconds by exchanging the low-KCl "load" solution with the high-KCl "evoke" solution. The "evoke" solution contained the same components as the "load" solution, except that it contained 100 mM KCl (the concentration of NaCl was accordingly lowered to 40 mM, to maintain isotonicity as the solutions were exchanged). Where appropriate, GDNF (50 ng/mL) was added during the

last 15-minute wash, as well as during the evoke phase. Where indicated, U0126 pre-treatment (10μM U0126 in DMSO vehicle, final concentration of DMSO: 0.01%) was introduced during the first wash, and maintained throughout all subsequent steps of the assay. At the end of the 100-second evoke phase, the medium was collected in liquid scintillation vials containing 15 mL of scintillant. Cells were then lysed in 10% SDS in phospho-buffered saline (PBS), and collected, as with the evoke phase, for liquid scintillation counting. A liquid scintillation counter was used to quantitate [^{3}H]-DA from the media (DA_{evoked}) and the cellular lysate ($DA_{cellular}$). Dopamine release was defined as the ratio of DA_{evoked}/DA_{total}, with DA_{total} representing the sum total of DA_{evoked} and the $DA_{cellular}$.

Preparation of midbrain slices for acute, ex vivo GDNF treatment – Male Sprague Dawley rats were deeply anesthetized with isoflurane before euthanasia by rapid decapitation. The brains were quickly removed and immediately placed in ice-cold artificial cerebral spinal fluid (aCSF; 126 mM NaCl, 1.2 mM KCl, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 2.4 mM $CaCl_2$, 18 mM $NaHCO_3$, 11 mM glucose) saturated with oxygen (95% O_2 and 5% CO_2 mixture). Horizontal slices (150 μm) containing the midbrain regions were prepared with a Leica vibratome in ice-cold aCSF. The ventral midbrain, containing the VTA and the substantia nigra (SN), were dissected from the slices, and let rest in oxygen-saturated aCSF (as above) at room temperature for 45 minutes prior to treatment. Slices were treated with 400 ng of GDNF for an additional 45 minutes in oxygen-saturated, room temperature aCSF. At the end of the treatment, the slices were mechanically homogenized in RIPA buffer (described below) in preparation for Western blot analysis.

Culture of primary midbrain neurons – A litter of Sprague Dawley rats (P0- P1) were sacrificed by rapid decapitation, and the ventral midbrain region was dissected out. Tissues were pooled and incubated in dissection solution containing papain for 30-45 minutes at 37°C. At the end of this incubation period, the papain solution was removed and replaced with Steve medium (Minimum Essential Media containing 5% BSA, 20 mM D-glucose, and serum extender) containing trypsin inhibitor. Cells were mechanically dissociated, counted, and plated at a density of 2e5 cells/mL on poly-D-lysine-coated plates, or CC2-coated glass chamber slides in Neurobasal-A media (NB-A) supplemented with B-27 and GlutaMax for 14 days in vitro (DIV), with 50% of the media changed on DIV1, DIV7, and DIV10. On DIV1, 10μM cytosine arabinoside (AraC) was added to inhibit mitotic (glial) growth. On DIV13, all of the medium was exchanged for Earle's Balanced Salt Solution, which we found lowers basal ERK1/2 activity, enabling us to detect the activation of ERK1/2 in response to GDNF (Appendix B, Figure A-2). The following day, neurons were either fixed for TH and MAP2 staining, or treated with GDNF and lysates analyzed by Western blot.

Immunocytochemistry – Neurons were washed in PBS and fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Excess PFA was removed over two 10-minute washes with PBS. Neuron membranes were permeabilized in a 10-minute wash in PBS containing 0.1% Triton X-100 (PBS-Triton), and blocking was conducted over the course of a 4-hour incubation with 3% normal donkey serum (NDS) in PBS-Triton. Cells were incubated in primary antibody prepared in 1% NDS in PBS-Triton overnight at 4°C (TH, 1:500; MAP2, 1:2000). The next day, cells were washed three times in PBS-Triton, followed by a 2-hour room temperature incubation in the dark

with AlexaFluor-conjugated secondary antibodies (1:500 dilution). Slides were washed in PBS twice, and water once, before mounting in VectaShield containing 4'6-diamidino-2-phenylindole (DAPI). Neurons were visualized with a laser-scanning confocal microscope. An estimate of the TH^+, and thus dopaminergic, neurons was determined as the ratio of TH^+ neurons to total number of nuclei over at least six visual fields at 63X magnification in three independently-prepared neuron cultures.

Generation of K_v4.3 and Synapsin Ib over-expression adenoviruses – cDNA constructs of rat K_v4.3 and enhanced green fluorescent protein (eGFP)-conjugated rat Synapsin Ib ($_{eGFP}$SynIb) were obtained from the Koh (University of Nevada, Reno) and Augustine (Duke University) laboratories, respectively (Gitler et al., 2004b; Sergeant et al., 2005). The K_v4.3 cDNA was subcloned into the bicistronic pShuttle2-mCherry construct, which expresses the mCherry reporter under a separate promoter, while the $_{eGFP}$SynIb construct was subcloned into the pShuttle2 vector. The respective expression cassettes were then subcloned into the Adeno-X viral genome and adenoviral particles were packaged and amplified in HEK293 cells according to the Adeno-X Expression System 1 protocol. Virus was purified with the Adeno-X Maxi Purification kit, and titered using the Adeno-X Rapid Titer kit.

Limited-access drinking in the dark two-bottle choice drinking in mice – Mice were trained to consume intoxicating amounts of alcohol in the limited access to 10% alcohol drinking in the dark two-bottle choice procedure for three weeks. This drinking paradigm has been shown to illicit excessive voluntary alcohol consumption (Logrip et al., 2009). Briefly, mice were given 4-hour access to a bottle of 10% alcohol (v/v, in tap water) in addition to a bottle of tap water beginning 2 hours into the dark cycle. Water-

only control subjects were provided with two bottles of tap water during this 4-hour period. Mice were sacrificed immediately following the end of the last drinking session, and the ventral midbrain was dissected, immediately homogenized in RIPA buffer containing protease and phosphatase inhibitors, and used for Western blot analysis (described below).

Western blot analysis – Cells were lysed and collected in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 120 mM NaCl, 1% NP-40, 0.1% deoxycholate, and 0.5% SDS) containing protease and phosphatase inhibitors. RIPA with 2.5% SDS was used to prepare samples being used for $K_v4.3$ detection. Cell lysates or tissue samples were briefly sonicated and allowed to rest on ice for 30 minutes. Protein concentrations were determined using the BCA Protein Assay kit. Equal amounts of total protein from each sample were resolved on NuPAGE 4-12% Bis-Tris gels, and transferred onto nitrocellulose membranes (Millipore). For phosphorylated and total protein detection, membranes were first probed for the phosphorylated form of the protein, then stripped (25 mM glycine-HCl, 1% SDS, pH 3, for 30 minutes at room temperature) and reprobed for the total protein. Membranes were blocked for 1 hour in 5% nonfat dry milk prepared in PBS with 0.1% Tween-20 (PBS-T) The primary antibodies were diluted in 1% nonfat dry milk in PBS-T as follows: pRet, 1:1000; Ret, 1:2000; pERK1/2, 1:2000; ERK2, 1:2000; pK_v, 1:250; $K_v4.3$, 1:2000; pSynI, 1:500 (slices) and 1:1000 (SH-SY5Y); SynI, 1:500; GFP, 1:50,000; GAPDH, 1:5000. HRP-conjugated secondary antibodies (1:1000 in 1% milk/PBS-T) were used to detect immunoreactivity via an enhanced chemiluminescent reaction. Images were developed on

Kodak film, and digitally scanned for densitometric quantification using NIH ImageJ software.

Statistics – The biochemical data were analyzed by Student's t-test or one-way analysis of variance (ANOVA). For the [^{3}H]-DA release assay, the data were analyzed by two- or three-way ANOVAs, and significant main effects or interactions were further investigated using the Bonferonni post-test.

Results

GDNF enhances evoked dopamine release in vitro in an ERK1/2-dependent mechanism – We used the [^{3}H]-DA release assay to test the effect of GDNF and GDNF-activaed ERK1/2 on evoked dopamine release in the human dopaminergic-like SH-SY5Y cell line. This assay has been used successfully in our lab to model the effects of cellular signaling pathways on KCl-evoked dopamine release (Gibb et al., 2011). Differentiated SH-SY5Y cells were treated with GDNF (50 ng/mL, for 15 minutes) before a 100-second, 100 mM KCl evoke. We found significant main effects of both the 100 mM KCl Evoke [$F(1,8) = 111.97$, $p < 0.001$] and the GDNF Treatment [$F(1,8) = 7.18$, $p < 0.05$], as well as a significant interaction [$F(1,8) = 9.53$, $p < 0.02$]. *Post hoc* comparisons revealed a significant difference between the control- and GDNF-treated cells in the 100mM KCl Evoke group ($p < 0.01$), but not in the No Evoke group. This indicates that GDNF significantly enhances the KCl-evoked dopamine release (Figure 4-1A). Next, we set out to determine the contribution of GDNF-activated ERK1/2 signaling to this effect. To do so, we blocked the activation of ERK1/2 by pre-treatment with the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor, U0126 (10 μM in DMSO

vehicle, included in all of the washes, as well as the evoke phase). As before, we found significant main effects of both the 100 mM KCl Evoke [$F(1,16) = 65.52, p < 0.001$] and

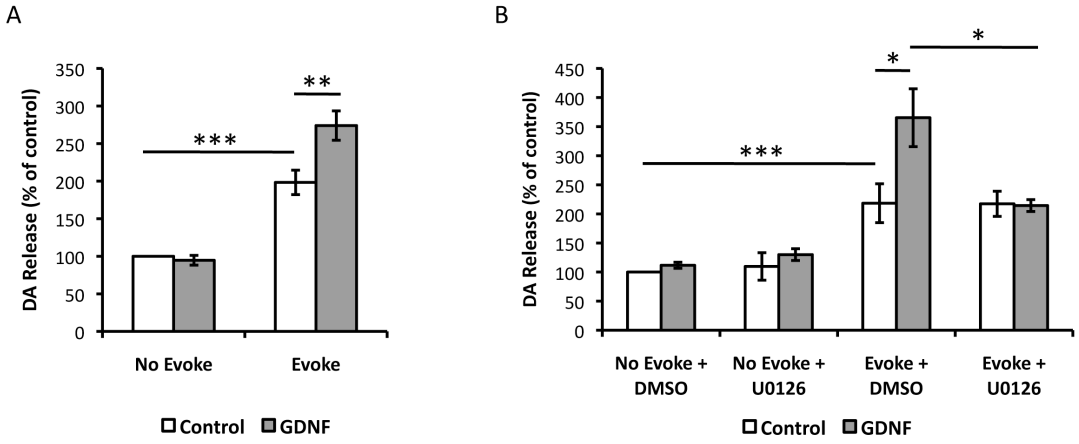

Figure 4-1. GDNF enhances KCl-evoked dopamine release in an ERK1/2-dependent mechanism. A, GDNF augments dopamine (DA) release evoked by KCl. Release of radio-labeled DA was evoked over a 100-second period in the presence of 100 mM KCl with and without GDNF (50 ng/mL). **B,** GDNF-enhanced DA release is ERK1/2-dependent. 100 mM KCl was used to evoke radio-labeled DA release in GDNF- or Control-treated cells with and without a U0126 (10 μM) pre-treatment. For both **A** and **B,** DA release is represented as a percentage of control (No Evoke, no GDNF, DMSO vehicle pre-treatment), n = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, between indicated treatment groups.

GDNF Treatment [$F(1,16) = 6.38, p < 0.05$], as well as a two-way interaction between the Evoke and the U0126 Pre-treatment [$F(1,16) = 6.67, p < 0.05$], and a three-way interaction between the Evoke, GDNF Treatment, and U0126 Pre-treatment [$F(1,16) = 5.19, p < 0.05$]. Bonferonni *post hoc* comparisons revealed significant differences between the control- and GDNF-treated cells in the Evoke group ($p < 0.05$), and between the DMSO- and U0126-pre-treated groups in the Evoke + GDNF group ($p < 0.05$). Therefore, inhibition of ERK1/2 activation by GDNF signaling attenuated the GDNF-mediated enhancement of evoked release, but not that of evoked release in the absence of GDNF.

The A-type potassium channel Kv4.3 is not a phosphorylation target of GDNF-activated ERK1/2 – We next set out to test whether the A-type potassium channel $K_v4.3$, is a downstream target of GDNF-activated ERK1/2. Since the specific, ERK1/2-mediated phosphorylation of Kv4.3 has yet to be investigated, no antibody against the phosphorylated form of this protein is available. We therefore tested whether the commercially available antibody that recognizes Thr^{607}-phosphorylated $K_v4.2$ ($pK_v4.2(Thr^{607})$) can detect $K_v4.3$ that is phosphorylated at the homologous ERK1/2 consensus site. To do so, we used Western blot analysis of rat striatum and ventral midbrain samples to co-detect $pK_v4.2(Thr^{607})$ and $K_v4.3$. In agreement with previous studies (Serodio and Rudy, 1998), $K_v4.3$ is detected in the ventral midbrain, but not striatal, tissues (Figure 4-2A). Additionally, the $pK_v4.2$ antibody recognizes a band co-localized with $K_v4.3$ (Figure 4-2A), suggesting that this antibody most likely recognizes

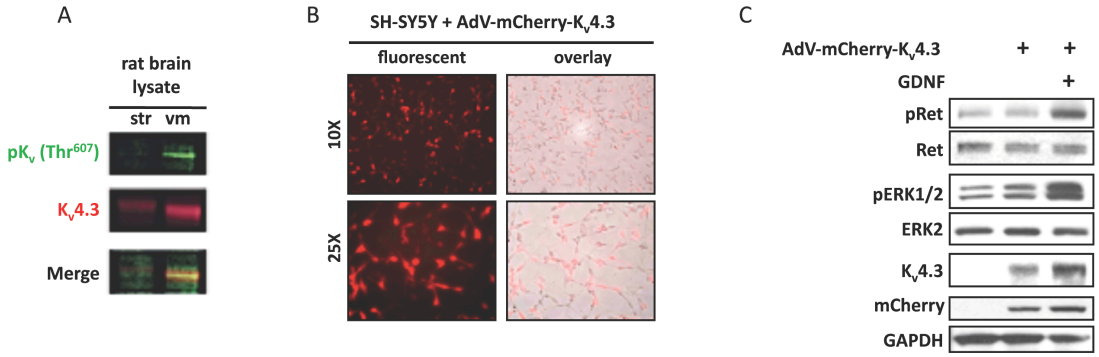

Figure 4-2. Validation of pK_v antibody and adenoviral-mediated over-expression of $K_v4.3$ in SH-SY5Y cells. A, The $pK_v(Thr^{607})$ antibody recognizes phosphorylated $K_v4.3$ in rat striatal (str) and ventral midbrain (vm) tissues. Western blot analysis with fluorescent secondary antibodies was used to co-detect pKv (green) and Kv4.3 (red), the co-localization of which (Merge) suggests that the pK_v antibody can recognize ERK1/2-phosphorylated $K_v4.3$. **B,** Differentiated SH-SY5Y cells were infected with AdV-mCherry-$K_v4.3$ (2e6 ifu/mL, for 72 hours) and live-imaged to verify the expression of the red fluorescent mCherry reporter. **C,** Over-expression of $K_v4.3$ via adenovirus does not affect the GDNF signaling pathway. As in **B,** SH-SY5Y cells were infected with AdV-mCherry-$K_v4.3$ for 72 hours. Cells were then treated with GDNF (50 ng/mL for 5 minutes). Western blot analysis was used to, 1) verify the expression of both the $K_v4.3$ and mCherry proteins, and 2) evaluate the GDNF-mediated phosphorylation of the Ret receptor and ERK1/2.

$K_v4.3$ that is phosphorylated at Thr^{624}, which is homologous to the Thr^{607} site in $K_v4.2$. Because the SH-SY5Y cell line does not express the $K_v4.3$ channel subunit, we constructed an adenovirus with an mCherry reporter that over-expresses $K_v4.3$ in these cells (Figure 4-2B & C). Importantly, adenoviral-driven over-expression does not affect the GDNF signaling pathway, as demonstrated by the phosphorylation (and thus activation) of the Ret receptor, as well as ERK1/2, in the presence of GDNF (50 ng/mL for 5 minutes, Figure 4-2C).

We next tested whether $K_v4.3$ is a substrate of GDNF-activated ERK1/2. To do so, we treated SH-SY5Y cells over-expressing $K_v4.3$ with 50 ng/mL GDNF. As shown in Figure 4-3A, we did not detect the phosphorylation of $K_v4.3$. Interestingly, however, longer exposures to GDNF (30-60 minutes) appeared to induce a down-regulation of the $K_v4.3$ channel subunit (Figure 4-3B), suggesting that, while $K_v4.3$ may not be a direct substrate of GDNF-activated ERK1/2 at this site, GDNF signaling might be responsible for a targeted degradation of this channel subunit.

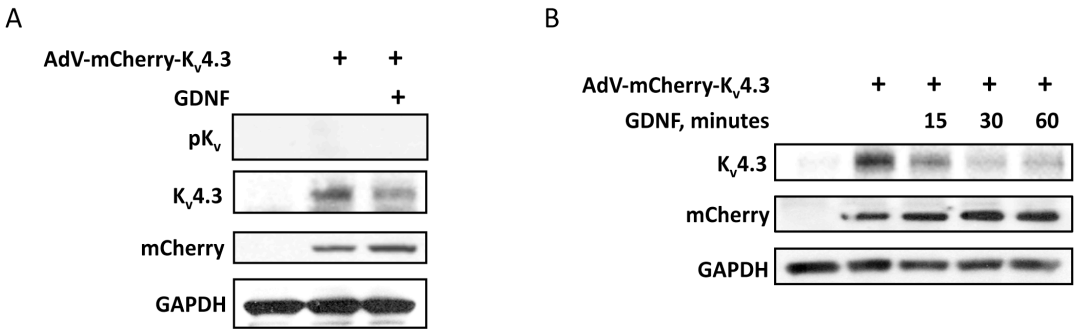

Figure 4-3. $K_v4.3$ is not a phosphorylation substrate of GDNF-activated ERK1/2. A, SH-SY5Y cells adenovirally over-expressing Kv4.3 were treated with GDNF (50 ng/mL for 5 minutes), and Western blot analysis was performed to determine whether Kv4.3 is phosphorylated as a result. Total Kv4.3 and mCherry were also detected, to verify adenoviral infection and expression of the channel. Figure is representative of 4 independent experiments. **B,** Prolonged treatment of SH-SY5Y cells over-expressing Kv4.3 with GDNF (50 ng/mL) may result in a degradation of the channel, as determined by Western blot. Blots were also probed for mCherry, as a measure of equal viral infection in each sample. Figure is representative of 2 independent experiments.

The synaptic vesicle protein Synapsin I is novel phosphorylation target of GDNF-activated ERK1/2 – We next set out to determine whether SynI is a target of GDNF signaling. To begin, we tested whether GDNF treatment of *ex vivo* ventral midbrain slices or neuron cultures with GDNF would elicit a phosphorylation of Synapsin I at its ERK1/2 consensus sites. We prepared horizontal rat midbrain slices (Figure 4-4A), for treatment with GDNF (400 ng/mL) for 45 minutes. This longer treatment time allowed the GDNF to penetrate the slice and activate the GDNF signaling pathway (Wang et al., 2010). Using an antibody against the ERK1/2-phosphorylated form of SynI, we observed a repeatable increase in the phsphorylation of the lower molecular weight SynIb isoform (Figure 4-4B). Likewise, in rat primary midbrain neuron cultures (approximately 30-40% TH^+, Figure 4-4C), we also observed an increase in the

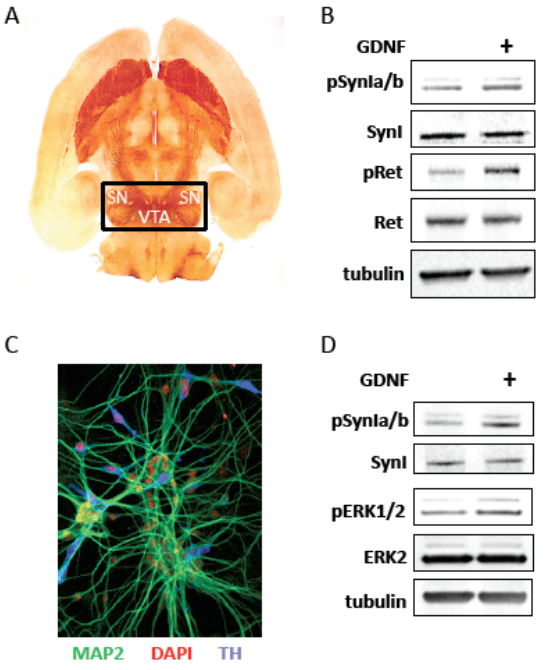

Figure 4-4. SynIb is a novel target of GDNF signaling in the rat ventral midbrain. A & B, SynIb is a phosphorylation target of GDNF signaling in rat midbrain slices. Midbrain slices (**A,** horizontal slice stained for TH; box indicates region of prepared slice, which includes the substantia nigra [SN] and the ventral tegmental area [VTA]) were prepared from rat and treated with GDNF (400 ng/mL for 45 minutes). Western blot analysis of pRet levels was used to verify activation of the GDNF signaling pathway. Phosphorylated SynIa/b was detected using antibodies specific for the ERK1/2-phosphorylated form of the protein. Western blot is representative of 4 independent experiments. **C & D,** SynIb is a phosphorylation target of GDNF signaling in rat primary midbrain neurons. Neuron (green, $MAP2^+$) cultures containing ~40% TH^+ (blue) cells were treated with GDNF (50 ng/mL for 10 minutes). Activation of the GDNF signaling pathway was verified by detection of pERK1/2 by Western blot analysis. As in **B,** pSynIa/b was detected using antibodies that recognize ERK1/2-phosphorylated SynI forms of both the SynIa and SynIb isoforms. Blot is representative of 3 independent neuron preparations.

ERK1/2 phosphorylation of the SynIb isoform (Figure 4-4D). Together, this indicates that SynIb is likely to be phosphorylation target of GDNF-activated ERK1/2. In order to further investigate this possibility in depth, we utilized the human dopaminergic-like SH-SY5Y cells. As shown in Figure 4-5A, treatment of SH-SY5Y cells with GDNF (50

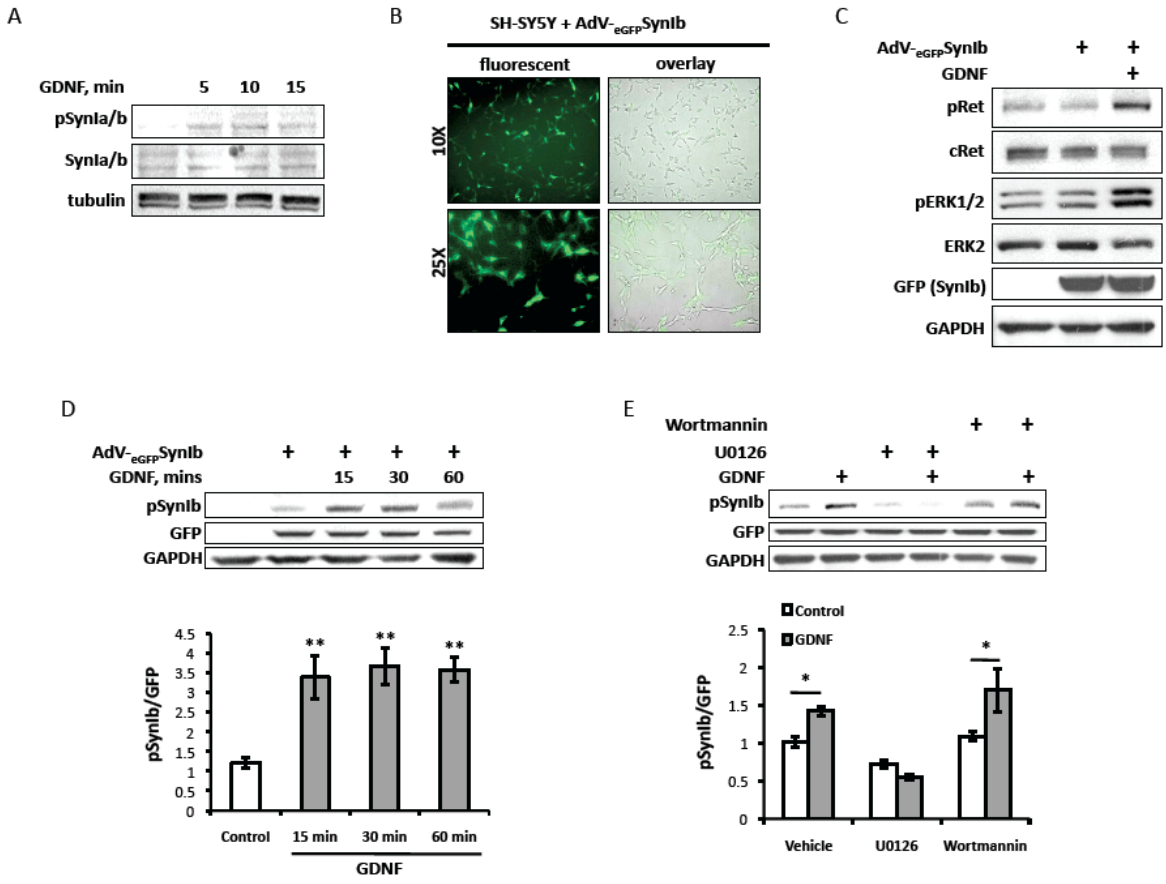

Figure 4-5. SynIb is a target of GDNF-activated ERK1/2. A, Single demonstration of GDNF-mediated phosphorylation of SynIb, as determined by Western blot analysis, in SH-SY5Y cells. **B-E,** Differentiated SH-SY5Y cells were infected with AdV-eGFPSynIb (2e6 ifu/mL for 72 hours). **B,** Cells were live-imaged to verify the expression of the fluorescent eGFP tag. **C,** The GDNF signaling pathway remains intact after over-expression of eGFPSynIb. Cells were treated with GDNF (50 ng/mL for 5 minutes). Western blot analysis was used to, 1) verify the expression of SynIb via its eGFP tag, and 2) evaluate the GDNF-mediated phosphorylation, and thus activation, of its receptor, Ret, and downstream kinase, ERK1/2. **D & E,** SynIb is a phosphorylation target of GDNF-activated ERK1/2. For **D,** SH-SY5Y cells were treated with GDNF (50 ng/mL), and in **E,** Cells were pre-treated with U0126 (10 μM) or wortmannin (10 nM) for 10 minutes before a 15-minute GDNF treatment. Western blot analysis was used to verify ERK1/2-phosphorylated SynIb. Data is presented as the average pSynIb/GFP ± SEM, n = 3 in dependent experiments. *$p < 0.05$, **$p < 0.01$, between control- and GDNF-treated groups.

ng/mL) elicits the phosphorylation of SynIb, although the expression of SynI in these cells is very low, impeding the replication of this finding in this model system. Therefore, we constructed an adenovirus to over-express eGFP-tagged SynIb ($_{eGFP}$SynIb; Figure 4-5B). Importantly, as with the over-expression of $K_v4.3$, over-expression of $_{eGFP}$SynIb did not adversely affect the GDNF signaling pathway (Figure 4-5C). We found that treatment of SH-SY5Y cells over-expressing $_{eGFP}$SynIb induced a significant phosphorylation of SynIb at its ERK1/2 site (Figure 4-5D). Furthermore, as shown in Figure 4-5E, we verified that this phosphorylation event is indeed ERK1/2-activity dependent, as it was blocked by pre-treatment with the MEK inhibitor, U0126 (10 μM), but not the phosphoinositide-3-kinase (PI3K) inhibitor, wortmannin (10 nM). Together, these *ex vivo* and *in vivo* findings illustrate that SynIb is a novel target of GDNF-activated ERK1/2.

Alcohol exposure lowers SynIa/b phosphorylation – We identified SynIb as a novel target of GDNF signaling in the ventral midbrain of rat. As discussed in Chapter 2, the GDNF system is subject to alcohol- and drug-induced alterations (Green-Sadan et al., 2003; Messer et al., 2000; Ron and Janak, 2005; Semba et al., 2004). Interestingly, Messer and colleagues noted that chronic cocaine and morphine exposure resulted in a decrease in the level of phosphorylated, and thus activated, Ret in the VTA, suggesting that drugs of abuse may down-regulate the GDNF signaling pathway (Messer et al., 2000). Because SynIb is a phosphorylation target of GDNF signaling, we therefore asked whether voluntary alcohol drinking would result in a decrease in levels of phosphorylated SynI in the ventral midbrain. To do so, we trained mice to voluntarily consume intoxicating amounts of alcohol in the limited-access two-bottle choice drinking in the dark procedure (Logrip et al., 2009). After three weeks of alcohol drinking in this model,

the ventral midbrain was collected, and the level of ERK1/2-phosphorylated SynIa/b was determined by Western blot. As shown in Figure 4-6, basal levels of pSynIa/b were significantly decreased in mice that had access to alcohol, as compared to water controls. This suggests that long-term consumption of physiologically intoxicating levels of alcohol negatively regulates this phosphorylation target of the GDNF pathway.

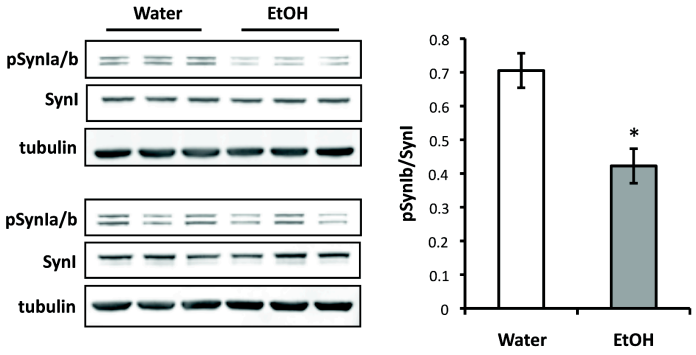

Figure 4-6. Excessive alcohol consumption lowers basal pSynI levels in the ventral midbrain of mice. Mice drank alcohol in the limited-access two-bottle choice drinking in the dark paradigm for three weeks. At the end of the last drinking session, the ventral midbrain was collected, and pSynIa/b levels were determined by Western blot analysis. Data are represented as the mean pSynIb/SynI ± SEM, n = 6 mice per group. *$p < 0.05$, as compared to water controls.

Discussion

This chapter outlines efforts to determine the molecular mechanism of the GDNF-ERK1/2-meidated reduction in alcohol consumption. We found that GDNF enhances evoked dopamine release in a cell culture model, and that this enhancement involved the ERK1/2 signaling pathway (Figure 4-1). As a result, we utilized a candidate-based approach to test two potential targets of GDNF-activated ERK1/2 that may influence neurotransmission: $K_v4.3$ and SynI. We found that the synaptic vesicle protein SynIb is a novel target of GDNF signaling in the midbrain (Figure 4-4), and, specifically, is a substrate of GDNF-activated ERK1/2 (Figure 4-5). Interestingly, while we did not observe any direct phosphorylation of the $K_v4.3$ channel subunit, we did observe what

may be a GDNF-induced degradation of this protein (Figure 4-3B). It remains to be determined whether this effect is genuine, and if it is ERK1/2-dependent.

Behavioral implications of the GDNF-mediated augmentation of evoked dopamine release – As mentioned in Chapter 1, activation of the mesolimbic pathway by alcohol positively reinforces alcohol consumption, partly by increasing dopamine release from the VTA to the NAc (Yim and Gonzales, 2000). However, long-term exposure to alcohol decreases dopaminergic tone in the limbic system (Zhou et al., 1995), as well as in the ventral midbrain (Lanca, 1994). As a result, the motivation to continue drinking alcohol in order to sustain rewarding levels of dopamine is an attractive hypothesis. Koob and LeMoal's allostatic hypothesis further elaborates upon this hypothesis by suggesting that continued alcohol intakes is sought by an individual as an effort to alleviate anhedonia (to which a deficit in dopamine can contribute), thus restoring their emotional affective state back to a previous, homeostatic setpoint (Koob and Le Moal, 2001). Indeed, a recent study conducted by our group shows that GDNF, through the likely activation of its signaling pathway, very neatly reverses the alcohol-induced allostatic decrease in dopamine levels in the mesolimbic circuit (Barak et al., 2011). Together with previous work demonstrating that GDNF is a potent inhibitor of alcohol consumption (Carnicella et al., 2008), this indicates that one possible mechanism for the GDNF-mediated reduction in alcohol consumption is via its ability to positively influence dopamine transmission. In this chapter, we used a cell culture model to establish that GDNF-mediated signaling augments dopamine release in an ERK1/2-dependent manner. This finding lends additional support to our working hypothesis in which the GDNF-

induced decrease in alcohol intake is mediated by a GDNF-ERK1/2-signaling mechanism that alters dopamine neurotransmission.

K_v4.3 is not a direct target of GDNF-activated ERK1/2 at Thr^{624} – We hypothesized that the A-type potassium channel subunit, K_v4.3, is a potential target of GDNF-activated ERK1/2. The ERK1/2-mediated phosphorylation, and thus inactivation, of the A-type channels containing this subunit might then contribute to an increase in neuronal excitability, resulting in an increased probability of dopamine release and a subsequent decrease in alcohol consumption. Although we did not observe any K_v4.3-associated phosphorylation event in response to GDNF treatment, it does not preclude the possibility that this channel subunit is a genuine target of GDNF-activated ERK1/2 signaling. For instance, we observed a consistent trend towards a decrease in K_v4.3 protein levels in response to longer GDNF treatments, both in SH-SY5Y cells over-expressing an untagged K_v4.3 (Figure 4-3B), as well as in cells over-expressing GFP-tagged K_v4.3 (Appendix B, Figure A-3). This strongly suggests that K_v4.3 may be targeted for degradation as a result of GDNF signaling. Whether this targeted degradation is ERK1/2-dependent remains to be tested. Additionally, we tested just one possible ERK1/2 phosphorylation site of K_v4.3. According to the GPS2.1 phosphorylation site prediction software (Xue et al., 2008), K_v4.3 contains at least four additional putative ERK1/2 phosphorylation sites, two of which are homologous to the ERK1/2 phosphorylation sites that were identified on K_v4.2 (Schrader et al., 2005). Thus, the possibility that GDNF-activated ERK1/2 targets and phosphorylates K_v4.3 at any of these residues warrants further investigation.

SynIb is a novel substrate of GDNF-activated ERK1/2, and is hypophosphorylated after long-term, excessive alcohol drinking – Here, we report the identification of a novel target of GDNF-activated ERK1/2 signaling, SynIb. The precise effect of this phosphorylation event on dopamine release, however, is yet to be determined. Synapsins are general regulators of neurotransmitter release (Hilfiker et al., 1999), but a closer inspection reveals differential roles for synapsins, depending upon at which site they are phosphorylated (Chi et al., 2003), what type of neurons they are expressed in (Gitler et al., 2004a), as well as the neurotransmitter compliment of the synaptic vesicles they are associated with (Kile et al., 2010). With regard to this last point, recent evidence points to a negative regulation of catecholamine (including dopamine) release by synapsins (Kile et al., 2010; Villanueva et al., 2006), however, nothing is yet known of the specific contribution of ERK1/2 phosphorylation of SynI to dopamine release. One possibility, at first counter-intuitive, may be that ERK1/2 phosphorylation of SynIb in the midbrain attenuates local dopamine release. If so, the GDNF-activated ERK1/2 phosphorylation of this protein that we observed (Figure 4-3) would lower the somatodendritic release of dopamine, releasing the dopamine D2 receptor (D2R)-mediated auto-inhibition of the dopaminergic neurons, thus contributing to an increase in dopamine release to the NAc. The other possibility is that ERK1/2 phosphorylation of SynIb increases dopamine release. This seems less likely to fit with our overall hypothesis, however, as an increase in dopamine overflow in the VTA would effectively lower the dopamine from the VTA to the NAc, due to the afore-mentioned D2R-mediated auto-inhibition. Nevertheless, an interesting recent report indicated that high-frequency stimulation of the mossy fiber (MF)-CA3 connections in hippocampal

slices caused a spatio-specific activation of ERK1/2 in the MF axon terminals, which was accompanied by an increase in ERK1/2-phosphorylated SynI (Vara et al., 2009). Thus, it is possible that activation of the GDNF signaling pathway in the VTA might result in activation of ERK1/2, and subsequent phosphorylation of SynI, in the terminals of the VTA dopaminergic neurons that project to the NAc. In this case, a positive regulatory effect of ERK1/2 phosphorylation of SynI on dopamine release could explain the increase in dopamine in the NAc that occurs after the delivery of GDNF to the VTA (Barak et al., 2011). We therefore propose to use site-directed mutagenesis in order to test the contribution of ERK1/2 phosphorylation of SynIb on GDNF-enhanced dopamine release *in vitro*. By comparing the effect of over-expressing wild-type SynIb or a non-ERK1/2-phosphorylatable SynIb point mutant on GDNF-enhanced dopamine release, we will be able to establish whether ERK1/2-phosphorylated SynIb is a positive, or negative, regulator of dopamine release in the presence of GDNF.

Summary

This chapter describes our efforts to identify the molecular mechanism of the GDNF-activated ERK1/2-mediated reduction in alcohol consumption. We found that a GDNF-activated ERK1/2-containing mechanism causes an enhancement of evoked dopamine release. This finding is the first step towards bridging two previous reports from our lab, 1) that GDNF lowers alcohol in an ERK1/2-dependent mechanism (Carnicella et al., 2008), and 2) that GDNF reverses the alcohol-induced allostatic dopamine deficiency (Barak et al., 2011). While it remains to be determined whether, in fact, the GDNF-mediated reduction in alcohol drinking is the result of the reversal of the

alcohol-induced decrease in dopamine, here, we are able to clearly attribute the actions of GDNF on dopamine release to the activation of the ERK1/2 signaling pathway by GDNF.

Experiments to identify molecular targets of GDNF-activated ERK1/2 that could affect neurotransmitter release revealed that SynIb is a novel target of this signaling pathway both in *in vitro* and in *ex vivo* models of the ventral midbrain. As an integral part of synaptic vesicle machinery, the ERK1/2 phosphorylation of SynIb is likely to play an important role in GDNF-enhanced dopamine release. Further investigation is needed to determine the precise contribution of this phosphorylation event. Separately, while the $K_v4.3$ subunit of the A-type may not be a direct target of GDNF-activated ERK1/2, it may be targeted for degradation as a result of GDNF signaling, and may thusly affect GDNF-induced neurotransmission.

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CHAPTER 5

PHARMACOLOGICAL INDUCTION OF GDNF BY THE FDA-APPROVED DRUG, CABERGOLINE

Abstract

We previously showed that glial cell line-derived neurotrophic factor (GDNF) is a potent inhibitor of alcohol consumption (Carnicella et al., 2008). GDNF's inability to cross the blood-brain barrier precludes it from being directly developed as a possible treatment for alcohol dependence (Boado and Pardridge, 2009; Kastin et al., 2003). Therefore, we set out to determine whether pharmacological up-regulation of GDNF would decrease alcohol-drinking and -seeking behaviors *in vivo*. Cabergoline is an FDA-approved drug that has been shown to increase GDNF levels and secretion *in vitro* (Ohta et al., 2004). We determined that cabergoline increases GDNF levels, and activates its signaling pathway, both *in vitro* (in the dopaminergic-like SH-SY5Y cell line) and *in vivo*. Subsequently, we found that systemic administration of cabergoline reduced operant alcohol self-administration in rats. Importantly, alcohol-seeking, as well as the reacquisition of alcohol self-administration after a period of abstinence, were also attenuated by systemic cabergoline. Intra-ventral tegmental area (intra-VTA) infusion of cabergoline decreased operant alcohol self-administration in rats, localizing cabergoline's actions to this brain area. Finally, systemic treatment of GDNF heterozygous knockout mice with cabergoline did not affect alcohol consumption, as compared with wild-type mice, indicating that cabergoline lowers alcohol-drinking and -seeking behaviors via GDNF. Together, these findings indicate that cabergoline, via the up-regulation of the GDNF signaling pathway, is a potential treatment for alcohol dependence.

Introduction

Currently, there remains a desperate need for pharmacotherapies for the treatment of alcohol dependence and its associated phenotypes, such as excessive alcohol intake, craving, and relapse. While there are three FDA-approved medications for the treatment of alcohol addiction (disulfuram, acamprosate, and naltrexone), these medications have had limited success in the treatment of alcoholism. This is generally attributed to a low level of patient compliance as a result of unpleasant adverse side effects, as well as a small effect size (Assanangkornchai and Srisurapanont, 2007; Bouza et al., 2004; Johnson, 2008). As a result, there remains a pressing need for the development of more efficacious pharmacotherapies for the treatment of alcohol addiction.

Cabergoline (marketed under the brand names Cabaser and Dostinex) is an ergot derivative prescribed primarily for the treatment of hyperprolactinemia. Cabergoline is principally a dopamine D2 receptor-like (D2R-like) agonist, although it has also been shown to bind to serotonergic (Newman-Tancredi et al., 2002b) and adrenergic receptors (Newman-Tancredi et al., 2002a), as well as to the dopamine D1 receptor, albeit weakly (Millan et al., 2002). Interestingly, cabergoline was found to induce the expression and secretion of GDNF in cultured mouse astrocytes (Ohta et al., 2004; Ohta et al., 2003). We recently demonstrated that the anti-addiction properties of the naturally-occurring alkaloid, ibogaine, lowered alcohol self-administration by up-regulating GDNF and its signaling pathway in the VTA of rats (He et al., 2005). In line with this finding, infusion of GDNF into the rat VTA precipitates a rapid and long-lasting reduction in alcohol operant self-administration via the activation of the extracellular signal-regulates kinase 1/2 (ERK1/2) pathway (Carnicella et al., 2008). Importantly, this intra-VTA application

of GDNF also attenuated alcohol-seeking, as well as alcohol self-administration in a relapse model (Carnicella et al., 2008). As a result, we hypothesized that pharmacologic up-regulation of GDNF and its signaling pathway could lower alcohol consumption, as well as alleviate the risk of relapse. Because cabergoline was shown to induce GDNF expression, we tested whether cabergoline, via up-regulation of GDNF and it signaling pathway could lower alcohol-drinking and -seeking behaviors.

Materials & Methods

Materials – Cabergoline was a generous gift from Pfizer Inc. Anti-Ret, anti-phospho-Ret (pRet, Tyr1062), anti-MAPK extracellular signal-regulated kinase 2 (ERK2), anti-phospho-ERK1/2 (pERK1/2, Tyr 204), anti-tubulin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Protease inhibitors were purchased from Roche. Quinpirole, retinoic acid and phosphatase inhibitors were purchased from Sigma. The BCA Protein Assay Kit was purchased from Pierce Biotechnology. TRIzol reagent and pre-cast Bis-Tris SDS-PAGE gels and associated buffers were purchased from Invitrogen. The Emax GDNF ImmunoAssay System, Reverse Transcription System and 2X PCR Master Mix were purchased from Promega. Wheatgerm Agglutinin Scintillation Proximity Assay (SPA) beads were purchased from Amersham Biosciences. [^{35}S]-Guanosine 5`-(γ-thio)triphosphate ([^{35}S]-GTPγS) (250μCi; 9.25MBq) was supplied by Perkin-Elmer.

Animals – Male Long-Evans rats (300-350 grams at the start of experiments) were purchased from Harlan. Male C57BL/6J mice (6 – 8 weeks old at start of experiments) were purchased from Jackson Laboratories. Heterozygous GDNF KO mice

($GDNF^{+/-}$), on a DBH/CD1 genetic background, were a generous gift from Drs. Barry Hoffer and Andreas Tomac, NIDA. Male $GDNF^{+/-}$ and their wild-type littermates ($GDNF^{+/+}$) were generated by in-house mating of the $GDNF^{+/-}$ mice. Mouse genotypes were determined by PCR analysis of products derived from tail DNA. Animals were housed individually under a 12-hr light/dark cycle (lights on, 7:00 a.m. for the rats, 10:00 p.m. for the mice, i.e. reverse dark cycle) with food and water available *ad libitum*. All animal procedures in this report 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.

Cell culture – The dopaminergic-like SH-SY5Y cell line was plated at a density of 2e5 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10% FBS), penicillin/streptomycin, and non-essential amino acids. Cells were differentiated into a neuronal phenotype by supplementing a 1% FBS DMEM solution containing 10 μM retinoic acid (RA) for three days, at which point the medium was changed again, to 1% FBS DMEM without RA for a further 18-24 hours to remove residual RA. At this point, cells were treated with 60 μM cabergoline in DMSO (final concentration of DMSO: 0.1%).

Reverse transcription polymerase chain reaction (RT-PCR) – SH-SY5Y cells, VTA, or substantia nigra (SN) tissues were mechanically homogenized in Trizol reagent and total RNA was isolated, according to the manufacturer's protocol. mRNA was selectively reverse transcribed from 500 ng of total RNA using the Reverse Transcription System with the oligo (dT) primer at 42° C for 30 minutes. For the PCR, amplifications of *GDNF* and the housekeeping gene, *Glyceraldehyde-3-phosphate dehydrogenase*

(GAPDH), were conducted with the resulting cDNA using the following primers: rat *GDNF* upstream: 5'- GAC GTC ATG GAT TTT ATT CAA GCC ACC -3'; rat *GDNF* downstream: 5'- CTG GCC TAC TTT GTC ACT TGT TAG CCT -3'; human *GDNF* upstream: 5'- TGC CAG AGG ATT ATC CTG ATC AGT TCG ATG -3'; human *GDNF* downstream: 5'- GAT ACA TCC ACA CCT TTT AGC GGA ATG CTT -3'; rat/human *GAPDH* upstream: 5'- TGA AGG TCG GTG TCA ACG GAT TTG GC -3'; rat/human *GAPDH* downstream: 5'- CAT GTA GGC CAT GAG GTC CAC CAC -3'. For *GDNF,* 33-35 amplification cycles were used, while 27 cycles were used for *GAPDH.* PCR products were resolved on a 1.8% agarose gel supplemented with 0.05% EtBr for visualization under UV light. Images were captured using Eagle Eye 2 software. Band intensities were quantified using NIH ImageJ software.

Western blot analysis – Cells were lysed and collected in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 120 mM NaCl, 1% NP-40, 0.1% deoxycholate, and 0.5% SDS) containing protease and phosphatase inhibitors. Tissue samples were mechanically homogenized in RIPA buffer containing phosphatase and protease inhibitors. Protein concentrations were determined using the BCA Protein Assay kit. Equal amounts of total protein from each sample were resolved on NuPAGE 4-12% Bis-Tris gels, and transferred onto nitrocellulose membranes (Millipore). For phosphorylated and total protein detection, membranes were first probed for the phosphorylated form of the protein, then stripped (25 mM glycine-HCl, 1% SDS, pH 3, for 30 minutes at room temperature) and reprobed for the total protein. Membranes were blocked for 1 hour in 5% nonfat dry milk prepared in PBS with 0.1% Tween-20 (PBS-T) Primary antibodies were diluted in 1% nonfat dry milk in PBS-

T as follows: pRet, 1:1000; Ret, 1:2000; pERK1/2, 1:2000; ERK2, 1:2000; tubulin, 1:5000. HRP-conjugated secondary antibodies (1:1000 in 1% milk/PBS-T) were used to detect immunoreactivity via an enhanced chemiluminescent reaction. Images were developed on Kodak film, and digitally scanned for densitometric quantification using NIH ImageJ software.

Enzyme-linked immunosorbant assay (ELISA) – Cells were collected and lysed in RIPA buffer. Total GDNF protein was measured using the Emax GDNF ImmunoAssay System, according to the manufacturer's recommended protocol.

Quinpriole-stimulated D2R-like [^{35}S]GTPγS binding assay – Midbrain and striatal tissues were dissected from mice and homogenized on icein homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM MgCl2, pH 7.4; 1g brain tissue/20 mL buffer), and after centriugation, resuspended in HME assay buffer (100 mM HEPES, 8 mM MgCl2, 4 mM EDTA, 10 ug/ul saponin, and protease inhibitor cocktail; pH 7.5). Binding assays were performed in 96-well plates on ice with each reaction containing [^{35}S]GTPγS (50 pM), cell membrane (10 ug protein), GDP (30 uM), and SPA beads (0.5 mg) with HME assay buffer. Assay plates were shaken for one hour at 25°C, then centrifuged (1500 rpm for 5 minutes), before [^{35}S]GTPγS-stimulated binding was assessed using the NWT TOPCOUNTER. Single dose-response curves of quinpirole-dopamine receptor stimulated [^{35}S]GTPγS binding were performed with quinpirole (0.1 nM – 1 mM dose range) with each midbrain or striatal membrane preparation run in triplicate.

Limited-access two-bottle choice drinking paradigm – Mice were allowed continuous access to water. After acclimatization to the reverse dark cycle, one bottle

containing 10% (v/v) alcohol, saccharin (0.3% w/v), or quinine (0.03 mM) in tap water was made available for four hours per day, five days a week, beginning one hour after the start of the dark cycle (i.e.: 11:00 a.m. – 3:00 p.m.). The placement of the alcohol, saccharin, or quinine bottle was alternated daily to control for the development of a side preference. All behavioral experiments were conducted after one week of training on this schedule. A bottle containing water in a cage without animals was used to evaluate spillage, which was always 0.1 mL.

Operant self-administration – Rats were habituated to drinking alcohol in their home cages by exposing them to 10% alcohol in tap water (v/v) mixed with a decreasing concentration of sucrose (10%, 5% and 0%, w/v). After three weeks, operant ethanol self-administration training commenced. The self-administration chambers contained two levers: an active (alcohol) lever, for which presses resulted in delivery of 0.1 ml of a 10% ethanol solution, and an inactive lever, for which responses were counted as a measure of nonspecific behavioral activity, but no programmed events occurred. No discrete cues were used at any time during the self-administration procedure. After 2-3 nights in the chambers to allow acquisition of a lever-press response for 10% ethanol under a fixed ratio 1 (FR1; one press delivers one reward), 60-min sessions were conducted 5 days a week, with the schedule requirement increasing to FR3 over the first week. As the level of presses on the inactive lever was low after acquisition (< 10 presses), and the activity on this lever was not affected by any of the experimental treatments, this measure was excluded from the figures and the analysis for better clarity. After 2 months of training, surgery to implant cannulae was conducted or systemic injections began. For the operant self-administration test in extinction, rats were given an i.p. injection of cabergoline 3

hours before the beginning of a weekly 1-hour test in which responses on the alcohol-associated lever were measured, but no alcohol reward was delivered. To assess the reacquisition of alcohol self-administration, rats experienced daily, 1-hour extinction sessions for 17 days. On the test day, rats were given an intraperitoneal (i.p.) injection of cabergoline 3 hours before the reacquisition test session. An alcohol prime (0.2 mL of 10% alcohol) was delivered at the beginning of the test session (non-contingent to the lever press) to reinstate lever pressing for an alcohol reward on an FR3 schedule, as in the self-administration procedure. After 1 week of reacquisition of alcohol self-administration, rats experienced 10 additional extinction sessions, and a second reacquisition test session was conducted with the drug treatments reversed.

Operant responding during extinction after abstinence following a long history of excessive voluntary alcohol consumption – Excessive voluntary alcohol consumption by rats was induced over a period of 6 weeks according to the previously described intermittent-access to 20% alcohol two-bottle choice paradigm (Carnicella et al., 2009; Simms et al., 2008). Rats were then trained to self-administer a 20% alcohol solution in the operant chambers, in 30-minute sessions, as described above. After 3 months of training, rats experienced a 10-day withdrawal period. The motivation to seek alcohol after this period of abstinence was assessed during a 15-minute extinction session in the operant chambers, 3 hours following the i.p. administration of drug. After the test, rats self-administered alcohol for 1 week, followed by an additional 10-day withdrawal period. At this point, a second extinction test session was conducted, with the drug treatments reversed.

Preparation of cabergoline for in vivo studies – For systemic intraperitoneal (i.p.) injections, cabergoline was prepared as a suspension in a solution of saline containing 0.25% methylcellulose and 3% Tween-80 for rats, or 0.25% methylcellulose and 0.6% Tween-80 for mice. For the intra-VTA or intra-SN microinjections, cabergoline was prepared in a phospho-buffered saline (PBS) containing 5% dimethyl sulfoxide (DMSO). All cabergoline treatments were administered 3 hours prior to the start of the behavioral test session.

Surgery & microinjection – Rats were anesthetized continuously with isofluorane. Bilateral guide cannulae were aimed dorsal to the VTA (5.4 mm posterior to bregma, 1.0 mm mediolateral, 8.0 mm ventral to the skull surface), or the substantia nigra pars compacta (SNc, 5.4 mm posterior to bregma, 2.6 mm mediolateral, 6.8 mm ventral to the skull surface), according to Paxinos and Watson (Paxinos and Watson, 1998). One week after recovery, subjects resumed the operant self-administration training, and microinjections were begun once operant responding reached stable levels. Cabergoline or vehicle was infused to gently restrained rats over a 2-minute period, 3 hours before the start of the test session. Injection cannulae extended 0.5 mm beyond the guide cannulae tip, and were left in place for an additional 2 minutes. All subjects received each treatment in a counterbalanced design, with 1 injection/week, allowing time for lever pressing for alcohol to return to baseline between treatments. Locations of cannulae were verified in 60 um coronal sections stained with thionin. Only data from subjects with correctly inserted injectors were included in the data analysis.

Statistics – The biochemical data were analysed by Student's t-test or two-way analysis of variance (ANOVA). Behavioral experiments were conducted in within- or

mixed within-subjects design analyzed by one- or two-way ANOVAs with repeated measures. Significant main effects or interactions of the ANOVAs were further investigated using the Student-Newman-Keuls test or the method of contrasts. For the [^{35}S]GTPγS binding assay, the E_{max} and EC_{50} were determined by linear regression using a curve with variable slope and data were analyzed by two-way ANOVA.

Results

Cabergoline increases GDNF and activates its signaling pathway in vitro and in vivo – Cabergoline was found to induce GDNF expression and secretion in cultured mouse astrocytes (Ohta et al., 2004). We tested whether systemic administration of cabergoline to rats would result in increased *GDNF* expression in the midbrain regions. Cabergoline (1 mg/kg in saline vehicle containing 0.25% methylcellulose and 3% Tween-80) was delivered i.p. 1.5 hours before collection of the VTA and SN tissues. *GDNF*

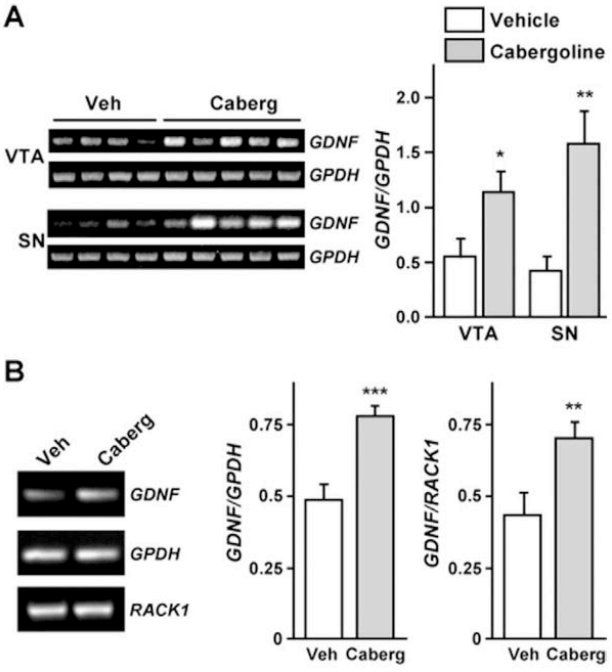

Figure 5-1. Cabergoline increases *GDNF* levels in the rat midbrain and in SH-SY5Y cells. A, Rats were given an i.p. injection of cabergoline (1 mg/kg), and *GDNF* expression in the VTA and SN were assessed 1.5 hours later. **B,** Differentiated SH-SY5Y cells were treated with 60 μM cabergoline for 1.5 hours before measuring *GDNF* levels. *GPDH* and *RACK1* expression were used as internal controls. Bar graphs represent the *GDNF*/*GPDH* or *GDNF*/*RACK1* ratios ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n = 4-5.

mRNA was significantly elevated in both the VTA and SN in those subjects that had received cabergoline, as opposed to the control subjects that received vehicle only (Figure 5-1A). We next utilized the human dopaminergic-like SH-SY5Y cells to determine whether treatment with cabergoline resulted in increased GDNF protein levels. As in the rat midbrain, treatment of the SH-SY5Y cells with cabergoline induced an up-regulation of *GDNF* mRNA levels (Figure 5-1B). GDNF protein levels were detected by ELISA, and were found to increase significantly after treatment with cabergoline (Figure 5-2A). Next, we tested whether up-regulation of GDNF in SH-SY5Y cells after treatment of cabergoline resulted in the activation of the GDNF signaling pathway. We measured the phosphorylation, and thus activation, of the GDNF receptor Ret, and the extracellular signal-regulated kinases (ERKs) 1 and 2, downstream targets of GDNF-activated Ret. We found increased phosphorylation of both the Ret receptor, and the ERKs 1 and 2

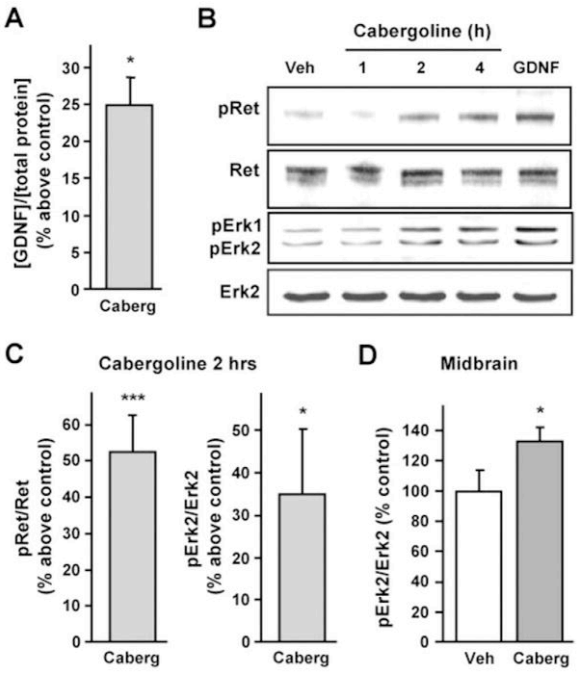

Figure 5-2. Cabergoline increases GDNF protein levels in SH-SY5Y cells and induces the activation of the GDNF pathway *in vitro* and *in vivo*. A, GDNF protein levels in differentiated SH-SY5Y cells were assessed by ELISA following a 3-hour treatment with 60 μM cabergoline. GDNF levels are expressed as a percentage increase over the control GDNF/total protein ratio ± SEM, n = 3. **B & C,** Activation of the GDNF signaling pathway in SH-SY5Y cells following a 2-hour treatment with 60 μM cabergoline. GDNF treatment (50 ng/mL for 15 minutes) was used as a positive control. Data are shown as the percentage above control of the pRet/Ret or pERK2/ERK2 ratios ± SEM, n = 5. **D,** ERK2 phosphorylation in the rat midbrain 3 hours after the i.p. administration of 1 mg/kg cabergoline. Bar graph depicts the ratio of pERK2/ERK2 ± SEM, n = 6. *$p < 0.05$, ***$p < 0.001$.

following treatment with cabergoline (Figure 5-2B & C). Phosphorylated levels of ERKs 1 and 2 were also elevated in the rat midbrain following systemic administration of cabergoline (Figure 5-2D). Together, these data strongly indicate that cabergoline treatment up-regulates GDNF levels, and activates its signaling pathway in the midbrain.

Cabergoline lowers alcohol self-administration and seeking in rats – To evaluate the effects of cabergoline on alcohol consumption in rats, we used the operant alcohol self-administration procedure. Rats that had been trained to self-administer alcohol in this model were systemically administered cabergoline 3 hours before the test session. Cabergoline dose-dependently lowered operant responding (lever presses) for alcohol (Figure 5-3A) [$F(4,28) = 4.87$, $p < 0.01$], but not for sucrose (Figure 5-3B) [$F(4,32) = 1.40$, $p < 0.26$]. Seeking for alcohol was determined by measuring lever

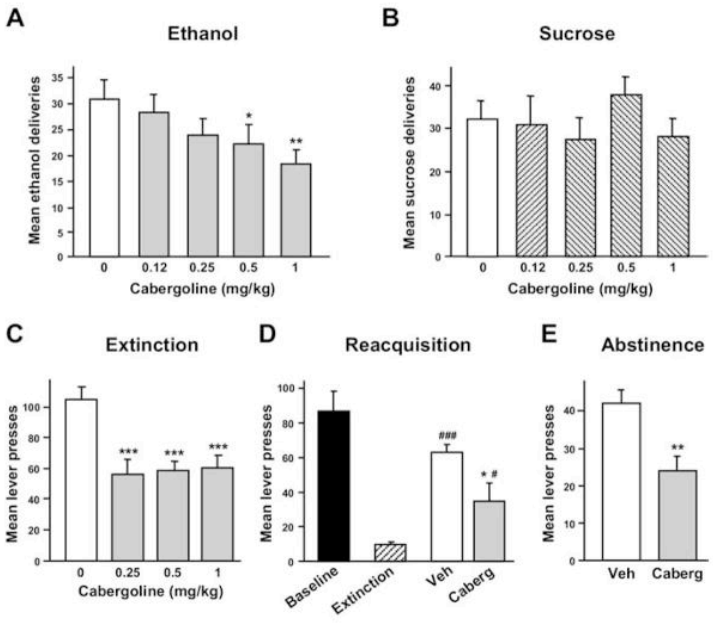

Figure 5-3. Cabergoline decreases operant self-administration-related behaviors in rats. Vehicle or cabergoline (0.12, 0.25, 0.5, or 1 mg/kg) was injected i.p. 3 hours befre the beginning of the test sessions. **A,** Cabergoline decreased the number of alcohol deliveries, n = 8. **B,** Sucrose self-administration was unaffected by cabergoline, n = 9. **C,** Cabergoline decreased the number of presses on the alcohol lever during an extinction session, n = 8. **D,** Cabergoline reduced reacquisition of operant alcohol self-administration after a period of extinction. Baseline represents the mean lever presses over the last 4 days of self-administration training, and extinction represents the mean lever presses during the final extinction session, n = 8. **E,** Cabergoline decreased the number of presses on the alcohol lever during an extinction session of 15 minutes that followed 10 days of abstinence from alcohol in rats with a history of excessive voluntary alcohol consumption, n = 8. All data are shown as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with vehicle controls, and #$p < 0.05$, ###$p < 0.001$, compared with extinction.

presses for alcohol during extinction, when no alcohol reward is delivered. Systemic administration of cabergoline reduced the number of lever presses for alcohol (Figure 5.3C) [$F(3,21) = 15.60, p < 0.001$].

Cabergoline lowers reacquisition of alcohol self-administration and seeking after a period of abstinence – The risk of relapse presents an enormous challenge for recovering alcoholics. Thus, we tested whether cabergoline could effectively reduce the propensity to relapse in a rat model. First, we determined the effect of systemic cabergoline (1 mg/kg), or vehicle, on the reacquisition of alcohol self-administration. Reacquisition is the rapid resumption of operant responding that occurs when the reward is made available again after a period of extinction (Bouton, 2002), and is a useful tool for discerning the ability of a therapeutic to extinguish drug-related behaviors (such as craving and relapse). Moreover, we have previously found that GDNF reduces the reacquisition of alcohol self-administration, indicating that cabergoline, via GDNF, may also do the same. We found that systemic cabergoline markedly reduced the reacquisition of operant responding for alcohol [$F(3,21) = 20.40, p < 0.001$], while a rapid, albeit partial, reacquisition of alcohol self-administration was observed in the control vehicle-injected rats (Figure 5-3D). We next measured the effect of cabergoline on lever pressing for alcohol during an extinction test that followed 10 days of abstinence from alcohol. This procedure is used to assess the motivation of rats to seek alcohol after a period of abstinence (Epstein et al., 2006; Le and Shaham, 2002). In control vehicle-injected rats, re-exposure to the self-administration chambers after a period of abstinence resulted in a high rate of operant responding on the alcohol-associated lever within a short, 15-minute time period (Figure 5-3E). Administration of cabergoline significantly attenuated this

increase [$T(7) = 3.66$, $p < 0.01$]. Together, these data suggest that the motivation to consume and seek alcohol in these models of relapse is reduced by treatment with cabergoline.

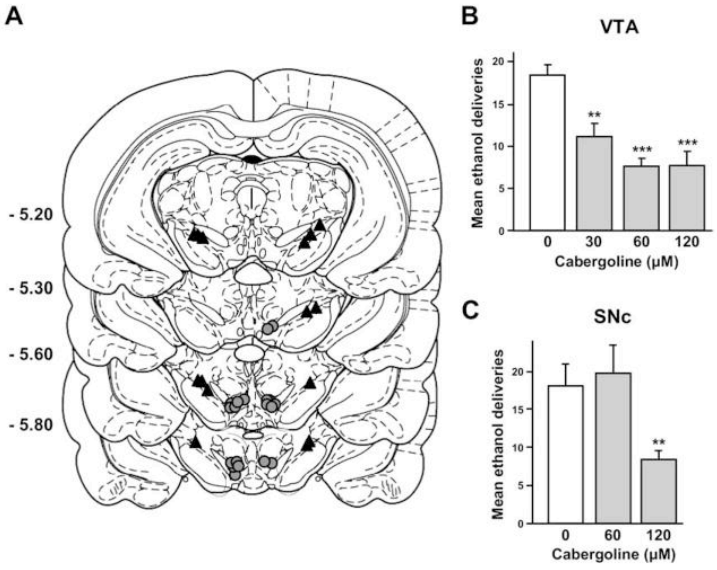

Figure 5-4. Intra-VTA injections of cabergoline decreases operant-responding for alcohol in rats. Vehicle or cabergoline (30, 60, or 120 μM) was infused into the VTA or SNc 3 hours before the test sessions. **A,** Schematic representation of the injection cannulae placements in coronal sections (Paxinos and Watson, 1998). The locations of the injector tips are represented by gray circles, and black triangles show the VTA and SNc microinjection sites. Numbers indicate the distance posterior to bregma in millimeters. **B,** Cabergoline infused into the VTA, decreased alcohol self-administration, n = 10. **C,** The highest concentration of cabergoline decreased alcohol self-administration when infused into the SNc, n = 9. Data are shown as the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to vehicle.

Microinjection of cabergoline into the rat VTA reduces alcohol self-administration – Because systemic administration of cabergoline increases *GDNF* levels in the VTA, the site of GDNF's anti-alcohol actions, we set out to determine whether cabergoline would act directly in the VTA to lower alcohol self-administration. Intra-VTA infusion of cabergoline significantly reduceded operant responding for alcohol in a dose-dependent manner (Figure 5-4A) [$F(3,27) = 12.61$, $p < 0.001$]. To localize cabergoline's effects to the VTA, we also tested whether infusion of cabergoline into the neighboring dopaminergic midbrain region, the SN pars compacta (SNc), would lower alcohol self-administration. We found that only the highest concentration of cabergoline

tested (120uM) lowered operant responding for alcohol [$F(2,16) = 6.35$, $p < 0.01$]. Therefore, cabergoline's effects on alcohol self-administration are more potent in the VTA.

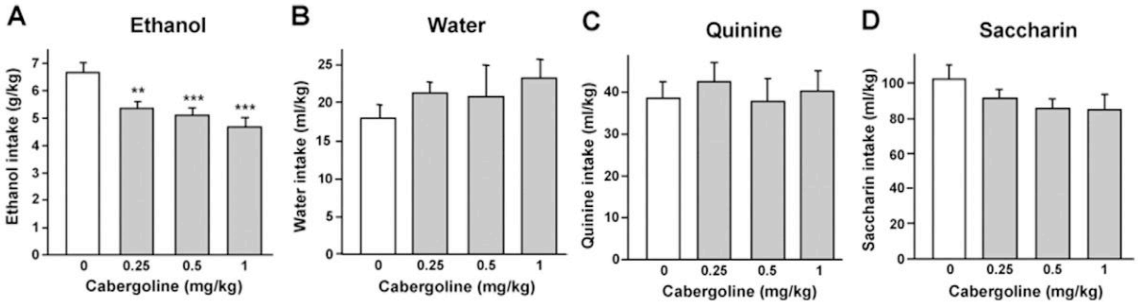

Figure 5-5. Cabergoline decreases alcohol consumption in mice. Vehicle or cabergoline (0.25, 0.2, or 1 mg/kg) was administered i.p. to mice 3 hours before the start of the test session. Cabergoline lowered voluntary alcohol intake in a dose-dependent manner during a 4-hour access period **(A)**, but not water **(B),** quinine **(C),** or sucrose **(D)** intake. For each experiment, n = 12. Data are shown as the mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with vehicle.

Cabergoline does not affect midbrain GDNF expression, or alcohol intake, in GDNF heterozygous knockout mice – Based on our findings above, we hypothesized that cabergoline lowers alcohol-drinking behaviors via GDNF. To test this possibility, we investigated whether cabergoline treatment would up-regulate *GDNF* and decrease alcohol-drinking in GDNF heterozygous knock-out mice ($GDNF^{+/-}$), which express significantly less *GDNF* than their wild-type ($GDNF^{+/+}$) littermates (Figure 5-6A, white bars). As in rats, systemic administration of cabergoline increased *GDNF* expression in the midbrain of the $GDNF^{+/+}$ mice; however, the cabergoline treatment did not induce *GDNF* expression in their $GDNF^{+/-}$ littermates (Figure 5-6A) [Genotype, $F(1,32) = 21.83$, $p < 0.001$; Treatment, $F(1,32) = 2.28$, $p > 0.14$; Genotype × Treatment interaction, $F(1,32) = 5.50$, $p < 0.05$]. Remarkably, systemic administration of cabergoline lowered

alcohol intake in the GDNF$^{+/+}$ mice, but not in their GDNF$^{+/-}$ littermates (Figure 5.6B). Two-way ANOVA analysis revealed no interaction [Genotype, $F(1,27) = 18.67$, $p < 0.001$; Treatment, $F(1,27) = 8.26$, $p < 0.01$; Genotype × Treatment interaction, $F(1,27) = 1.03$, $p < 0.32$], however, analysis of the data using the method of contrasts showed a significant difference between the vehicle- and cabergoline-treated GDNF$^{+/+}$ mice [$T(13) = 4.28$; $p < 0.001$], but not for the GDNF$^{+/-}$ mice [$T(13) = 1.01$, $p < 0.33$].

We further determined that the inactivity of cabergoline (a D2R-like agonist) on *GDNF* expression or alcohol intake in the GDNF$^{+/-}$ mice was not a result of changes in D2R expression or function in these mice. As shown in Figures 5-6C and 5-6D, quinpirole-stimulated, dose-dependent [^{35}S]GTPγS binding to the D2R was similar in

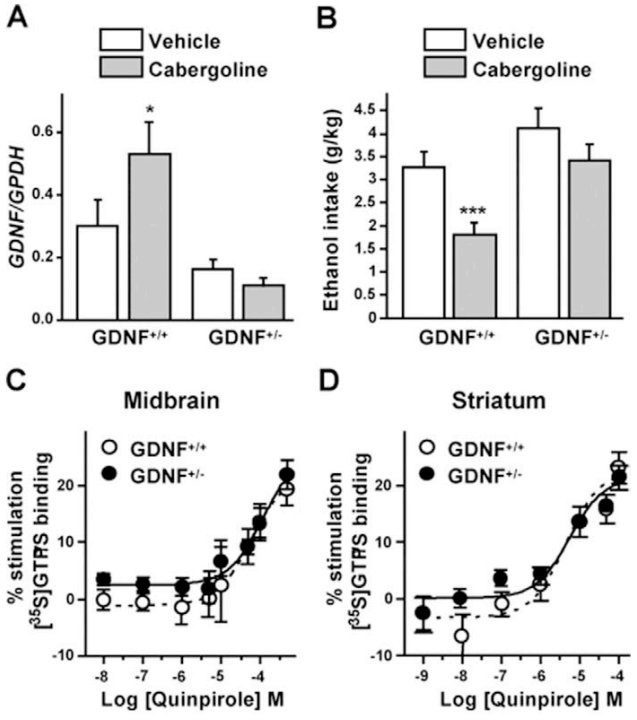

Figure 5-6. Cabergoline fails to increase midbrain *GDNF* expression levels or to decrease alcohol consumption in GDNF heterozygote knock-out mice. A, Cabergoline (1 mg/kg) was injected i.p. 1.5 hours before the dissection of the midbrain. *GDNF* was increased in the GDNF$^{+/+}$ mice, but not in their GDNF$^{+/-}$ littermates. Data are presented as the mean *GDNF/GPDH* ratio ± SEM, n = 8-10. **B,** Cabergoline (1 mg/kg) injected i.p. 3 hours before the test session lowered alcohol intake in the GDNF$^{+/+}$, but not the GDNF$^{+/-}$ mice, n = 14. **C & D,** Quinpriole-stimulated dopamine D2 receptor (D2R)-like [^{35}S]GTPγS binding measured in midbrain (C) and striatal (D) membrane homogenates prepared from GDNF$^{+/+}$ and GDNF$^{+/-}$ mice. The E_{max} and EC_{50} values were similar for the GDNF$^{+/+}$ and GDNF$^{+/-}$ mice (midbrain: E_{max}, 28 ± 2% and 24 ± 2%; EC_{50}, 59 ± 19 μM and 85 ±17 μM; striatum: E_{max}, 23 ± 3%, and 21 ± 2%; EC_{50}, 3.8 ± 0.2 μM, and 3.6 ± 0.3 μM; for GDNF$^{+/+}$ and GDNF$^{+/-}$ mice, resectively), n = 3. Data are shown as the mean ± SEM. *$p < 0.05$, ***$p < 0.001$, compared with vehicle.

membrane homogenates prepared from either $GDNF^{+/+}$ or $GDNF^{+/-}$ mice [for the midbrain: Treatment, $F(6,122) = 12.53$, $p < 0.001$; Genotype, $F(6,122) = 2.24$, $p = 0.14$; Treatment × Genotype interaction, $F(6,122) = 0.18$, $p = 0.98$; for the striatum: Treatment, $F(6, 123) = 59.37$, $p < 0.001$; Genotype, $F(1,123) = 0.62$, $p = 0.16$; Treatment × Genotype interaction, $F(6,123) = 2.10$, $p < 0.35$], indicating that the lower *GDNF* levels in the $GDNF^{+/-}$ mice do not alter the level of D2R-like activity. Therefore, the effects of cabergoline on alcohol-drinking and -seeking behaviors are very likely to be mediated by GDNF.

Discussion

Here, we demonstrate the ability of cabergoline to up-regulate *GDNF* mRNA and activate its signaling pathway, both *in vitro* and *in vivo* (Figures 5-1 and 5-2). Moreover, we found that systemic cabergoline lowered alcohol intake, as well as the propensity to seek alcohol, in rats (Figure 5-3). Additionally, intra-VTA infusion of cabergoline potently lowered alcohol intake, but intra-SNc infusion of cabergoline only lowered alcohol consumption at the highest dose tested, indicating that cabergoline's anti-alcohol actions are likely to be localized to the VTA (Figure 5-4). Furthermore, we demonstrated that cabergoline does not induce *GDNF* expression or lower alcohol intake in the $GDNF^{+/-}$ mice (Figure 5-6). Thus, we propose that cabergoline may alleviate alcohol consumption and relapse via the up-regulation of *GDNF*, and subsequent activation of its signaling pathway, in the VTA (Figure 5-7). These findings significantly add to a growing body of evidence, extensively discussed in this dissertation, indicating that

GDNF can be targeted to regulate alcohol consumption-related behaviors, such as relapse, to treat alcohol dependence (Carnicella and Ron, 2009; Ron and Janak, 2005).

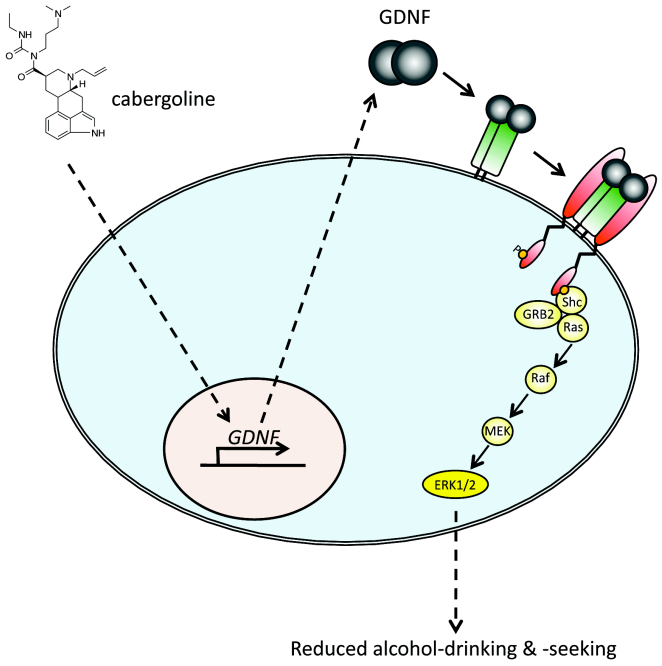

Figure 5-7. Cabergoline reduces alcohol-drinking and –seeking via an up-regulation of *GDNF* and activation of its signaling pathway. Treatment with cabergoline causes an up-regulation of *GDNF* expression in the VTA, which leads to an increase in the GDNF protein. Subsequently the GDNF signaling pathway is induced, determined by the phosphorylation, and thus activation, of the Ret receptor and the ERK1/2 pathway. This contributes to a reduction in alcohol consumption, as well as alcohol-seeking behaviors.

Cabergoline is a specific drug treatment for alcohol-seeking – Rats systemically treated with cabergoline did not change their self-administration of sucrose, a naturally-rewarding substance (Figure 5-3B). This suggests that cabergoline acts on alcohol-specific processes, rather than those that encode general rewarding and/or motivational mechanisms. This finding is similar to our previous report showing that rats treated with the anti-addiction agent, ibogaine, have reduced alcohol, but not sucrose, intake (He et al., 2005). We also previously demonstrated that the intra-VTA infusion of GDNF does not affect sucrose self-administration, but potently lowers self-administration of alcohol (Carnicella et al., 2008). This relatively selective mode of action by cabergoline is in marked contrast to acamprosate and naltrexone, two drugs that are currently approved for the treatment of alcohol craving, both of which lower alcohol and sucrose consumption in

rodents (Escher and Mittleman, 2006; Steensland et al., 2007). This non-specificity in acamprosate's and naltrexone's actions indicates a general effect on motivation, which possibly underlies the reported naltrexone-associated dysphoria (Crowley et al., 1985; Hollister et al., 1981), and contributes to the lack of patient compliance for both medications (Bouza et al., 2004; Johnson, 2008). Importantly, we found that cabergoline decreases the motivation to seek alcohol (Figure 5-3C), and also attenuated operant responding for alcohol in two animal models of relapse: reacquisition after a period of extinction (Figure 5-3D) and alcohol-seeking after a period of abstinence from excessive voluntary drinking (Figure 5-3E). Thus, cabergoline appears to have a selective effect on alcohol consumption, and most relevantly, blocks alcohol-seeking, as well as relapse behaviors. Together, this indicates that cabergoline may be a more effective treatment than the medications for alcohol dependence and relapse that are currently available.

The ability of cabergoline to reduce alcohol consumption is localized to the VTA – Our results also indicate that the VTA is the primary site of action for cabergoline (Figure 5-4B), which is in line with our previous findings localizing GDNF's anti-alcohol actions to the VTA (Carnicella et al., 2008). We did, however, observe that a high concentration of cabergoline infused into the neighboring SNc also lowered alcohol self-administration (Figure 5-4C). Diffusion of the drug from the SNc to the VTA could account for this effect. Furthermore, infusion of GDNF into the SNc does not alter alcohol self-administration (Carnicella et al., 2008), strongly suggesting that cabergoline's site of action is likely to be the VTA. Yet, we cannot exclude the possibility of a SNc-derived contribution to cabergoline's actions, as neurons from this brain region project to the dorsal striatum, which is implicated in addiction behaviors,

including the self-administration of drugs of abuse and alcohol (Everitt and Robbins, 2005; Jeanblanc et al., 2009; Jeanblanc et al., 2006; Vanderschuren et al., 2005; Volkow et al., 2006; Wang et al., 2007).

Cabergoline-induced GDNF: Where does it come from, and how? – Of additional interest is the cellular source of, as well as the mechanism underlying, the cabergoline-induced GDNF. If the findings described in Chapter 3 of this dissertation are any indication, it is possible that activation of the D2R, a molecular target of cabergoline (Newman-Tancredi et al., 2002a), underlies the up-regulation of *GDNF* in response to this drug. Indeed, *in vitro* blockade of the D2R in mouse astrocytes partially inhibited the up-regulation of *GDNF* by cabergoline (Ohta et al., 2004). Moreover, GDNF is expressed in both neurons and astrocytes (Airaksinen and Saarma, 2002), and both of these cell types express the D2R (Bal et al., 1994; Beaulieu and Gainetdinov, 2011; Khan et al., 2001). Thus, further investigation into whether cabergoline's actions at the D2R result in the induction of *GDNF in vivo,* and in which VTA cell population this occurs, is warranted.

Cabergoline: Safety and efficacy – Cabergoline is approved for marketing in several countries, including the United States, for the treatment of hyperprolactinemia (Colao et al., 2006; Webster et al., 1994). Interestingly, it is also used as an adjunctive or monotherapy for the alleviation of the symptoms that accompany Parkinson's disease (PD) (Bonuccelli, 2003; Jankovic and Stacy, 2007). In PD patients, the high doses of cabergoline used (2-6 mg/day) have been reported to contribute to the risk of cardiac valvulopathy (Kars et al., 2008). The doses used in this study (0.25 - 0.5 mg/kg) to effectively reduce alcohol intake, however, are much lower. Moreover, comparably low

doses in humans being treated for hyperprolactinemia (0.25 – 3.5 mg/week) for several years have only shown an association between moderate valvular regurgitation and the highest doses of cabergoline (Bogazzi et al., 2008; Colao et al., 2008), suggesting that low doses that effectively lower alcohol consumption carry a low risk of this side effect. Furthermore, a pilot study demonstrated that a weekly dose of 0.5 mg per week significantly reduced cocaine use in human cocaine addicts, as determined by analysis of cocaine metabolite levels in urine samples, as well as usage self-reports (Shoptaw et al., 2005). Together, these findings present a strong possibility for cabergoline to be safely and effectively used to lower both alcohol and drug use.

Summary

In conclusion, we found that cabergoline is a selective and effective drug treatment for alcohol-drinking and -seeking behaviors in rodents. Moreover, we established that cabergoline's anti-alcohol actions are mediated by the up-regulation of *GDNF* and the subsequent activaton of its signaling pathway. Together with our previous reports, this strongly supports the idea that a targeted activation of the GDNF pathway may be a valuable strategy for the treatment of alcohol addiction. In addition, while further studies are needed to evaluate cabergoline's effectiveness in human alcoholics, this study highlights the strong possibility that this drug may be used as a selective and efficacious medication for the treatment of alcohol abuse and dependence.

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CHAPTER 6

GENERAL CONCLUSIONS & FUTURE DIRECTIONS

Half of science is putting forth the right questions.

Sir Francis Bacon

This dissertation represents a multi-faceted approach that sheds new light on the mechanisms both upstream and downstream of glial cell line-derived neurotrophic factor (GDNF), and their implications for the regulation of alcohol consumption. Here, I will discuss the findings described in the previous four chapters as a whole, and in the context of future alcohol addiction research.

Fluctuating GDNF Expression in the Ventral Tegmental Area: An Alcohol-Induced Biochemical Neuroadaptation

A seminal finding of this work is the identification of *GDNF* as a novel alcohol-responsive gene, whose expression in the ventral tegmental area (VTA) of rats in the context of alcohol changes as a function of drinking history. During early exposures to alcohol, *GDNF* is induced in the VTA of rats. Deprivation after a long history of excessive alcohol consumption causes *GDNF* levels to fall below normal baseline levels. At this point, another bout of alcohol drinking restores *GDNF* levels back to normal, basal levels. We propose that fluctuating *GDNF* levels are an alcohol-induced biochemical neuroadaptation that, although initially acting as a homeostatic control against the development of excessive drinking, over time constitutes one component of the alcohol-induced negative affective state, thus incentivizing continued alcohol intake.

GDNF and the homeostatic regulation of alcohol intake – Short-term voluntary alcohol drinking, in addition to a single acute administration of alcohol, induces the expression of *GDNF* in the VTA of rats. Using shRNA to attenuate this increase, we determined that this serves to regulate the escalation of alcohol intake in a model of voluntary excessive drinking. Thus, alcohol up-regulates *GDNF,* which activates its signaling pathway, and acts as a homeostatic regulator of the progression of excessive drinking.

In addition, we identified a possible molecular mechanism for this alcohol-mediated up-regulation of *GDNF* in the VTA. A single systemic administration of alcohol caused a rapid increase in the expression level of a transcription factor, Zif268, whose genomic targets include *GDNF*. Importantly, this increase in *Zif268* expression preceded the up-regulation of *GDNF,* suggesting that alcohol first increases levels of the transcription factor, which may then act to induce the expression of its target gene. We also observed a rapid increase in *Zif268* levels in the rat VTA following the activation of the dopamine D2 receptor (D2R) via a systemic administration of a D2R agonist. Furthermore, *in vitro* D2R activation up-regulates *Zif268* in a Gβγ-ERK1/2-dependent mechanism, and is followed by an increase in GDNF mRNA and protein levels. These results posit an interesting function for the alcohol-induced somatodendritic release of dopamine within the VTA (Perra et al., 2011). While it is well-known that this local release of dopamine increases D2R-mediated autoinhibition, we propose that an additional molecular consequence is the Zif268-dependent up-regulation of *GDNF*. Therefore, we propose a mechanism in which alcohol, through an increase in the somatodendritic release of dopamine and the subsequent the activation of D2Rs in the

VTA, mediates the up-regulation of *GDNF* via a D2R-Gβγ-ERK1/2-Zif268 pathway. What follows is the activation of the GDNF pathway, which then functions as a homeostatic mechanism, activated by alcohol, to control the development of excessive drinking.

Reduced basal levels of GDNF during deprivation from long-term excessive alcohol consumption – After a long history of repeated bouts of excessive alcohol drinking and deprivation, we found that *GDNF* levels in the rat VTA are abnormally low during deprivation. Interestingly, *GDNF* levels are restored to former baseline levels when alcohol is consumed following a deprivation period. As a result, we propose that the deprivation-associated allostatic decrease in *GDNF* partially incentivizes continued alcohol intake, as this behavior will return *GDNF* levels to previous, normal levels.

We also observed a concomitant decrease in the levels of the Pitx3, a transcriptional activator of *GDNF* expression. We therefore suggest that a progressive loss of Pitx3 as a result of excessive alcohol intake is the molecular mechanism underlying the deprivation-associated down-regulation of *GDNF*. Pitx3, by regulating the expression of dopamine neuron-associated genes (GDNF, tyrosine hydoxylase [TH], and the dopamine transporter [DAT]) is a noted contributor to the maintenance of dopaminergic neuron health (Li et al., 2009). This is particularly interesting, as it is well-established that withdrawal after chronic alcohol consumption results in a decrease in the activity of the nucleus accumbens (NAc)-projecting VTA dopamine neurons (Bailey et al., 2001; Diana et al., 1993; Shen, 2003; Shen et al., 2006), subsequently reducing dopamine levels in the NAc (Diana et al., 1993; Rossetti et al., 1992a; Rossetti et al., 1992b; Weiss et al., 1996), which is thought to contribute to alcohol craving (Koob,

2003; Tupala and Tiihonen, 2004). Thus, as they are important to the maintenance of dopaminergic neuron health, a reduction in Pitx3 and/or GDNF in the VTA during deprivation might contribute to this reduced dopaminergic activity, which, in turn, leads to craving and relapse.

Interestingly, a recent study in our lab demonstrated that intra-VTA infusion of GDNF reversed the reduction in dopamine release caused by withdrawal from excessive alcohol drinking (Barak et al., 2011). Taken together with the findings discussed here, this suggests that not only does the deprivation-associated down-regulation of *GDNF* contribute to the overall alcohol-induced allostasis of the mesolimbic system, but that the restoration of this pathway, either by the up-regulation of *GDNF* after a bout of alcohol drinking, or by the infusion of the exogenous protein, restores the activity of this system. In other words, the reduced levels of *GDNF* provide not only a cause of continued, excessive drinking, but also highlights its signaling pathway as a possible target for its treatment.

GDNF-Mediated Signaling: Effects & Pharmacotherapeutic Value

Effects of GDNF signaling – One aim of this dissertation project was to determine the molecular mechanism underlying the rapid, GDNF-mediated reduction in alcohol consumption (Carnicella et al., 2008). Because this effect was found to be extracellular-regulated kinase 1/2 (ERK1/2)-dependent (Carnicella et al., 2008), we sought to determine the effects of GDNF-activated ERK1/2. Using an *in vitro* model of dopaminergic neurons, we found that GDNF enhances evoked dopamine release in an ERK1/2-dependent mechanism. We also identified the synaptic vesicle protein, Synapsin

Ib (SynIb), as a novel phosphorylation target of GDNF-activated extracellular signal regulated kinase 1/2 (ERK1/2) in both *ex vivo* midbrain and *in vitro* dopaminergic model systems. As SynIb is a regulator of neurotransmitter release, we propose that GDNF-mediated ERK1/2 phosphorylation of SynIb plays a role in GDNF-induced dopamine release. As mentioned above, we recently reported a GDNF-mediated increase in mesolimbic dopamine, which reversed the alcohol withdrawal-associated dopamine deficiency in this circuit (Barak et al., 2011). The results presented here indicate that this is likely to occur in an ERK1/2-dependent mechanism, and may include the phosphorylation of SynIb. Moreover, in light of the knowledge that chronic alcohol reduces mesolimbic dopamine during withdrawal (Diana et al., 1993), and that this hypodopaminergic state can contribute to the motivation to consume alcohol (Koob, 2003; Tupala and Tiihonen, 2004), these findings suggest that the GDNF-mediated reduction in alcohol intake is caused, in part, by its ability to increase in dopamine release

Therapeutic value of the GDNF signaling pathway – We determined that pharmacological activation of the GDNF signaling pathway, via the cabergoline-mediated up-regulation of *GDNF,* in the VTA effectively reduced alcohol consumption, as well as alcohol seeking and relapse behaviors (Carnicella et al., 2009). This study testifies to the therapeutic potential of this signaling pathway. Importantly, as described above, because GDNF has the ability to enhance dopamine release, it also has the potential to be rewarding itself. However, as GDNF itself did not induce conditioned place preference (CPP, a measure of reward) (Barak et al., 2011), it is unlikely that it presents an abuse liability. Thus, GDNF and its signaling pathway represent a family of

drug targets that may be safely used to pharmacologically reduce alcohol intake and craving.

Overall Summary

Together, our previous studies and the findings presented herein highlight the role of GDNF and its signaling pathway in the regulation of alcohol consumption. Clearly, GDNF is a key player in an endogenous pathway that regulates the development of excessive alcohol intake in response to early drinking experiences. Moreover, this pathway is likely to be activated as a direct result of the effect of alcohol on dopaminergic activity in the VTA. Chronic, excessive alcohol drinking then results in the breakdown in this pathway during a period of deprivation, which may contribute to the overall alcohol withdrawal-induced hypodopaminergic state of the mesolimbic system. Furthermore, *GDNF* expression is returned to former baseline levels after a bout of alcohol consumption, temporarily restoring this pathway, and thus promoting persistent cycles of alcohol drinking, withdrawal, and relapse. Importantly, the findings presented here also support an overall working hypothesis wherein the activation of the GDNF signaling pathway restores dopaminergic tone, via modifications in synaptic vesicle machinery, whereby alcohol consumption is reduced. Finally, a comprehensive molecular and behavioral study establishes that pharmacological activation of GDNF and its signaling pathway possesses great therapeutic potential for the treatment of alcohol dependence.

Future Directions

The data represented in this dissertation give rise to a number of interesting questions. For example, while we have established that acute alcohol exposure up-regulates *GDNF* expression in the rat VTA, it remains unclear what the source of this newly-expressed *GDNF* is. Both neurons and astrocytes have been shown to express *GDNF* (Airaksinen and Saarma, 2002). Additionally, both of these cell types express the D2R (Bal et al., 1994; Beaulieu and Gainetdinov, 2011; Khan et al., 2001), the activation of which in the presence of alcohol we suggest can result in *GDNF* expression. It would therefore be of great interest to determine whether neurons or astrocytes, or both cell types, contribute to the increase in *GDNF* in the presence of alcohol.

Similarly, we observed that the alcohol-mediated alterations in *GDNF* are localized to the rat VTA, and not the NAc. This finding was surprising, as the main source of *GDNF* is the striatum, including the NAc (Trupp et al., 1997). Moreover, preliminary experiments conducted in mice suggest that voluntary alcohol intake up-regulates *GDNF* in the NAc (Appendix B, Figure A-4). This observation raises two major questions: 1) do alcohol-induced alterations in *GDNF* expression occur in a regional pattern that is species-specific, and if not, 2) why was no change in *GDNF* expression after alcohol consumption in the NAc of rats observed? A recent report identified two splice isoforms of the GDNF precursor (pro-GDNF) in cultured cortical neurons that display differing secretion rates and mechanisms (Lonka-Nevalaita et al., 2010). An interesting possibility to consider is that alcohol causes complementary, splice isoform-specific changes in *GDNF* expression in the NAc. Thus, while overall levels of *GDNF* are unchanged, the relative proportion of each splice variant may have shifted. Because

of the differences in secretion dynamics or each splice variant, it would be of interest to determine what effect, if any, such a shift would have on alcohol consumption.

This dissertation highlights two possible molecular mechanisms involving the Zif268 and Pitx3 transcription factors that underlie alcohol-mediated changes in *GDNF* expression. There are, however, a multitude of mechanisms, in addition to transcriptional machinery, that regulate the pattern of gene expression. Currently, there is a bourgeoning interest in the study of epigenetic modifications, such as histone acetylation and methylation and DNA methylation, and their effects on gene expression. With regard to GDNF, histone hyperacetylation at the *GDNF* promoter has been shown to up-regulate *GDNF* expression in mixed cultures of neurons and astrocytes (Wu et al., 2008). Additionally, both DNA methylation and histone acetylation are implicated in the stress-induced increase in *GDNF* expression, which contributed to an adaptive response to stress (Uchida et al., 2011). Most interestingly, differences in the epigenetic status, and not DNA sequence polymorphisms, of the *GDNF* promoter region between a stress-adaptable mouse strain and a stress-inadaptable mouse strain regulated the expression of *GDNF* in response to stress, and defined the ability of one mouse strain, and not the other, to adapt to it (Uchida et al., 2011). This seminal study highlights the importance of the epigenetic status of the *GDNF* promoter region in response to a stimulus. Incidentally, there is a growing body of evidence demonstrating that drugs of abuse, including alcohol, alter the epigenetic landscape in neurons (Maze and Nestler, 2011; Wong et al., 2011). Alcohol has been shown to affect both DNA methylation in cultured neurons (Zhou et al., 2011), as well as histone acetylation and methylation in a neuroblstoma cell line (D'Addario et al., 2011). It is as yet unknown whether alcohol will

induce changes in the epigenetic status of the *GDNF* promoter region, and what, if any, affect such changes would have on either the development or the persistence of excessive alcohol consumption.

Conversely, the basal epigenetic landscape of the *GDNF* promoter may also define an individual's susceptibility to consume alcohol to excess. The presence of a GDNF-mediated homeostatic mechanism that regulates the progression of excessive alcohol consumption also reasonably supposes that varying levels of endogenous GDNF can contribute to an individual's propensity to drink too much. Interestingly, a recent study of the inter-individual variation in experimental alcohol drinking models revealed that variations in basal gene expression (including those genes that function in epigenetic modifications) were correlated with the level of alcohol intake (Wolstenholme et al., 2011). Testing whether differences in basal levels of *GDNF* expression are correlated with alcohol intake, and whether these changes are due to underlying variations in the epigenetic status of its promoter, would therefore be of great interest.

Concluding Remarks

Here, we highlight a role for GDNF and its signaling pathway in the response to, and treatment of, excessive alcohol consumption. *GDNF* is up-regulated as a result of alcohol drinking to regulate the progression of excessive consumption. The pathway underlying this increased expression likely involves D2R-mediated signaling and a subsequent Gβγ- and ERK1/2-dependent activation of the Zif268 transcription factor. Importantly, long-term excessive alcohol intake results in an abnormally low level of *GDNF* expression during deprivation, which is reversed after a bout of alcohol drinking.

Furthermore, we identify a novel target of GDNF-activated ERK1/2, SynIb, which may mediate the reduction of alcohol consumption by GDNF-ERK1/2 signaling. Finally, we demonstrate that *GDNF* expression can be pharmacologically induced to lower alcohol consumption, as well as alcohol-seeking and relapse behaviors, in rodents. Alcohol dependence and abuse constitute an enormous financial and societal burden. These data reveal not only the importance of GDNF in the progression, persistence, and prevention of alcohol dependence, but also present new and exciting avenues of examination towards a deeper understanding of, and therapeutic possibilities for, this disease.

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APPENDIX A: LIST OF COMMON ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
CPP	conditioned place preference
D2R	dopamine D2 receptor
DAT	dopamine transporter
ERK1/2	extracellular signal-regulated kinases 1&2
GABA	γ-amino butyric acid
GDNF	glial cell line-derived neurotrophic factor
GDNF-/-	GDNF homozygous knock-out
GDNF+/-	GDNF heterozygous knock-out
Gβγ	G protein β and γ subunits
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
NAc	nucleus accumbens
PFC	prefrontal cortex
PI3K	phosphoinositide 3-kinase
Pitx3	paired-like homeodomain transcription factor-3
Ret	rearranged during transfection
SN	substantia nigra
SNc	substantia nigra *pars compacta*
SynI	synapsin I
SynIb	synapsin I isoform b
TH	tyrosin hydroxylase
VTA	ventral tegmental area
Zif268	zinc-finger protein 268

APPENDIX B: SUPPLEMENTAL FIGURES

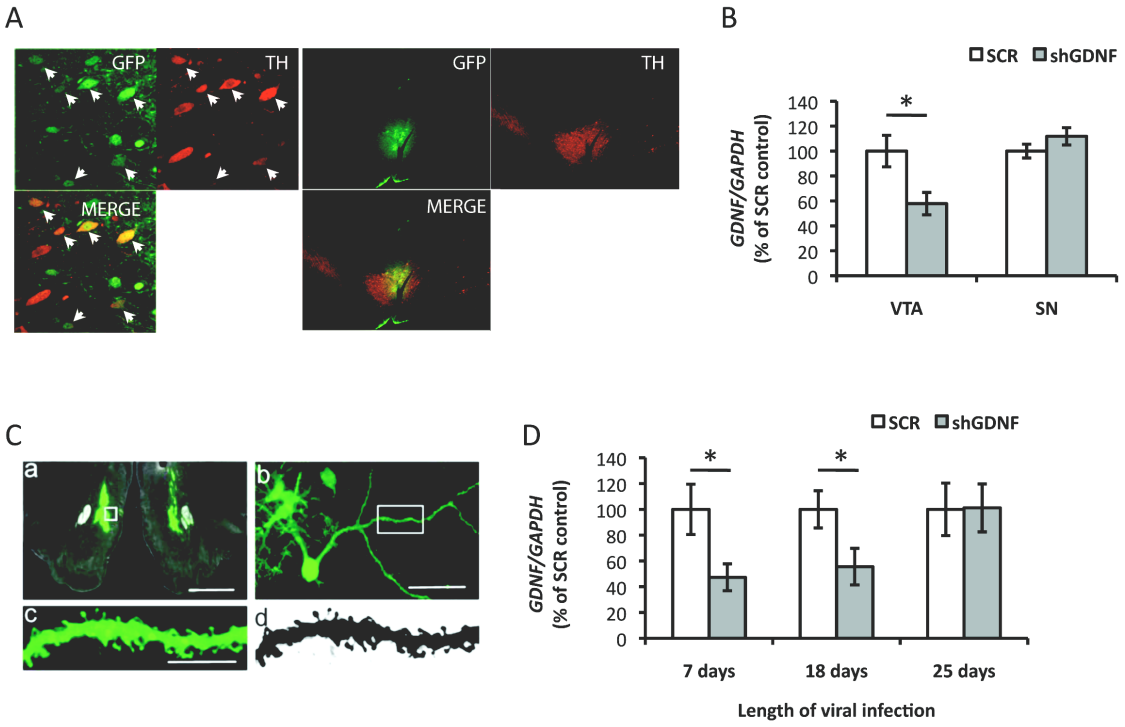

Figure A-1. *In vivo* validation of adenoviral-mediated *GDNF* knock-down. A & B, Adenovirus expressing shRNA targeted against GDNF (shGDNF) or a control adenovirus expressing a scrambled control sequence (SCR) was infused into the VTA of rats (1.2 µL/side, 1.3e8 ifu/ml). **A,** Adenovirus expressing shGDNF infects dopaminergic neurons in the ventral tegmental area (VTA). Immunohistochemistry of ventral midbrain slices was performed 5 days post-infection in order to detect tyrosine hydroxylase (TH, a marker of dopaminergic neurons, red) and green fluorescent protein (GFP, to localize viral infection, green). **B,** Infusion of adenovirus expressing shGDNF into the VTA decreases *GDNF* expression in the VTA, but not the very proximal substantia nigra (SN). *GDNF* mRNA levels were quantified by qRT-PCR on 14 days post-virus infusion. Data are expressed as mean ± SEM of *GDNF/GAPDH* expression, n = 6 animals per group. *$p<0.05$, compared to SCR control. **C & D,** Adenoviral-mediated down-regulation of *GDNF in vivo* is time-dependent. **C,** Confirmation of adenoviral infection in the nucleus accumbens (NAc) by GFP immunofluorescence, 18 days after infusion of the virus (2 µL/side, 3.2e9 ifu/mL). The white boxes in **a** and **b** indicate the position of the medium spiny neuron shown in **b** and the position of the spiny dendrite shown in **c** and **d** (**c** and **d** are the same dendrite represented in color and in black and white, respectively). Scale bars: **a**, 2mm; **b**, 50 µm; **c**, 10 µm. Image reproduced from: (Wang et al., 2010) **D,** Adenoviral-mediated down-regulation of *GDNF* expression in the NAc is detectable by one week (7 days) after viral infusion, and is transient. *GDNF* levels were quantified by qRT-PCR 7, 18, and 25 days post-viral infusion. Data is expressed as the mean *GDNF/GAPDH* ± SEM, n = 4-6 animals per group. *$p < 0.05$, as compared to SCR control.

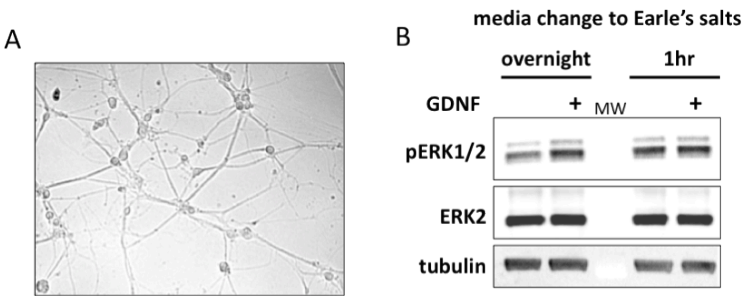

Figure A-2. Overnight incubation of primary neuron cultures from the ventral midbrain in Earle's salt solution reduces basal levels of activated ERK1/2. A & B, Neurons were prepared from the ventral midbrain regions of P0 rat pups. Neurons were cultured for 14 days in vitro (DIV). **A,** Representative light image of cultured primary ventral midbrain neurons, 25X magnification. **B,** An overnight incubation of cultured neurons in Earle's Balanced Salt Solution (EBSS) reduced the level of basal ERK1/2 activity. Cell culture medium was exchanged for 100% EBSS overnight (~18 hours) or 1 hour before treatment with 50 ng/mL GDNF for 15 minutes. Detection of phosphorylated ERK1/2 (pERK1/2) by Western blot was used to determine ERK1/2 activity. Tubulin was used as a loading control.

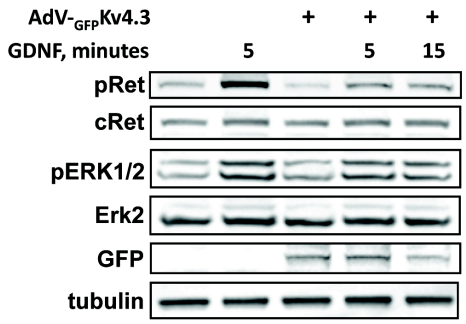

Figure A-3. Long-term GDNF treatment of SH-SY5Y cells over-expressing GFP-tagged K_v4.3 might cause a reduction of $_{GFP}K_v$4.3 levels. Differentiated SH-SY5Y cells were infected with adenovirus over-expressing green fluorescent protein (GFP)-tagged K_v4.3 (1.5e7 ifu/mL for 72 hours), then treated with GDNF (50 ng/mL) for 5 or 15 minutes. Western blot analysis was used to detect levels of phosphorylated Ret receptor (pRet) and phosphorylated ERK1/2 (pERK1/2), which were used to verify activation of the GDNF signaling pathway. Levels of K_v4.3 were determined by probing for its GFP tag. Tubulin was used as a loading control. Image is representative of four similar timecourse experiments.

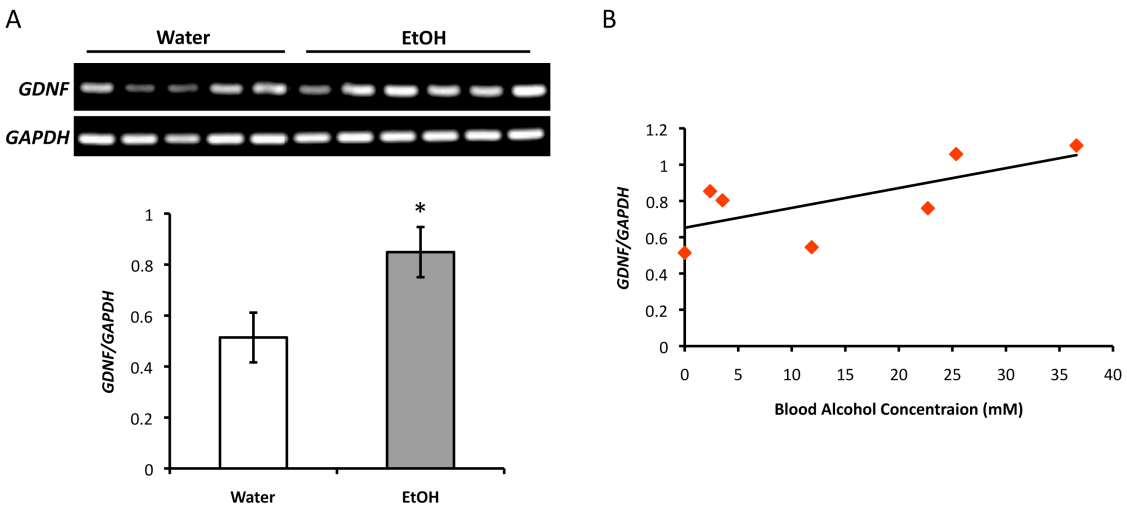

Figure A-4. Short-term, excessive alcohol intake in mice elevates *GDNF* expression in the nucleus accumbens. A & B, Mice (male C57BL/6) consumed alcohol (EtOH) in the limited-intermittent-access to 20% alcohol drinking in the dark schedule. In brief, a 20% alcohol solution was made available, in addition to water, for four hours every other day. Access to the alcohol bottle began two hours following the beginning of the dark cycle. Mice experienced a total of four alcohol-drinking sessions over the course of one week. **A,** *GDNF* expression levels in the nucleus accumbens (NAc) are significantly increased after voluntary short-term excessive alcohol intake. NAc tissues were collected immediately following the end of the last alcohol-drinking session. Semi-quantitative RT-PCR was used to measure *GDNF* mRNA. Bar graph represents the mean *GDNF*/*GAPDH* ± SEM, n = 5-6 per group. *$p < 0.05$, as compared to the water-only controls. **B,** Relative *GDNF* expression levels tend to correlate with blood alcohol concentration (BAC, in mM). BAC was assayed by gas chromatography from trunk blood collected immediately following the end of the last alcohol-drinking session. Note that the water-only (0 mM BAC) controls are grouped as a single point and assigned the value of the average *GDNF*/*GAPDH* for that group. Pearson's two-tailed correlation analysis revealed a strong trend towards a positive correlation between *GDNF*/*GAPDH* levels and BAC ($R = 0.7031$, $df = 5$, $p = 0.078$).

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