ROLE OF BDNF-TRKB SIGNALING IN COCAINE ADDICTION

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DEDICATION

There are a number of people who were instrumental in my graduate studies, and without whom this dissertation would not have come to fruition:

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ROLE OF BDNF-TRKB SIGNALING PATHWAY IN COCAINE ADDICTION

by

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Cocaine addiction results in neuroadaptations and drug-induced neuroplasticity that promote changes in protein expression and neuron morphology. Cocaine-induced increases in dopamine ultimately alter dopamine signaling in brain regions modulating reward and motivation, specifically the nucleus accumbens (NAc), and downstream proteins. One protein of particular interest is brain-derived neurotrophic factor (BDNF), a modulator of cell survival, viability, and plasticity. Cocaine has been shown to increase BDNF mRNA and protein levels in the NAc shell. In addition, intra-NAc infusions of BDNF have been demonstrated to increase cocaine intake and motivation for cocaine. These increases in BDNF also lead to activation

of its receptor, tropomyosin receptor kinase B (TrkB). Studies indicate that the loss of TrkB specifically in the NAc shell reduced the reinforcing effects of cocaine using a self-administration paradigm, and also psychomotor effects of cocaine on activity; however, the contributions of each signaling pathway are unknown. Chapter 3 examined the creation of a cell-type specific herpes simplex viral (HSV) vector system to over-express wildtype TrkB or its docking mutants. *In vivo* and cell culture experiments indicated very weak viral expression, while cocaine self-administration testing produced inconsistent and inconclusive results. Chapter 4 examined cocaine-induced BDNF-TrkB receptor signaling using an adeno-associated viral vector system to over-express wildtype TrkB and its signaling mutants, more generally across NAc cell types. Initial self-administration testing suggested that overexpression of kinase dead TrkB (TrkB K571N) in the NAc shell increased the threshold dose required to maintain self-administration on the dose-response test and reduced motivation for cocaine. Subsequent behavioral testing did not confirm these results. Preliminary tissue staining demonstrated similar levels of viral infectivity between AAV-GFP and AAV-TrkB WT; however, subsequent tissue staining demonstrated very weak to no viral expression, consistent with the lack of consistent behavioral results. Finally, Chapter 5 utilized a transient but efficacious HSV vector system to over-express wildtype TrkB and its signaling mutants during cocaine-induced activation of the BDNF-TrkB receptor signaling pathway. Self-administration testing suggested that the kinase dead TrkB viral mutant (HSV-TrkB K571N) inversely affected cocaine taking and motivation for cocaine. In contrast to

the cell-specific HSV vectors tested earlier, immunohistochemical techniques indicated stronger and consistent expression of these HSV-TrkB viruses; however, TrkB signaling-specific protein expression was not found. These findings indicate an inconsistency between behavioral results and viral expression, yet suggest that further experimentation is warranted.

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PRIOR PUBLICATIONS

Simmons D, **Buzin N**, Larson E, Graham DL, Edwards S, Bachtell RK, Neve R, von Zastrow M., Self D (in preparation). Modulation of mu opioid receptors in nucleus accumbens by beta endorphin release during cocaine self-administration.

Larson EB, Grahamn DL, Arzaga RR, **Buzin NR**, Webb J, Green A, Bass CE, Neve RL, Terwilliger EF, Nestler EJ, and Self DW. (2011). CREB-mediated increases in cocaine-taking and -seeking behavior are associated with increased BDNF levels in the nucleus accumbens shell. J. Neuroscience, 31 (45), 16447-16457.

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6-OHDA – 6-hydroxydopamine

AAV – Adeno-associated virus

AMPA – Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF– Brain-Derived Neurotrophic Factor

C – Celsius

cAMP – cyclic Adenosine Mono Phosphate

cDNA – complementary deoxyribonucleic acid

CMV – Cytomegalovirus promoter

CREB – cAMP Response Element-Binding Protein

D1 – Dopamine D1 receptor

D2 – Dopamine D2 receptor

DA – Dopamine

DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acide

Dyn – Dynorphin promoter

ECL – Enhance Chemiluminesence

Enk – Enkephalin promoter

ERK – Extracellular Receptor Kinase

FR# – Fixed Ratio #

GABA – Gamma Aminobutyric Acid

GFP – Green Fluorescent Protein

GFAP – Glial Fibrillary Acidic Protein

HEK – Human Embryonic Kidney cells

HEPES – N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

HSV – Herpes Simplex Virus

ip – intraperitoneal

IRES – Internal Ribosomal Entry Site

iv – intravenous

L-DOPA – L-3,4-dihydroxyphenylalanine

LTD – Long-Term Depression

LTP – Long-Term Potentiation

mRNA – messenger Ribonucleic Acid

MSN – Medium Spiny Neuron

NAc – Nucleus Accumbens

NGS – Normal Goat Serum

NMDA – N-methyl-D-aspartate receptor

OFC – Orbital Frontal Cortex

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PFC – Prefrontal cortex

PI3K – Phosphoinositide 3-kinase

PLCγ – Phospholipase C-gamma

PR – Progressive Ratio

PVDF – Polyvinylidene fluoride

SA – Self-Administration

sc – subcutaneous

SDS – Sodium Dodecyl Sulfate

TrkB – Tropomyosin-Receptor-Kinase B

TTBS – Tween Tris Base Saline

UTR – Untranslated Region

VTA – Ventral Tegmental Area

WT – Wild Type

CHAPTER ONE

BDNF-TRKB SIGNALING IN COCAINE ADDICTION

Cocaine Addiction

Addiction is marked by an escalation in drug intake and compulsive drug-taking despite adverse economic, physiological, psychological and social consequences. These behaviors are further exacerbated during periods of abstinence (withdrawal) from the drug, where cravings for the drug increase and drug-seeking becomes uncontrollable (Grimm et al., 2001; Lu et al., 2004b; Self, 2004). Furthermore, substance dependence is defined by increased time spent on activities necessary for obtaining the drug, as well as persistent and usually unsuccessful attempts to cease substance use (*Diagnostic and Statistical Manual of Mental Disorders-IV-TR*, 2000). This compulsive drug use can lead to the engagement of motivational circuits by three stimuli that induce relapse in animal models and are thought to cause craving in humans: 1) drug priming, 2) drug-conditioned Pavlovian cues, and 3) stressful situations.

Cocaine addiction, in particular, creates a significant economic and health-related burden in the United States. The popularity of cocaine rose between the 1980s and 1990s (NIDA, 2010), and as of 2008, there were 1.9 million cocaine users (SAMHSA, 2009). In the brain, cocaine inhibits the dopamine (DA) transporter and blocks reuptake of dopamine into presynaptic terminals, increasing extracellular

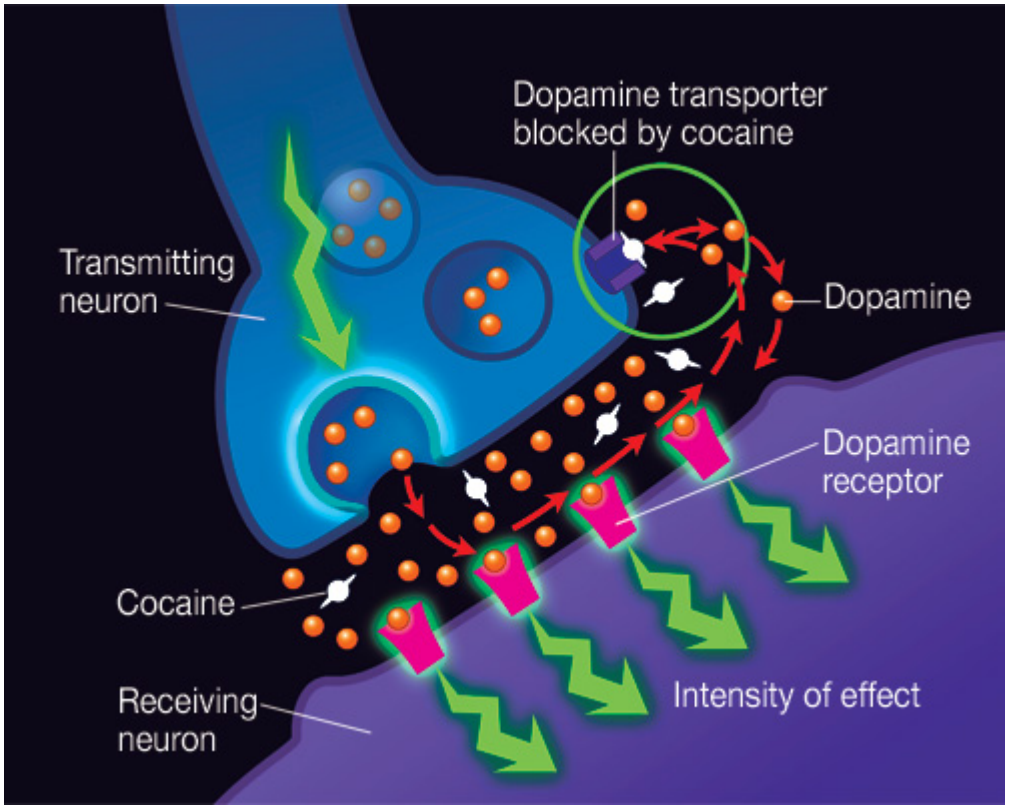

Figure 1.1 The mechanism of action of cocaine. This figure illustrates the ability of cocaine to prevent the reuptake of dopamine through the blockade of the dopamine transporter. The result is an increase in extracellular dopamine, which modulates the activities of brain regions involved in the reward pathway (NIDA, 2010).

dopamine concentration (Figure 1.1) (Nestler & Malenka, 2004; Ron & Jurd, 2005; Self, 1998). Additionally, cocaine blocks the transporters for serotonin and noradrenaline, and increases their extracellular concentration (Elliott & Beveridge, 2005). Thus, through its indirect modulation of neurotransmitters, cocaine alters brain regions involved in reward, memory, and emotion. Although addiction research has provided great insight into the cellular, molecular, and behavioral complications of cocaine use, the transition from recreational drug use to an addicted state is ill-defined. In order to define this transition, addiction research examines drug-induced changes that occur in the reward pathway of the brain.

The Reward Pathway

The cocaine-induced increase in dopamine release affects brain regions comprising the reward pathway, a network of brain regions implicated in mediating molecular and cellular changes and modulating behavioral responses to both drugs of abuse and natural rewards (Figure 1.2) (Dackis & O'Brien, 2001; Nestler, 2004). Dopaminergic projections originating from the ventral tegmental area (VTA) are involved in the reinforcing properties of cocaine and natural rewards (Dackis & O'Brien, 2001; Phillips et al., 2003; Self, 2004), and demonstrate a characteristic burst firing activity that cocaine mimics through the blockade of the dopamine transporter (Grace & Bunney, 1983). These dopaminergic projections terminate in the nucleus accumbens (NAc), on gamma-Aminobutyric acid (GABA)-containing medium spiny neurons, and increase dopamine levels in response to many drugs of

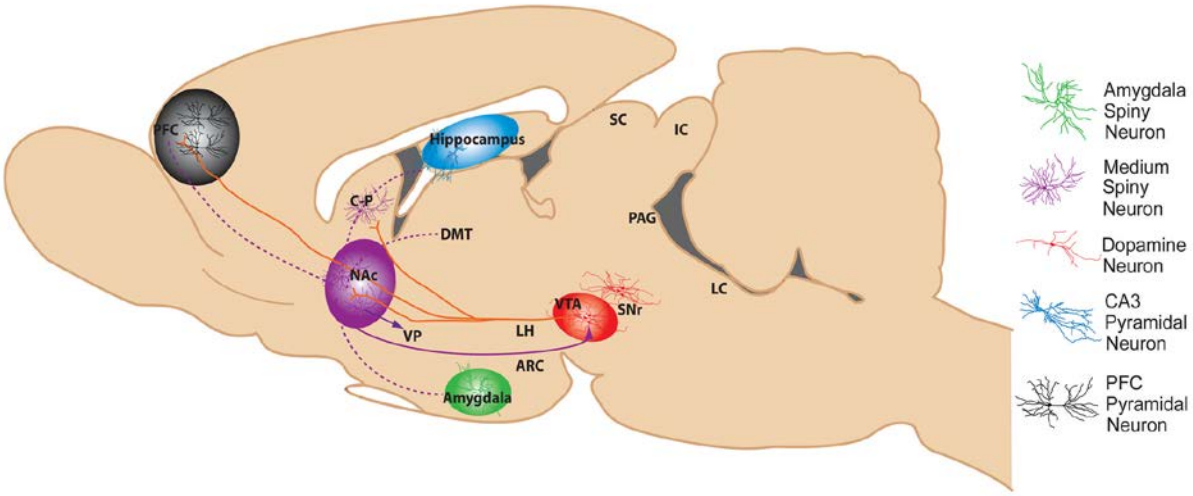

Figure 1.2 Reward circuitry underlying addiction. VTA dopaminergic neurons terminate in the PFC and NAc. The GABAergic medium spiny neurons of the NAc innervate the VTA. The dotted lines represent glutamatergic neurons that project to the NAc, hippocampus, and amygdala. Drugs of abuse lead to electrophysiological and morphological alterations in these neuronal populations. Adapted from Russo et al. (2009).

abuse, including cocaine, amphetamine, and alcohol (Carlezon & Wise, 1996; Leshner & Koob, 1999; Wise, 1996). The dopaminergic projections of the VTA also extend into the amygdala and lateral hypothalamus. In addition to dopaminergic innervations from the VTA, the NAc also receives glutamatergic input from the prefrontal cortex (PFC), orbital frontal cortex (OFC), and anterior cingulate cortex (Nestler, 2004; Russo et al., 2009).

The convergence of these neural circuits on the NAc leads to sensorimotor integration and the activation of goal-directed responses to conditioned stimuli, by assessing the salience of the reinforcer (Salamone et al., 1997). Mesolimbic DA projections from the VTA to the NAc are implicated in mediating other reward-related behaviors involved in drug abuse; specifically, DA signaling in the NAc is important for regulating both drug-taking and -seeking behaviors. Similarly, DA is implicated in food reward, which is suggested to occur through the NAc microcircuits that affect goal-directed behavior (Bernal et al., 2008; Cacciapaglia et al., 2011). Additionally, by blocking dopamine1 and 2 (D1 and D2) receptor subtypes, cocaine, amphetamine, and nicotine conditioned taste aversion is abolished (Di Chiara et al., 2004). Intra-NAc infusions of D1 or D2 receptor antagonists have been shown to increase cocaine self-administration (SA) under free access conditions as compensation for reduced cocaine effects (Bachtell et al., 2005; Caine et al., 1995; McGregor & Roberts, 1993; Phillips et al., 2003). Before lever-pressing for cocaine there is an increase in dopamine that coincides with the initiation of drug-seeking, and cocaine seeking can be induced through electrical stimulation of dopamine

release (Phillips et al., 2003). After lever pressing, there are increases in dopamine coinciding with cocaine-related injection cues (Phillips et al., 2003). Furthermore, administration of D1 or D2 dopamine antagonists into the core and shell of the NAc decreased the reinforcing efficacy of cocaine (Bari & Pierce, 2005). Dopamine receptors also have necessary and sufficient roles in reinstating cocaine-seeking. Intra-NAc infusions of D1 and D2 agonists or dopamine reinstate cocaine seeking in rats, while subtype-selective antagonists attenuate cocaine seeking (Bachtell et al., 2005; Cornish & Kalivas, 2000).

The dopamine system is altered in human cocaine addicts. Drug addicted subjects show decreases in DA D2 receptor and decreases in DA release (Volkow et al., 1997). In addition, cocaine leads to stimulation of dopamine in the NAc of animals; interestingly, the NAc neurons have been shown to fire in anticipation of cocaine (Carelli & Ijames, 2000). These findings suggest that the drive for drug-taking is disparate from the euphoria perceived when initially administering the drug. However, aversive stressful events can also alter mesolimbic dopamine release in humans, as seen by decreased [^{11}C] raclopride (a synthetic D2 receptor antagonist) binding on a positron emissions test (Pruessner et al., 2004).

While the NAc is primarily involved in relapse and the reinforcing effects of cocaine, other brain regions associated with the reward pathway modulate the conditioned learning underlying addiction. The amygdala is involved in stimulus-reward associations, which are important for the acquisition of motivational salience for cocaine-related cues. For example, both a D1 antagonist and pharmacological

blockade of the baso-lateral amygdala either prior to the acquisition or during the reactivation of a cocaine-conditioned stimulus attenuates reinstatement to cue-induced cocaine-seeking behavior (Berglind et al., 2006; Lee et al., 2005; Rogers & See, 2007; See, 2005). These behavioral responses are mimicked through modulation of the hippocampus, a brain region important for learning and memory. Inactivation of ventral hippocampus by microinjections of GABA receptor agonists attenuated cue-induced and cocaine-primed reinstatement, behaviors associated with relapse (Rogers & See, 2007). The dorsal hippocampus, however, has been shown to mediate stimulus-stimulus associations related to contextual learning (Fuchs et al., 2005). Additionally, the PFC, OFC, and anterior cingulate cortex are important for executive functioning and emotional responses to drugs of abuse. During withdrawal, the orbitofrontal cortex in drug abusers is hypoactive; however, during craving the orbitofrontal cortex becomes hypermetabolic in proportion to the craving (Volkow & Fowler, 2000), suggesting that the OFC regulates the compulsivity and drive of addicts. Together, these brain regions mediate the craving and relapse aspects of cocaine addiction; while, the mesolimbic DA projections from the VTA to the NAc also mediate reward related behaviors.

The neurocircuitry involved in the reward pathway is central to the initiation, maintenance, and relapse of drug-seeking behaviors. Cocaine, in particular, potentiates this system by increasing the release of dopamine into the NAc. Inputs from brain regions like the amygdala, hippocampus, and cortical regions regulate the emotional and conditioned responses associated with cocaine; whereas, the

dopaminergic input from the VTA to the NAc is crucial for goal-directed behaviors. Through the assessment of the salience of environmental stimuli, the NAc regulates whether an animal's behavior is reinforced by cocaine, the amount of effort expended to maintain drug intake, and the propensity of relapse.

The Nucleus Accumbens and its subregions

The nucleus accumbens acts as the interface between adaptive and goal-directed behaviors by integrating memory, motivation, and emotion. The nucleus accumbens is located in the ventral striatum, with direct connections to the dorsal striatum—receiving inputs from sensory and motor cortices. The ventral striatum receives most innervations from the amygdala and hippocampus, areas of the brain that process affect-related information (Heimer et al., 1991a; Heimer & Van Hoesen, 2006).

The accumbens can be divided into two sub-regions: the core and the shell. The subregions of the accumbens receive afferent projections from the prefrontal cortex (Brog et al., 1993; Zahm & Brog, 1992), the basolateral amygdala (Brog et al., 1993; Wright et al., 1996), the subiculum of the hippocampus (Brog et al., 1993; Groenewegen et al., 1996; Zahm & Brog, 1992), and the VTA (Zahm & Brog, 1992); although the pattern of these innervations is different between sub-regions. The core is innervated by the insular and prelimbic cortices and the dorsal subiculum of the hippocampus (Groenewegen et al., 1996). Efferents from the core project to the ventral pallidum, subthalamic nucleus, and the substantia nigra (Brog et al., 1993; Heimer et al., 1991b).

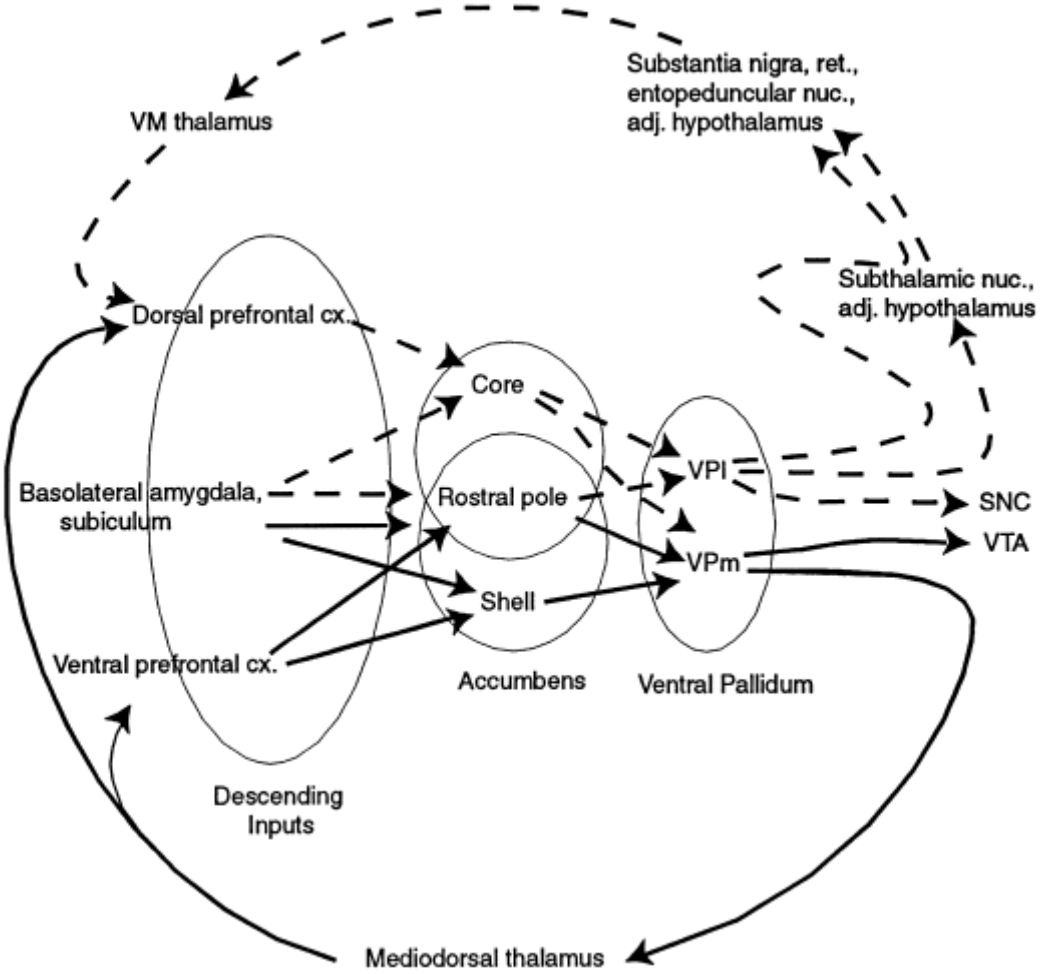

Figure 1.3 Afferents and efferents of the nucleus accumbens. The nucleus accumbens receives input from the amygdala, hippocampus, and cortical structures. The information relating to motivation, affect, and memory is integrated into goal-directed responses. The two sub-regions of the NAc—the core and shell—project to different regions of the ventral pallidum and receive differential inputs from overlapping brain regions. Adapted from (Zahm, 2000).

In contrast, the ventral hippocampus (Groenewegen et al., 1996), amygdala (Wright et al., 1996), and the VTA innervate the shell. Additionally, the shell innervates the ventromedial part of the subcommissural ventral pallidum, which projects to the thalamic mediodorsal nucleus (Heimer et al., 1991b; Zahm et al., 1996), which then projects to the dorsal prelimbic and agranular insular cortex (Zahm et al., 1996). The inputs and outputs of the core and shell contribute to the variations in the types of behaviors they modulate. These behavioral responses are further differentiated based on the neuronal populations activated.

Cell distribution within the nucleus accumbens

The striatum consists of medium spiny projection neurons (MSNs), which release GABA and comprise 90-95% of the total neuronal population (Meredith et al., 1999). MSNs are quiescent cells, whose activity is dependent upon excitatory input from cortical and limbic regions as discussed above (Sesack & Grace, 2010). The morphology of the MSNs—size and density—within the accumbens differ based on region (Meredith et al., 1992). Corticostriatal glutamatergic afferents and mesostriatal dopaminergic afferents often synapse onto the same MSN dendritic spine (Dani & Zhou, 2004). In the core only one-half of the dopaminergic inputs contact the necks of spines, while in the shell only one-third of these terminals synapse on spines (Zahm & Brog, 1992). Shell neurons have fewer spines than do core neurons, and the dopaminergic inputs are made onto proximal dendrites rather than on spines (Dumartin et al., 2007).

Among these neurons are two distinct populations: MSNs containing D1 dopamine receptors constitute the direct output pathway and MSNs containing D2 dopamine receptors comprise the indirect output pathway. Within the striatum the D1 receptors are differentially distributed between the shell and core; however, a small percentage of neurons co-express both receptor subtypes (Le Moine & Bloch, 1995). In the medial shell, the D1 receptors co-localize with NMDA receptors on dendrites (Dumartin et al., 2007). D1 receptor-containing neurons co-express substance P, while cells expressing D2 receptors co-express enkephalin (Lu et al., 1998). The D1/substance P expressing cells of the NAc core and shell project to the ventral mesencephalon, while the D2/enkephalin expressing neurons preferentially innervate the ventral pallidum (Lu et al., 1998). MSNs in the indirect pathway have a greater probability of synaptic release and intrinsic excitability compared to D1-containing MSNs (Grueter et al., 2010). In the core, the D1-NMDA interactions have been implicated in controlling learning-related plasticity (Wolf, 2003), by altering glutamatergic activity.

In addition to MSNs, the striatum also contains interneurons that are important for the timing and pattern of firing of the dopaminergic neurons and MSNs. The interneurons in the neostriatum can be classified as one of three types of GABAergic interneurons, based on their expression of different neurochemicals:
1.) somatostatin and neuropeptide Y, 2.) parvalbumin, or 3.) calretinin (Kawaguchi et al., 1995). These interneurons comprise only 2% of the total neostriatal cell population (Rymar et al., 2004). Furthermore, the striatum also contains cholinergic

interneurons, which are the largest neurons in the neostriatum and primarily target MSNs. These neurons typically fire tonically and have been shown to fire in response to cues that predict saliency or reward (Aosaki et al., 1994).

Through the concerted efforts of dopaminergic signaling through the MSNs and the regulatory effects of interneurons, the striatum integrates the neurochemical inputs received from the aforementioned brain regions important for influencing memory, motivation, and motor responses related to reward. The integrated neurochemical responses to these external stimuli produce the goal-directed behaviors that contribute to the development of an addictive phenotype.

The NAc core and cocaine-related behaviors

As stated previously, the nucleus accumbens is composed of two sub-regions: the core and shell. Administration of drugs of abuse, specifically cocaine, leads to the dopamine receptor activation of the accumbens; however, the sub-divisions of this brain region are implicated in different behavioral responses to the drug. In response to repeated cocaine, the synapse to neuron ratio in animals increases in the core (49.1%) (Alcantara et al., 2011). Additionally, rats that developed psychomotor sensitization after receiving daily 15mg/kg injections of cocaine for 8 days show increased spine density 2 weeks after their last injection (Li et al., 2004). Behaviorally, the NAc core is associated with drug-induced behavioral sensitization and drug-seeking behaviors. The sensitization to cocaine, as well as to morphine, nicotine, and amphetamine is correlated with increased DA release in the core (Cadoni & Di Chiara, 2000). These finding are further supported by

pharmacological lesions in the core, using 6-hydroxydopamine (6-OHDA) that reduced amphetamine-induced locomotion (Sellings & Clarke, 2003). This reduction in locomotion is correlated with a loss of DA terminals in the core (Sellings & Clarke, 2003). Infusions of a D1 antagonist (SCH 23390) into the core attenuates discrete cue-induced heroin seeking (Bossert et al., 2007). However, these infusions reduce the intra- VTA nicotine aversion and potentiate nicotine reward sensitivity (Laviolette et al., 2008), but failed to affect the acquisition of morphine CPP (Fenu et al., 2006). Furthermore, lesions or antagonism of AMPA receptors in the core decrease cue-induced cocaine seeking (Di Ciano & Everitt, 2001; Di Ciano et al., 2008), while intra-NAc core infusions of GABA agonists attenuate cocaine-primed reinstatement of drug seeking (McFarland & Kalivas, 2001). Thus, through the increase of dopamine, the NAc core modulates locomotor responses to initiate drug-seeking after reward-related learning has occurred.

The NAc shell and cocaine-related behaviors

Dissociating the roles of the shell and core is important for understanding how the nucleus accumbens integrates inputs from a variety of brain regions. Rats will self-administer cocaine directly into the shell (Carlezon & Wise, 1996), as well as increase locomotor activity following an acute intracranial injection of cocaine into the NAc shell (Filip & Siwanowicz, 2001). Cocaine conditioned cues paired with natural reward (Bassareo et al., 2007; Pontieri et al., 1995) increase dopamine release in the shell, while 6-OHDA lesions of the NAc impair cocaine self-administration (Di Chiara, 2002). D1 and D2 antagonists decrease the reinforcing

effects of cocaine (Bachtell et al., 2005; Bari & Pierce, 2005). D2 antagonists have also been shown to reduce motivation for cocaine, as evidenced by a decrease in break point under a progressive ratio reinforcement schedule (Hubner & Moreton, 1991). These findings support the importance of dopamine-mediated increases in the NAc shell to modulate the initial reinforcing effects of cocaine.

Long-term neuroadaptations in the NAc shell promote the transition to an addictive phenotype. Following 5 or 21 days of withdrawal from chronic cocaine, behavioral sensitization is observed in rats receiving intra-NAc shell injections of cocaine (Filip & Siwanowicz, 2001). In addition, chronic cocaine has been demonstrated to reduce the excitatory postsynaptic currents at prefrontal cortical synapses that innervate medium spiny neurons in the NAc shell (Thomas et al., 2001). These findings suggest that behavioral sensitization may be the result of chronic cocaine-induced long-term depression of excitatory synapses in the NAc.

Morphologically, there are increases in dendritic spines and branching following chronic cocaine (Li et al., 2004; Robinson & Kolb, 1999), as well as increases in the synapse to neuron ratio in the shell of cocaine-treated (49.1%) rats (Alcantara et al., 2011). The increase in dendritic spines in the NAc has been shown to oppose cocaine-induced behavioral sensitization and is thought to act as a compensatory mechanism to reduce maladaptive behavioral responses (Pulipparacharuvil et al., 2008). These alterations in plasticity and dendritic spine formation are thought to involve neurotrophins, such as brain-derived neurotrophic factor (BDNF).

BDNF and the brain

Chronic cocaine intake and the propensity for relapse may increase the activity-dependent release of neurotrophins, which generally play major roles in cell survivability, cell to cell communication, dendritic outgrowth, and even cell death. Of particular interest is brain-derived neurotrophic factor (BDNF), a dimeric protein that is found in neuronal cell bodies, nerve terminals, and pre-terminal axons (Yan et al., 1997). BDNF is synthesized in the endoplasmic reticulum as pre-pro-BDNF and is then converted to pro-BDNF by removal of the signal peptide, and is further cleaved to generate mature BDNF (Park & Poo, 2013). The human BDNF gene contains nine promoters—promoter IV is highly responsive to neuronal activity (Hong et al., 2008).

The exact mechanisms and proteases underlying the cleavage of pro-BDNF to BDNF are unknown. BDNF can be post-transcriptionally regulated to alter the length of the BDNF transcript, which can differentially affect neurons (Figure 1.4). For example, genetic deletion of the long 3' UTR results in increased number of dendritic spines in the hippocampus and cortex (Kaneko et al., 2012; Lau et al., 2010). BDNF transcripts with different 5' UTR splice variants are segregated to distinct cellular compartments (e.g. somal versus distal dendrites) prior to translation and secretion (Baj et al., 2011).

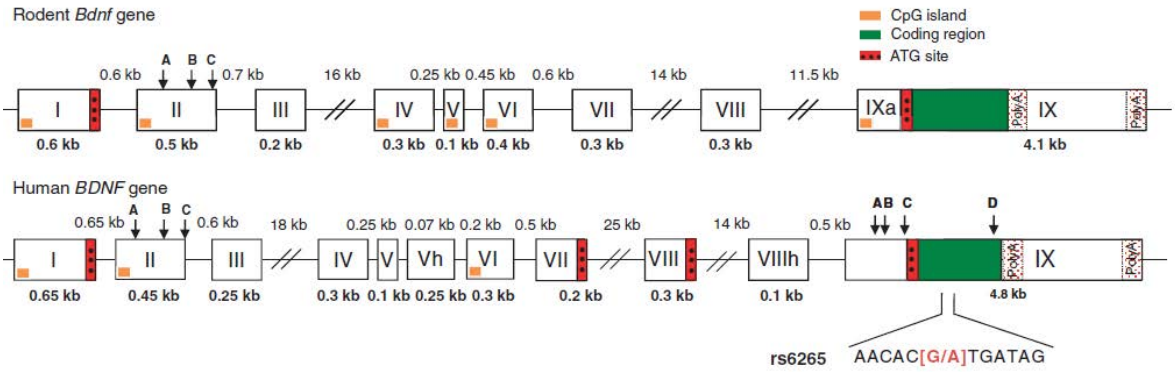

Figure 1.4 A comparison of BDNF gene structures in rodents and humans. BDNF is synthesized in the endoplasmic reticulum as pre-pro-BDNF and is then converted to pro-BDNF by removal of the signal peptide, and is further cleaved to generate mature BDNF. Both rodent and human genes contain nine exons that are post-translationally modified. Although the resulting transcripts produce differential effects in neurons, the promoter for exon IV is highly responsive to neuronal activity. Adapted from (Boulle et al., 2012).

BDNF secretion is triggered by membrane depolarization and by increases in cytoplasmic Ca^{2+} and cAMP levels (Lessmann et al., 2003). Secretion of BDNF occurs most often under high-frequency stimulation (Gartner & Staiger, 2002), capable of inducing long-term potentiation (LTP) at excitatory synapses, which is consistent with the requirement of BDNF for LTP induction (Korte et al., 1995). BDNF is released presynaptically in the hippocampus, with no evidence of postsynaptic storage of BDNF (Dieni et al., 2012). During development BDNF regulates the cortex by enhancing dendritic growth in an activity dependent manner (McAllister, 2002). In hippocampal neuronal cultures, exogenous BDNF promotes the formation of excitatory and inhibitory synapses (Vicario-Abejon et al., 1998). In addition, BDNF has been implicated in potentiating synaptic transmission (Kang & Schuman, 1995) both pre- and post-synaptically. Presynaptic deletion of BDNF leads to a similar impairment in LTP as a complete knockout of BDNF (Zakharenko et al., 2003) and induction and maintenance of LTP is increased with the application of BDNF (Kovalchuk et al., 2002). BDNF can increase neurotransmitter release through the indirect increase in intracellular Ca^{2+} via activation of PLCγ, through phosphorylation of synapsin and increased expression of Rab3 by mitogen-activated protein kinase (MAPK) (Alder et al., 2005), or by activating the motor myosin VI (Park & Poo, 2013). BDNF promotes changes in synaptic function, such as LTP and long-term depression (LTD). However, conflicting evidence suggests that pro-BDNF binding to p75NTR is necessary for LTD induction in the CA3-CA1 hippocampal synapses (Woo et al., 2005) while conditional BDNF knockout mice show no

abnormalities in LTD induction, suggesting that both pro-BDNF and BDNF may not be involved in LTD (Matsumoto et al., 2008). Homozygous and heterozygous BDNF knock-out mice have reduced LTP (Pozzo-Miller et al., 1999). BDNF release into the striatum occurs via projections from the prefrontal cortex and VTA (Altar et al., 1997; Guillin et al., 2001) (Tsai, 2007), as well as via intrinsic striatal neurons, based on previous studies (Altar et al., 1997; Graham et al., 2007).

BDNF mRNA levels are high in dopaminergic neurons (Baquet et al., 2005; Hofer et al., 1990; Maisonpierre et al., 1990; Seroogy et al., 1994), but are barely detectable in NAc neurons (Conner et al., 1997). However, compared to mRNA, there are higher levels of BDNF protein in the rat striatum (Graham et al., 2007; Radka et al., 1996). Neutrophins bind to two different classes of receptor proteins: neurotrophin receptor p75 ($p75^{NTR}$) and tropomyosin receptor kinase B (TrkB). The secreted protein is released as both mature BDNF and proBDNF. ProBDNF has a higher affinity for $p75^{NTR}$ and has been shown to activate the $p75^{NTR}$ to promote cell apoptosis (Baj et al., 2011). BDNF can bind to the $p75^{NTR}$, which has been shown in knockout mice to have deficits in LTD with unaffected LTP (Rosch et al., 2005; Woo et al., 2005), as well as negative structural differentiation (Zagrebelsky et al., 2005). In contrast, mature BDNF has a higher affinity for the TrkB receptor, which leads to alterations in dendritic spine outgrowth, cell survivability, and plasticity (Lu, 2003). The ability of BDNF to activate these receptors leads to a variety of behavioral responses.

BDNF and behavior

The secretion of BDNF modulates and mediates alterations in synaptic plasticity. These changes in plasticity not only affect the morphology and communication between neurons, but also regulate changes in behavioral responses. BDNF has been implicated in learning and memory, emotional memory, and mood disorders.

BDNF mRNA expression is correlated with performance on learning and memory behavioral tests. Specifically, transient increases in BDNF mRNA occur with hippocampus-dependent learning tasks, such as the Morris water maze, contextual fear conditioning, and passive avoidance tests (Hall et al., 2000; Tyler et al., 2002). When altering BDNF levels through the use of heterozygous BDNF mutant mice (Linnarsson et al., 1997) or treatment with anti-BDNF antibodies (Mu et al., 1999), water maze learning is impaired. Additionally, hippocampal-specific BDNF deletions in adult animals impair novel object recognition, spatial learning, and extinction of learned fear (Heldt et al., 2007). Since learning is a crucial component of an animal's ability to adapt to its environment, BDNF facilitates the retention of information necessary for processing those changes.

The processing and retention of emotional memories inform an animal's response to dangerous or predatory situations. Much like tasks for spatial learning and novel object recognition, fear conditioning also affects BDNF expression. Contextual fear conditioning increases BDNF expression specifically in CA1 of the hippocampus (Hall et al., 2000) and lateral amygdala (Rattiner et al., 2005), and

BDNF signaling is necessary for consolidation of contextual fear memories (Lee et al., 2004) and extinction (Chhatwal et al., 2006). Also, two hours after fear conditioning, there is an increase in BDNF transcripts containing exons I and III, specifically (Rattiner et al., 2004). In addition to changes in protein and mRNA levels, BDNF expression is also altered epigenetically through histone modification. Fear conditioning in mice enhances histone acetylation near the BDNF promoter during hippocampus-dependent fear learning and prefrontal cortex-dependent extinction learning (Rattiner et al., 2004). Thus, BDNF plays a prominent role in regulating limbic circuits important for processing fear.

The dysregulation in processing learned, emotional responses can lead to mood disorders, such as anxiety and depression, which are associated with altered levels of BDNF. Patients diagnosed with mood disorders and stress have decreased BDNF serum levels (Licinio & Wong, 2002), while human post-mortem tissue from untreated clinically depressed patients also show decreased levels of BDNF compared to anti-depressant treated patients (Chen et al., 2001). This decrease in BDNF is also demonstrated in animal models, where chronic and acute stress leads to a decrease in BDNF mRNA in the hippocampus (Duman, 2005); however, BDNF heterozygous knockout mice do not show depressive-like behaviors (Saarelainen et al., 2003). BDNF, though, is required for the development of experience-dependent social aversion, as evidenced by increases in BDNF protein levels 24 hours and four weeks after 10 days of social defeat stress (Berton et al., 2006). Interestingly, chronic administration of antidepressants increases BDNF

(Duman & Monteggia, 2006), signifying a potential mechanism for antidepressant efficacy.

Changes in BDNF levels are differentially correlated to the presence of mood disorders. For example increases in BDNF in the VTA and NAc (Krishnan et al., 2007) or decreases in BDNF in the hippocampus (Eisch et al., 2003) lead to depression-like symptoms. By reducing BDNF in the VTA, such as through VTA-specific deletions of BDNF, depression symptoms induced by social defeat stress are opposed (Berton et al., 2006; Krishnan et al., 2007).

BDNF is an important modulator of learning and memory and neuropsychiatric disorders. Signaling between brain regions regulates BDNF expression through changes in protein and mRNA levels, as well as modifications in mechanisms underlying its transcription. These alterations occur in many of the brain regions associated with the reward pathway, the integral system of brain regions that mediate alterations in motivation, reward, and drive. It is through the changes in expression level and the activation of subsequent signaling pathways that BDNF exerts its effects on more complex behaviors, like drug addiction.

BDNF in the PFC and cocaine addiction

BDNF exerts region specific neuroadaptations that result in differential behavioral responses. As stated previously, the PFC is one brain region comprising the reward pathway. Because the PFC regulates drive and decision-making processes, research in the addiction field has focused on changes in BDNF expression in the PFC and its effects on subsequent cocaine-related behaviors. A

single injection of cocaine has been shown to upregulate BDNF mRNA levels in the PFC two hours after the injection. This increase in mRNA levels translated into increases in BDNF protein levels (Fumagalli et al., 2007). Additionally, repeated injections of cocaine have been shown to potentiate the increase in BDNF mRNA levels in the PFC two hours after the last injection (Fumagalli et al., 2007). These studies suggest that the PFC is susceptible to cocaine-induced changes in BDNF, which may contribute to the addictive phenotype. Since this study only examined the effects of BDNF expression on experimenter-administered cocaine, it is possible that different modes of cocaine taking could affect functioning of the PFC. For example, 14 days of cocaine self-administration elevated BDNF protein expression 24 hours after the last session in both self-administering animals and animals passively receiving infusions (yoked) (Fumagalli et al., 2013). Interestingly, this increase in BDNF protein levels was accompanied by increases in BDNF exon I, but not exon IV, transcript levels (Fumagalli et al., 2013). These data indicate that the increase in BDNF protein is most likely a pharmacological response to cocaine, rather than an activity induced increase, since activation of exon IV is associated with activity-induced increases in BDNF transcription (Hong et al., 2008). Alternatively, PFC-specific infusions of BDNF into the dorsomedial prefrontal cortex following the last of 10 cocaine self-administration sessions attenuates cocaine-seeking during reinstatement (Berglind et al., 2007; Berglind et al., 2009). These data suggest that cocaine-induced alterations in BDNF regulate cortical signaling in a manner that is important for reducing drug relapse behaviors.

BDNF in the VTA and cocaine addiction

Much like the PFC, the VTA is also associated with the reward pathway, contributing dopaminergic projections to other brain regions like NAc. As stated previously, dopaminergic projections originating in the VTA are involved in the reinforcing properties of cocaine (Dackis & O'Brien, 2001; Phillips et al., 2003; Self, 2004). Thus, research has focused on the contributions of BDNF in the VTA to cocaine addiction.

Initial evidence for BDNF effects on dopamine in the VTA demonstrate that BDNF infusions into the VTA decrease the cocaine–induced increases in tyrosine hydroxylase levels, the enzyme responsible for catalyzing the conversion of L-tyrosine to L-DOPA – the precursor to dopamine (Berhow et al., 1995). Behaviorally, BDNF infused for two weeks into the VTA increases locomotor activity following repeated cocaine injections (Horger et al., 1999); however, subsequent cocaine injections did not continue to increase locomotor activity (Pierce et al., 1999). These findings suggest that BDNF in the VTA facilitates the development but not expression of behavioral sensitization.

Alterations in BDNF expression in the VTA have also been shown to play an important role in cocaine-seeking and relapse. BDNF infused into the VTA after cocaine training enhances cocaine seeking after withdrawal (Lu et al., 2004a). This enhancement in cocaine seeking is associated with time dependent increases (incubation) of BDNF protein levels following 30 and 90 days of cocaine withdrawal (Grimm et al., 2003). These increases in BDNF levels coincide with an increase in

excitatory postsynaptic potentials in VTA slices of rats 10 -15 days after cocaine withdrawal (Pu et al., 2006). Through alterations in the functional properties of the VTA and subsequent increases in cocaine seeking, BDNF primes the brain for relapse-related cues.

BDNF in the NAc and cocaine addiction

Much like the VTA, BDNF increases cocaine-related behaviors in the NAc. The third major component of the reward pathway, the NAc integrates input from the PFC, limbic regions, and VTA to regulate goal-directed behaviors. Specifically, the NAc is associated with mediating changes in the rewarding effects of cocaine and relapse behaviors to the drug. Since cocaine-induced BDNF alterations occur in other brain reward regions, the NAc is a likely candidate for modulation by BDNF with cocaine use. A single cocaine injection is sufficient to acutely increase BDNF mRNA in the NAc shell (Filip et al., 2006; Graham et al., 2007). During and after daily cocaine self-administration, BDNF mRNA and protein levels increase in the NAc (Graham et al., 2007). Previous studies have shown that supplementing these daily increases with intra-NAc BDNF infusions increase lever pressing for both drug and conditioned rewards, and leads to a delayed but prolonged increase in daily cocaine intake (Graham et al., 2007; Horger et al., 1999).

The behavioral effects of BDNF, however, are not limited to levels of cocaine intake. Graham et al. (2007) showed that 5 daily infusions of BDNF into the NAc shell were sufficient to increase the ratio of lever presses per cocaine injection a rat will perform to self-administer cocaine using a progressive ratio schedule of drug

reinforcement. This increase in lever press behavior suggests an increase in the animal's motivation for cocaine or cocaine reinforcement. BDNF protein levels increase in the NAc (Graham et al., 2007; Grimm et al., 2003) after withdrawal from cocaine, and continue for 90 days or longer (Corominas et al., 2007). The time course for the increase in BDNF in these regions coincides with the time course for enhanced cue-induced reinstatement (Graham et al., 2007; Grimm et al., 2003), implicating BDNF as a key mechanism in the in the propensity for drug relapse.

BDNF activation of TrkB receptors in cocaine addiction

The primary receptor target of BDNF is the TrkB receptor, which is expressed on accumbens and neostriatal medium spiny neurons, as well as other mesencephalic dopamine neurons (Corominas et al., 2007; Freeman et al., 2003). The binding of BDNF to TrkB causes the dimerization of two homodimeric subunits and the autophosphorylation of its tyrosine residues. The phosphorylation of these residues leads to the recruitment of proteins that activate different intracellular signaling cascades: Ras-Raf-Erk (extracellular signal-regulated kinase), PI3K (phosphoinositide 3-kinase)-Akt, and PLCγ (phospholipase C- gamma)-Ca^{2+} (Figure 1.5) (Huang & Reichardt, 2003). BDNF-mediated downstream effects of TrkB activation are of particular interest, since previous studies in cortical neurons have shown that the dynamics of TrkB phosphorylation resemble BDNF transcription following modulation of neuronal activity (Jia et al., 2008).

Chronic cocaine self-administration leads to a BDNF-mediated upregulation in full-length TrkB (Graham et al., 2009). Lentivirus vectors that overexpress TrkB in the NAc enhance cocaine place preference, delay extinction, and increase reinstatement of the place preference with cocaine priming (Bahi et al., 2008). These findings support previous studies that suggest BDNF in the NAc mediates cocaine-seeking (Graham et al., 2007) and the propensity for relapse (Graham et al., 2007; Grimm et al., 2003). More recently, cell-specific deletion of TrkB in D1 receptor-containing neurons was found to increase locomotor activity in rats, while deletion in D2 receptor-containing neurons reduced locomotor activity (Lobo et al., 2010). However, the D1-specific deletion of TrkB showed a reduction in the excitability of MSN neurons, and the D2-specific deletion of TrkB showed an increase in neuronal firing in response to current injections (Lobo et al., 2010). These data suggest that BDNF-TrkB signaling selectively exerts differential effects on behavior and neuronal functionality based on the neuronal population activated.

As stated above, phosphorylation of the TrkB tyrosine residues induces the activation of several intracellular signaling cascades. Previous studies have shown that point mutations can be introduced at specific sites on the TrkB receptor to selectively inhibit the functioning of each of these pathways. A point mutation that changes the tyrosine residue to a phenylalanine at site 490 interacts with Shc

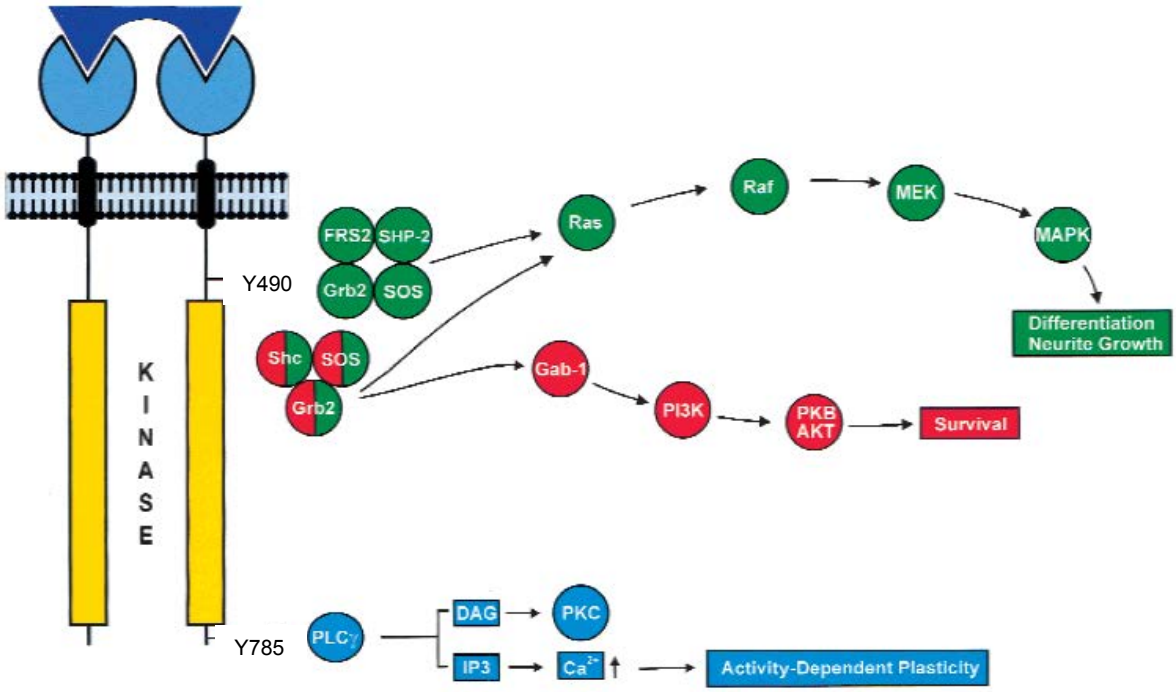

Figure 1.5 TrkB receptor and its signaling pathways. BDNF binds to the TrkB receptor, which causes homodimerization of the TrkB subunits. The TrkB receptor autophosphorylates, leading to the phosphorylation of other tyrosine residues along the receptor. Phosphorylation of the Y490 residue leads to phosphorylation of a series of adaptor proteins and activation of the Ras/Raf/MEK/MAPK pathway, implicated in neuron growth and differentiation. Additionally, another set of adaptor proteins activates the PI3K/AKT pathway, which is important for neurotrophin-induced cell survivability. Phosphorylation of the Y785 residue leads to the phosphorylation of the PLCγ signaling pathway, which regulates alterations in plasticity. Adapted from Bibel & Barde, 2000).

binding, preventing the activation of the Ras-Raf-Erk and PI3K-Akt pathways, but preserves the PLCγ pathway (Huang & Reichardt, 2003). Conversely, mutating phosphotyrosine-816 prevents the recruitment of PLCγ, but leaves the Ras-Raf-Erk and PI3K-Akt pathways intact (Liu & Meakin, 2002). In addition to specifically inhibiting the activation of a specific pathway, a point mutation changing the arginine at site 571 to an asparagine can effectively inhibit the ATP binding site, creating an overall kinase dead mutant (Eide et al., 1996). For all of the aforementioned mutations, the alternative pathway remains functional, when the subunits dimerize together. When heteromeric dimerization occurs between wildtype and mutant TrkB, the TrkB receptors become non-functional (Luikart et al., 2008). Thus, overexpression of TrkB signaling mutants inactivates the endogenous wildtype TrkB and leaves a pathway specific mutant TrkB receptor dimers.

PLCγ pathway and plasticity in addiction

BDNF-mediated TrkB activation can lead to the autophosphorylation of residue Y816 and the subsequent binding of PLCγ. Activation of this pathway is involved in neurotrophin-mediated neurotrophin release and synaptic plasticity (Bibel & Barde, 2000). PLCγ-mediated neuroplasticity produces long-term potentiation (LTP) in the hippocampus; however, overexpression of the Y816F mutation in TrkB receptors in the hippocampus reduces LTP, yet has no effect on signaling in the ERK/PI3K pathway (Gruart et al., 2007; Minichiello et al., 2002). This result suggests that the PLCγ-induced LTP and ERK/PI3K signaling pathways can be dissociated. In addition, PLCγ phosphorylation leads to protein kinase C activation,

increases in intracellular Ca^{2+}, and phosphorylation of cAMP response binding element (CREB) (Gartner et al., 2006; Minichiello et al., 2002). Ultimately, CREB mediates gene transcription and can target BDNF expression itself. Since BDNF increases during cocaine use have been shown to increase TrkB-mediated PLCγ phosphorylation (Graham et al., 2007), it is possible that the PLCγ pathway is mediating these effects.

ERK/PI3K pathway and dendritic spine formation in addiction

In addition to activating the PLCγ pathway, autophosphorylation of the TrkB receptor can activate the Erk/PI3K pathway, through the recruitment of Shc/FRS2 adaptor proteins. Evidence for the activation of this pathway during acute cocaine exposure are increases in ERK (Edwards et al., 2007; Kim & Kim, 2008; Lu et al., 2006) levels; whereas, chronic cocaine exposure increased PI3K (Corominas et al., 2007) and induced a 76-82% increase in ERK (Edwards et al., 2007) levels in the accumbens. These increases are thought to mediate the consolidation of the learned associations with the rewarding drug effects (Lu et al., 2006), and the establishment and maintenance of sensitization to cocaine (Izzo et al., 2002). As a result, pERK and PI3K activity increase during withdrawal (Corominas et al., 2007; Thomas et al., 2008) and in sensitized animals (Zhang et al., 2006) in the NAc shell and core. Thus, by inhibiting ERK/PI3K with pharmacological inhibitors, sensitized responding and conditioned, cocaine-associated memories are disrupted (Corominas et al., 2007; Izzo et al., 2002).

Previous studies have shown that 4 weeks of daily cocaine injections is sufficient to increase total dendritic spine growth in the NAc shell and core (Kolb et al., 2003; Robinson & Kolb, 1999). Because TrkB activation of the ERK/PI3K pathway induces dendritic spine formation and neuronal survival in other brain regions (Bibel & Barde, 2000), it is important to investigate the effect of TrkB activation of the ERK/PI3K pathway and dendritic spines on cocaine addiction. Recent studies suggest that the growth of these spines opposes behavioral sensitization (Pulipparacharuvil et al., 2008) and hippocampal-dependent learning and memory (Barbosa et al., 2008).

Both the BDNF and TrkB findings implicate the involvement of the TrkB receptor in escalating cocaine intake, cocaine reinforcement, and relapse mediated by stress, cocaine priming, and conditioned cocaine cues. Since overexpression of LV-TrkB increases cocaine place preference and deletions of TrkB in NAc neurons oppose these effects, it is important to study cocaine addiction in animals that are voluntarily self-administering cocaine, which more closely mimics human consumption of the drug. Furthermore, it is important to understand the contribution of both the PLCγ and ERK/PI3K pathways in cocaine addiction; since both downstream effectors of BDNF are important but distinct players in neuroplasticity and dendritic spine formation that occur with cocaine exposure. Due to the complexity of drug addiction and the interrelated pharmacological and psychological components contributing to this behavior, isolating each TrkB pathway and studying the resulting functional consequences on addictive behavior will increase the

understanding of the neuropathology leading to such prominent changes in drug-taking and drug-seeking behavior. Thus, this dissertation examines the role of TrkB and its distinct signaling pathways in cocaine intake, seeking, and relapse using a chronic, intravenous cocaine self-administration paradigm.

CHAPTER TWO

BASIC INTRODUCTION OF METHODOLOGY AND TECHNIQUES

Behavioral Techniques

The experiments delineated in this thesis use two techniques that assess the behavioral effects of cocaine in rodents: cocaine self-administration and locomotor sensitization. Cocaine self-administration experiments tested alterations in cocaine intake, cocaine reinforcement, and relapse to non-reinforced cocaine seeking following acquisition and extinction of self-administration. Locomotor sensitization studies analyze the unconditioned psychomotor stimulus effects of cocaine on locomotor activity. These behavioral techniques address different aspects of the addictive phenotype and provide the tools to examine the experimental questions discussed in later chapters. Furthermore, these paradigms are used to examine the selective contribution of TrkB signaling pathways in discrete addictive behaviors.

Cocaine self-administration paradigm

Cocaine self-administration studies conducted in this thesis involve procedures adapted from previous studies (Graham et al., 2007; Graham et al., 2009). Briefly, prior to and during food training, adult, male Sprague Dawley rats were food deprived. Animals were trained to self-administer sucrose pellets (45 mg) in operant chambers (Med Associates) using sucrose a fixed ratio 1 (FR1) reinforcement schedule. Food training ensured each animal had established an

association between lever pressing and the delivery of reward. Rats lever pressed for 100 pellets each day for 3 consecutive days in order to facilitate subsequent acquisition of cocaine self-administration training. Following successful completion of food training, animals were returned to their homecages for 3 days with *ad libitum* access to food and water. All animals were surgically implanted with indwelling, intravenous (i.v.) catheters into their right jugular vein, with Sialastic tubing, inserted as far as the right atrium of the heart. Rats received at least 1 week of recovery from catheter surgeries.

Following surgical recovery, animals were placed in operant chambers for cocaine self-administration training. At the start of each session, a house-light illuminated the chamber, indicating the availability of the drug. When an animal pressed the drug-paired lever, the house light turned off for a time-out period of 15 s and a drug injection of cocaine (0.5mg/kg/infusion over a 2.5 s duration) occurred while a cue light illuminated above the drug-paired lever. The cue-light remained illuminated for the duration of the drug injection. Following the time-out period, the house light became illuminated, indicating that drug was again available. Lever pressing on the inactive lever resulted in no consequence for the animals, and reflected a measure of non-specific activity.

Animals acquired cocaine self-administration by lever pressing cocaine (0.5mg/kg/infusion), during 3 h daily sessions 5 d a week on an FR 1 reinforcement schedule; whereby, one lever press resulted in the delivery of one injection of cocaine. Once animals stabilized cocaine intake on FR1, their training progressed

to a FR3 schedule where each infusion required 3 lever press responses. Animals stabilized on an FR3, meaning their mean cocaine intake did not differ by more than 10% for 3 consecutive days. Following FR3 stabilization, animals trained on an FR5 schedule under the same conditions as for FR3 training. In order to progress to behavioral testing, animals stabilized on an FR5, as previously defined for FR3 training. The training time period from acquisition on FR1 to stabilization on FR5 lasted approximately 4 weeks in daily 3 h sessions.

Locomotor Sensitization Assay

The locomotor sensitization assay used a circular chamber, consisting of 4 laser beam detection sensors at 90 degree angles around a 1.95 m perimeter that measured horizontal locomotion. Following a 10 d recovery period from stereotaxic surgery to infuse adeno-associated viruses into the NAc shell, animals were habituated to daily interperitoneal injections (i.p.) of saline in their home cages for 3 d. Animals were tested in the locomotor chambers for 4 h sessions for the subsequent 5 d. Each session consisted of a 2 h habituation period, followed by an injection of saline or cocaine, and a 2 h test period. On day one, all animals received a saline injection. For each subsequent day animals received either saline or cocaine (10, 20, 20, 20mg/kg, ip, respectively). Animals then remained in their home cages for 7 d of withdrawal. Following withdrawal, animals were tested in the locomotor chambers, as described above, and received a 10 mg/kg ip challenge or acute cocaine injection (for the saline group). For the remaining 2 d, the animals

received a subcutaneous (*sc*) injection of either a D1 (SKF81297, Sigma, St. Louis, MO) or D2 (Quinpirole, Sigma, St. Louis, MO) dopamine receptor agonist. For these tests, animals were placed in the locomotor chambers for 2 h of habituation, followed by hourly injections of one of the agonists given in ascending concentration order (0, 0.1, 0.3, 1.0 mg/kg, *sc*). On the subsequent day, the experimental test was completed for the remaining agonist. Data were plotted as measures of beam breaks in 10 min bins and 1 h bar graphs.

Biochemical Techniques

Western blot assays were used to determine cocaine-induced alterations in protein regulation in tissue and protein expression of DNA constructs transfected into HEK 293 cells. Immunohistochemistry provided visual confirmation of viral vector infection and infusion localization in tissue.

Western Blot Analysis

Animals used for western blot analysis were euthanized by rapid decapitation to preserve phosphorylated proteins. Brains were removed, chilled, and tissue punches of the NAc shell and core were collected from 1 mm brain slices and placed in individually labeled tubes. Tissue punches were immediately homogenized in homogenization buffer: 320mM sucrose, 5nM Hepes buffer (pH to 7.4), 50mM NaF, 1% SDS, 1:100 dilution each of phosphatase inhibitor cocktail I and III and protease inhibitor cocktail (Sigma, St Louis, MO). To determine protein concentrations of

each sample, the Lowry protein assay (BioRad) was used, and 20 ug samples were aliquoted and stored at -80 degrees C for future use. For cell culture experiments, cells were harvested in lamelli sample buffer (BioRad) with 2-mercaptoethanol (BioRad), sonicated, and boiled for 10 minutes. Remaining sample aliquots were stored at -80 ºC for future use.

Samples analyzed for changes in protein regulation were loaded into individual wells of 4-20% pre-cast, acrylamide gels (BioRad). Protein separation on the gels occurred in a running buffer: 10X Tris-Glycine-SDS (BioRad) diluted with 1X sertile water for a final volume of 1 L. Gels ran at 175v at room temperature for approximately 40 min. While the gel ran, transfer buffer was prepared: 200 ml Tris-Glycine (BioRad), 400 ml methanol, and 1400 ml sterile water (total volume of 2 L). Freshly prepared transfer buffer was cooled at 4ºC prior to use. Polyvinylidene fluoride (PVDF) membranes were soaked in 100% methanol for approximately 1 min, before being submerged in cooled transfer buffer for 15 min (or until the membrane sunk). In a separate container, blotting pads and filter paper were equilibrated to the transfer buffer. Acrylamide gels were transferred to PVDF members at 300 mA in an ice bath for 1.5 h. Following transfer, membranes were blocked in 10% milk in TTBS at room temperature for 45 min. Membranes were then incubated in primary antibody (TrkB, pTrkB, Erk, pErk, PLCγ, and pPLCγ) overnight at 4ºC. The following day, membranes were washed with TTBS at room temperature, and then were incubated with the respective secondary antibody for 1 h at room temperature. Membranes received another round of TTBS washes and

were then incubated in enhance chemiluminesence (ECL, Amersham) for 10 min at room temperature. ECL aids in the visualization of protein bands. X-ray films of protein blots validated the infectivity of each virus and confirmed expression of DNA plasmids.

Immunohistochemistry

This assay verified the placement of adeno-associated and herpes simplex viral infusions in animals running self-administration. Immunohistochemistry provided visual evidence of viral expression of TrkB, since visualizing endogenous TrkB with the primary and secondary antibodies described below, produced no fluorescence. Animals in the AAV-TrkB self-administration study were sacrificed following approximately 3 months of cocaine self-administration. In addition, staining was used to determine a time course for the onset of AAV expression. Rats were acutely infused with AAV-GFP, -TrkB^{WT}, or -TrkB^{KD} and returned to their homecage for either 14 or 21 days. For the animals infused with HSV-TrkB, rats were acutely infused with HSV-GFP, -TrkB^{WT}, or -TrkB^{KD} and returned to their homcage for either 2, 4, or 7 days. In both studies, animals were euthanized with chloral hydrate and transcardially perfused (8ml/ min) with ice-cold 1X PBS (BioRad) for 7 min followed by 4% paraformaldehyde for 15 min. Brains were dissected and stored in 4% paraformaldehyde overnight at 4C. Brains were then cryoprotected in 30% sucrose in PBS for 2-3 days at 4 ºC.

Brain sections were cut at 40 µm on a microtome and washed in 1X PBS. Slices were blocked in 0.3% Triton X (BioRad), 3% Normal Goat Serum (NGS;

Jackson Labs, Bar Harbor, Maine), and 1X PBS for 2 h at room temperature. Free-floating sections were incubated with chicken primary anti-GFP (1:5000; Aves Labs, Inc., Tigard, OR), rabbit primary anti-TrkB (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or mouse primary anti-Flag (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) overnight at 4ºC. Sections were washed in 1X PBS and labeled with CY2-Fluroescein-labeled-goat-anti-chicken (1:250; Aves Labs, Inc.), CY2-Goat-anti-rabbit (1:250; EMD Millipore, Billerica, MA), or CY3-conjugated AffiniPure sheep-anti-mouse (JacksonLabs). Following washes with 1X PBS, sections were incubated with DAPI (1:250; Roche Applied Science, Indianapolis, IN) for 30 min at room temperature. Sections were mounted onto electrostatically-charged slides, dehydrated, and coverslipped using DPX mountant.

Viral Vector Construction

pDyn-TrkB and pEnk-TrkB

In order to construct a herpes simplex virus (HSV) vector that expresses either a $TrkB^{WT}$ or $TrkB^{KD}$ (2471 bp), a flag-tagged TrkB gene was PCR cloned from pcDNA3.1 constructs containing wildtype TrkB, TrkBY490F, TrkBY785F, or TrkBK571N, as described previously (Hale et al., 2011). *pDyn-TrkB*. An upstream primer (5'-TAATGATATCACCATGAACTTTATCCCAGTCGACATTCC-3') and a downstream primer (5'– TAATGCGGCCGCCTAGCCTAGGATGTCCAGGT AGACGGG –3') introduced EcoRV and NotI cloning sites. The PCR product was gel purified and cloned into the pDyn-eGFP plasmid. pDyn-eGFP was generated as

previously described (Ferguson et al., 2011). To produce a construct that expressed TrkB under the control of the dynorphin promoter, the pDyn-eGFP construct was digested with EcoRV and NotI and ligated with the TrkB PCR products. *pEnk-TrkB*. An upstream primer (5' –TGGTACCGCTAGCGGCGCGCCACCATGAACTTTATCC CAGTCGACATTCC- 3') and a downstream primer (5'–TAATGGTACCGCGGCCG CACGCGTCTAGCCTAGGATGTCCAGGTAGACGGG–3') introduced KpnI cloning sites. The PCR product was gel purified and cloned into the pEnk-eGFP plasmid. The pEnk-eGFP clone was generated as described previously (Ferguson et al., 2011). To produce a construct that expressed TrkB under the control of the enkephalin promoter, the pEnk-eGFP construct was digested with KpnI and the TrkB PCR product was blunt-cloned into pEnk-eGFP. Restriction mapping was used to identify successfully ligated clones and the sequences were determined by the DNA Sanger Sequencing Core (UT Southwestern Medical Center, TX). The amplicons were packaged into viral vectors using a replication-deficient helper virus, as previously described (Neve et al., 1997). Packaged virus was suspended in 10% sucrose and 25 mM HEPES (7.3) in PBS.

pAAV-TrkB

The creation of the adeno-associated virus (AAV) vector that expresses either a wildtype TrkB, TrkBY490F, TrkBY785F, or TrkBK571N (2471 bp), required the flag-tagged TrkB gene to be PCR cloned from pcDNA3.1, as described previously (Hale et al., 2011). The TrkB PCR products were cloned into a pAAV construct,

containing a multiple cloning site, using the NheI and XbaI restriction sites. The CMV promoter of the pAAV construct was truncated by excising the beta-globin intron. This excision enabled the sufficient space for packaging of the TrkB cDNA insert. Following ligation of the TrkB cDNA and pAAV amplicon, the constructs were sent to the University of North Carolina at Chapel Hill Gene Therapy Center. Packaged virus was dialysed in 350mM NaCl and 5% D-Sorbitol in PBS.

pHSV-TrkB

Flag-tagged TrkB, TrkB Y490F, TrkB Y785F, TrkB K571N, or GFP in pcDNA3.1 constructs were obtained from the laboratory of Chris Cowan (McLean Hospital, Harvard University). TrkB cDNA was PCR cloned using an upstream primer (5' - CC GGATCC GCCGCCACC ATGAACTTTATCCCAGTCGACATTC - 3') and a downstream primer (5' - CC CTCGAGTAGCCTAGGATGTCCAGGTAGACG - 3') that introduced BamHI and XhoI cloning sites. Due to the location of the Y785F mutation, an alternate reverse primer, introducing the same restriction sites, was used (5' - CC CTCGAG CCGTCTGGGAGAAGAGGGAGTCTA - 3'). The PCR product was gel purified and cloned into an HSV-PrpUC amplicon and packaged using a 5dl1.2 helper virus. HSV-PrpUC amplicons containing TrkB, a TrkB signaling mutant, or GFP were packaged into a bicistronic herpes simplex viral vector, as previously described (Neve et al., 1997). Viruses were purified on a 10% sucrose gradient. Packaged virus was suspended in 10% sucrose and 25 mM HEPES (7.3) in PBS.

Cell Culture

Cell culture assays validated the expression of viral vectors and HSV and AAV amplicons containing flag-tagged TrkB inserts. These assays used either HEK 293 cells or striatal neuronal cultures. Following expression studies, virus and amplicon expression were further validated by western blot or immunoassay.

HEK293T cell transfections

HEK 293 cells (American Type Culture Collection) were plated in 6-well plates at a concentration of 500,000/well in medium consisting of 1X DMEM solution (Invitrogen; Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (50 µg/mL; Sigma Aldrich; St. Louis, MO)-streptomycin (50 units/mL; Sigma), and L-glutamine (4mM; Sigma Aldrich), warmed to 37ºC. Cells were transfected using a calcium phosphate technique, as previously described (Hale et al., 2011). HEK293T cells transfected with either the pAAV-TrkBs or pAAV-GFP were incubated for 24 hours at 37ºC. Following incubation, cells were harvested in lamelli sample buffer (BioRad) with 2-mercaptoethanol (BioRad), sonicated, and boiled for 10 min.

Dissociated striatal cultures

Embryonic striatal neurons (E18/19) were cultured from Long Evans rats (Charles River Labs; Wilmington, MA), as previously described (Pulipparacharuvil et al., 2008). Briefly, prior to dissociation, striatal tissues were digested with 10 unit/mL

papin (Worthington Biochemical Corp.; Lakewood, NJ) for 4 min at 37ºC. Dissociated neurons were plated at a concentration of 100,000/well on glass coverslips coated with PDL (Sigma) and laminin (Invitrogen). Coverslips plated with dissociated neurons were placed in 1x DMEM solution (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (50 µg/mL; Sigma Aldrich)-streptomycin (50 units/ml; Sigma), and L-glutamine (4mM; Sigma Aldrich), incubated at 37C/5% CO_2 for 24 h. Following incubation, the medium was changed to Neurobasal (Invitrogen), B27supplement (2% (v/v); Invitrogen), 1X penicillin-streptomycin, and L-glutamine (4mM; Sigma). Dissociated neurons were transfected with 2 µL of HSV-Dyn-GFP, HSV-GFP, or HSV-Dyn-TrkB-IRES2-GFP and harvested 6 d post-transfection. For immunocytochemistry experiments, plated dissociated neurons were washed in ice-cold PBS, and fixed in a solution of 1x 4% formaldehyde, 2% sucrose in PBS. Dissociated neurons were incubated in with the fixation solution at room temperature for 20 minutes, and then washed with PBS. To permeabilize the neurons, PBS with 0.4% (v/v) Triton X-100 (Biorad) was added to each well. Cells were incubated for 1 hour at room temperature and then washed with PBS. For western blot experiments, cells were harvested in Laemmli sample buffer (BioRad) with 2-mercaptoethanol (BioRad), sonicated, and boiled for 10 minutes.

CHAPTER THREE
Results

CELL-TYPE SPECIFIC VIRAL-MEDIATED OVEREXPRESSION OF TRKB SIGNALING MUTANTS

Introduction

Cocaine addiction creates a significant economic, psychological, and societal burden. The transition from recreational drug use to addiction is defined by an escalation in the amount of drug used and cravings during periods of withdrawal, leading to an increase in relapse (Berridge & Robinson, 1998; Wise, 2004). Neuroadaptations and drug-induced neuroplasticity define this transition and promote changes in protein expression and neuron morphology. In order to produce these alterations, cocaine acts by inhibiting the dopamine transporter, in addition to the transporters for norepinephrine and serotonin, and blocks reuptake of dopamine into presynaptic terminals; thereby, increasing the concentration of extracellular dopamine (Nestler & Malenka, 2004; Ron & Jurd, 2005; Self, 1998). These cocaine-induced increases in dopamine ultimately affect dopamine signaling, specifically in brain regions associated with modulating reward.

One brain region of particular interest in cocaine addiction is the nucleus accumbens. The NAc consists of MSNs which comprise approximately 90% of the total neuronal population (Meredith et al., 1999) within the brain region. More importantly, these neurons can be further distinguished by the dopamine receptor subtype they contain: D1 receptors and D2 receptors (Le Moine & Bloch, 1995).

Cells expressing D1 receptors co-express substance P and dynorphin (Dyn), while cells expressing D2 receptors co-express enkephalin (Enk) (Lu et al., 1998). In addition, the D1/substance P expressing cells project to the ventral mesencephalon, while the D2/enkephalin expressing neurons preferentially innervate the globus palidus (Lu et al., 1998). Due to the similarity in their morphology, it is visibly difficult to distinguish one cell type from another.

Although D1 and D2 receptors share physical and morphological similarities, the behavioral consequences associated with their selective activation has been demonstrated. Cocaine indirectly modulates the activation of these dopamine receptor subtypes to alter cocaine-related behaviors; however, the specific drug behaviors altered are dependent upon the sub-region of the NAc activated: the NAc core or shell. Systemic injections of cocaine produce behavioral sensitization, which has been demonstrated to increase dopamine release in the core (Cadoni & Di Chiara, 2000); while D1 and D2 antagonists decrease the reinforcing effects of cocaine (Bachtell et al., 2005; Bari & Pierce, 2005). Thus, these studies suggest that cocaine-induced dopamine signaling produces disparate behavioral responses. Work in the addiction field has moved toward methods that selectively activate each cell type to determine its contribution to cocaine addiction.

In addition to altering dopamine signaling, cocaine also affects the expression of proteins further downstream, which may account for many of the neuroplasticity- and morphology-related neuroadaptations occurring in the NAc. One protein of

particular interest is BDNF, a neurotrophic factor that modulates cell survivability, plasticity, dendritic outgrowth, and cell death. Cocaine has been shown to increase BDNF mRNA (Filip et al., 2006) and protein levels in the NAc shell (Graham et al., 2007). In addition, intra-NAc infusions of BDNF have been demonstrated to increase cocaine intake (Graham et al., 2007; Horger et al., 1999) and motivation for cocaine (Graham et al., 2007) using a self-administration paradigm, as well as increasing proteins levels of BDNF during withdrawal (Corominas et al., 2007; Graham et al., 2007; Grimm et al., 2003), suggesting BDNF also modulates relapse behaviors.

These increases in BDNF also lead to activation of its receptor, TrkB. Chronic self-administration increases BDNF-TrkB receptor-induced phosphorylation of PLCγ in the NAc shell, as evidenced by the blockade of this phosphorylation with intra-NAc shell infusions of anti-BDNF (Graham et al., 2007). Additional studies indicate that the loss of TrkB specifically in the NAc shell reduced cocaine place conditioning and the reinforcing effects of cocaine using a self-administration paradigm (Graham et al., 2009). More recently, research suggests that D2 receptor-specific deletions of TrkB reduced locomotor activity, but increased neuronal firing in response to current injections (Lobo et al., 2010).

In order to understand the contribution of BDNF-induced molecular changes to the escalation in drug intake and the increase in the propensity for relapse, this project examined the TrkB receptor and its distinct signaling pathways (PLCγ and ERK/PI3K) in cocaine addiction. A herpes simplex viral (HSV) vector system was

created to over-express wildtype TrkB or its docking mutants to prevent the activation of either PLCγ or ERK/PI3K pathways downstream of TrkB. The expression of each construct was under the control of cell-type specific promoters to limit expression to the dynorphin (D1)- containing neurons of the NAc shell. The ability to over-express TrkB and its signaling mutants was verified *in vivo* and by striatal cell cultures. The ultimate goal of this project was to study the effect of specific TrkB signaling pathways in distinct NAc shell cell types on cocaine addiction and relapse behaviors. The chapter describes the procedure for viral vector construction to over-express TrkB in the dynorphin (D1)-containing neurons of the NAc shell. Although an HSV vector was successfully created and validated prior to viral packaging, sufficient viral expression was not evident *in vivo* or primary striatal neuronal cultures.

Materials and Methods

Animals and intracranial viral infusions

Male Sprague-Dawley rats (weighing 250-300 grams) were ordered from Charles-River (Kingston, RI, USA) and individually housed in wire cages. Animals were under a 12:12 hour light:dark cycle (6:00 am to 6:00 pm) and fed *ad libitum.* Animal care was provided according to the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals and* IAACUC.

Animals were given acute infusions of HSV-Dyn-TrkB-IRES-GFP (containing either wildtype TrkB, TrkB K571N, TrkB Y490F,TrkB Y816F) or HSV-Dyn-GFP

controls, using a 5 µL Hamilton syringe with a 30 gauge injector tip directed at the NAc shell (+1.7 mm anterior to bregma, ±0.8 mm lateral, -6.7 mm ventral to dura). All viruses were infused at a rate of 0.1 µL/side/30 s, and injectors were held in place for an additional 2 min to allow for local diffusion into the NAc shell prior to injector removal. The total volume of virus injected was 2.0 µL/side. Following surgery, rats were given penicillin (60,000 IU/ 0.2mL, s.c.) to prevent infection, and ketoprofen analgesia was provided (5 mg/kg, s.c., twice a day for 3 d).

Cocaine self-administration training

Cocaine self-administration studies conducted in this thesis involved procedures adapted from previous studies (Graham et al., 2007; Graham et al., 2009). Briefly, prior to and during lever-press training for food, adult, male Sprague Dawley rats were food deprived. Animals were trained to self-administer sucrose pellets (45 mg) on a fixed ratio 1 (FR1) reinforcement schedule in operant chambers (Med Associates). Rats acquired lever-press behavior within 1-2 days, and subsequently self-administered 100 pellets each day for 3 consecutive days in order to progress to cocaine self-administration training. Food training ensured each animal had established an association between lever pressing and the delivery of a reward. Following successful completion of food training, animals were returned to their homecages for 3 days with *ad libitum* access to food and water. All animals were surgically implanted with indwelling, intravenous (i.v.) catheters into their right jugular vein, with Sialastic tubing, inserted as far as the right atrium of the heart. Rats received at least 1 week of recovery from i.v. catheter surgeries.

Following surgical recovery, animals were placed in operant chambers for cocaine self-administration training. At the start of each session, a house-light illuminated the chamber, indicating the availability of the drug. When an animal pressed the drug-paired lever, the house light turned off for a time-out period of 15 s and a drug injection of cocaine (0.5 mg/kg/infusion over a 2.5 s duration) occurred while a cue light was illuminated above the drug-paired lever for the duration of the drug injection. Following the time-out period, the house light became illuminated, indicating that drug was again available. Lever pressing on the inactive lever resulted in no consequence for the animals, and reflected a measure of non-specific activity.

Animals acquired cocaine self-administration by lever pressing cocaine (0.5mg/kg/infusion), during 3 h daily sessions 5 d a week on an FR 1 reinforcement schedule; i.e., one lever press resulted in one injection of cocaine followed by the timeout period. Once animals stabilized on FR1, their training progressed to an FR3 schedule, where each infusion required 3 lever press responses. Animals stabilized on FR3, meaning their mean cocaine intake did not differ by more than 10% for 3 consecutive days. Following FR3 stabilization, the FR requirement was increased to FR5 under the same conditions as for FR3 training. In order to progress to behavioral testing, animals stabilized on an FR5, as previously defined for FR3 training. The training time period from acquisition on FR1 to stabilization on FR5 lasted approximately 4 weeks.

Cocaine self-administration testing

Once animals stabilized on an FR5 reinforcement schedule, a between-session FR dose-response test was conducted. Rats received a total of 5 injection doses (0, 30, 100, 300, and 1000 µg/kg/injection). Each dose was given for 2 consecutive days, in a counterbalanced order, during 3-h sessions. After FR dose-response testing, animals were stabilized on the training dose (0.5mg/kg/infusion) prior to the start of the progressive ratio (PR) reinforcement schedule. For the PR dose-response testing, animals self-administered 1 of 2 injection doses (250 or 750 µg/kg), where the response requirement for successive injections increased by progressive increments (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, etc.) (Graham et al., 2007). Each injection dose was counterbalanced and presented for 2 consecutive days during 5-hour sessions. The break point was defined as the highest ratio of lever-presses/injection achieved before a 60 min period where no further reinforcements were earned.

Following PR schedule testing, animals were returned to their homecages for 7 days of withdrawal from cocaine self-administration. Extinction testing occurred in 5 daily, 3 h sessions in the absence of response-contingent cocaine and injection cues, during which non-reinforced drug-paired lever presses were recorded. The following week, stimuli-induced reinstatement of cocaine-seeking was tested in 5 daily, 3-hour sessions similar to extinction testing. During these reinstatement sessions, the reinstating stimuli were presented during the last h of each session using the following sequence of stimuli: cue light presentation (every 2 min for

1 h), immediately after an intraperitoneal (i.p.) cocaine priming injection (saline, 500, and 1,500 µg/kg, in counterbalanced order), and after 30 min of intermittent footshock stress (1.0 mA in 0.5 s with random intervals averaging 30 s) (Graham et al., 2007). Non-reinforced responding at both drug-paired and unpaired levers was analyzed for each 1-h reinstatement test.

Dissociated striatal cultures

Embryonic striatal neurons (E18/19) were cultured from Long Evans rats (Charles River Labs; Wilmington, MA), as previously described (Pulipparacharuvil et al., 2008). Briefly, prior to dissociation, striatal tissues were digested with 10 unit/mL papin (Worthington Biochemical Corp.; Lakewood, NJ) for 4 minutes at 37 ºC. Dissociated neurons were plated at a concentration of 100,000/well on glass coverslips coated with PDL (Sigma) and laminin (Invitrogen). Coverslips plated with dissociated neurons were placed in 1x DMEM solution (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (50 µg/mL; Sigma Aldrich)-streptomycin (50 units/mL; Sigma), and L-glutamine (4 mM; Sigma Aldrich), incubated at 37C/5% CO_2 for 24 hours. Following incubation, the medium was changed to Neurobasal (Invitrogen), B27supplement (2% (v/v); Invitrogen), 1X penicillin-streptomycin, and L-glutamine (4mM; Sigma). Dissociated neurons were transfected with 2 µL of HSV-Dyn-GFP, HSV-GFP, or HSV-Dyn-TrkB-IRES2-GFP and harvested 6 d post-transfection. For immunocytochemistry experiments, plated dissociated neurons were washed in ice-cold PBS, and fixed in a solution of 1x 4% formaldehyde, 2% sucrose in PBS. Dissociated neurons were incubated in with the

fixation solution at room temperature for 20 minutes, and then washed with PBS. To permeabilize the neurons, PBS with 0.4% (v/v) Triton X-100 (Biorad) was added to each well. Cells were incubated for 1 h at room temperature and then washed with PBS. For western blot experiments, cells were harvested in lamelli sample buffer (BioRad) with 2-mercaptoethanol (BioRad), sonicated, and boiled for 10 minutes.

Western blot analysis

Samples analyzed for changes in protein regulation were loaded into individual wells of 4-20% pre-cast, acrylamide gels (BioRad). Protein separation on the gels occurred in a running buffer: 10X Tris-Glycine-SDS (BioRad) diluted with 1X sterile water for a final volume of 1 L. Gels ran at 175v at room temperature for approximately 40 min. While the gel ran, transfer buffer was prepared: 200 ml Tris-Glycine (BioRad), 400 ml methanol, and 1400 ml sterile water (total volume of 2 L). Freshly prepared transfer buffer was cooled at 4ºC prior to use. Polyvinylidene fluoride (PVDF) membranes were soaked in 100% methanol for approximately 1 min, before being submerged in cooled transfer buffer for 15 min (or until the membrane sunk). In a separate container, blotting pads and filter paper were equilibrated to the transfer buffer. Acrylamide gels were transferred to PVDF members at 300 mA in an ice bath for 1.5 h. Following transfer, membranes were blocked in 10% milk in TTBS at room temperature for 45 min. Membranes were then incubated overnight at 4ºC in primary antibody: anti-Flag (1:2,000, Millipore,

Billerica, MA), anti-GFP (1:2,000, Invitrogen, Grand Island, NY), and anti-Beta Tubulin (1:100,000, Upstate, Billerica, MA). The following day, membranes were washed with TTBS at room temperature, and then were incubated for 1 h at room temperature with the respective secondary antibody: Goat anti-Rabbit (1:50,000, Biorad, Hercules, CA) for the anti-GFP primary antibodies and Goat anti-Mouse (1:25,000, Biorad, Hercules, CA) for the anti-Flag and anti-ß-Tubulin primary antibodies. Membranes received another round of TTBS washes and were then incubated in enhanced chemiluminesence (ECL, Amersham) for 10 min at room temperature. ECL aids in the visualization of protein bands. X-ray films of protein blots validated the infectivity of each virus and confirmed expression of DNA plasmids.

Immunohistochemistry

Rats were given acute infusions of HSV-Dyn-GFP, -TrkB WT, or –TrkB K571N, -TrkB Y490F, or –TrkB Y816F and returned to their homecage for either 7, 14, or 21 days. Animals were euthanized with chloral hydrate and transcardially perfused (8mL/ min) with ice-cold 1X PBS (BioRad) for 7 min followed by 4% paraformaldehyde for 15 min. Brains were dissected and stored in 4% paraformaldehyde overnight at 4 ºC. Brains were then cryoprotected in 30% sucrose in PBS for 2-3 days at 4 ºC. Brain sections were cut at 40 µm on a microtome and washed in 1X PBS. Slices were blocked in 0.3% Triton X (BioRad), 3% Normal Goat Serum (NGS; Jackson Labs, Bar Harbor, Maine), and 1X PBS for 2 h at room temperature.

For experiments using striatal cultures, plated dissociated neurons were washed in ice-cold PBS, and fixed in a solution of 4% formaldehyde, 2% sucrose in 1X PBS. Dissociated neurons were incubated in with the fixation solution at room temperature for 20 minutes, and then washed with PBS. To permeabilize the neurons, PBS with 0.4% (v/v) Triton X-100 (Biorad) was added to each well. Cells were incubated for 1 h at room temperature and then washed with PBS.

Free-floating sections or plated dissociated neurons were incubated with chicken primary anti-GFP (1:5000; Aves Labs, Inc., Tigard, OR) or mouse primary anti-Flag (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) overnight at 4 ºC. Sections and slides were washed in 1X PBS and labeled with Cy2-Fluroescein-labeled-goat-anti-chicken (1:250; Aves Labs, Inc.), Cy2-Goat-anti-rabbit (1:250; EMD Millipore, Billerica, MA), or Cy3-conjugated AffiniPure sheep-anti-mouse (JacksonLabs). Following washes with 1X PBS, sections and slides were incubated with DAPI (1:250; Roche Applied Science, Indianapolis, IN) for 30 minutes at room temperature. Sections and slides were mounted onto electrostatically-charged slides, dehydrated, and coverslipped using DPX mountant.

Cloning of pDyn-TrkB-IRES-eGFP amplicons

Overview of pDyn-TrkB amplicon construction

To examine the role of TrkB and its signaling pathways in cell-type specific striatal cultures, viral vector constructs using the pDyn gene promoter were created to target the striatopallidal neurons. Ferguson et al. (2011) constructed an HSV vector that

expressed GFP under the control of the dynorphin promoter, a 2 Kb fragment upstream of the dynorphin gene. In order to prevent HSV promoter-driven expression in non-targeted neuronal populations, the dynorphin promoter and GFP were inserted in the reverse orientation of the endogenous HSV promoter (Ferguson et al., 2011). Onset of gene expression was determined to occur 7 to 10 days post-infusion and viral expression was determined at 10 days, although behavioral effects were seen up to approximately 3 weeks later. Since HSV viruses with the IE/4 promoter generally begin to express approximately 2 to 3 days post-infusion and are no longer detected by 7 days post-infusion, using the dynorphin promoter would extend protein expression and enable the use of longer behavioral paradigms, like self-administration, while also allowing for cell-type specific expression.

Using the pDyn-GFP construct created in Ferguson et al. (2011), a cloning strategy was designed to express the TrkB receptor and its signaling pathway mutants under the control of the dynorphin promoter (Figure 3.1). Since the ability to fuse TrkB to GFP, allowing co-expression of TrkB and GFP in a single cell, had not been previously accomplished, an internal ribosomal entry site (IRES) was cloned into the pDyn-TrkB-GFP construct (Figure 3.2). Although expression of TrkB and GFP would not necessarily co-localize within a cell, the expression of GFP enabled the use of an alternative antibody for expression validation studies. TrkB antibodies for immunohistochemistry were inconsistent in their ability to detect the TrkB protein; however, a method for its detection was previously published (Eisch et al., 2003).

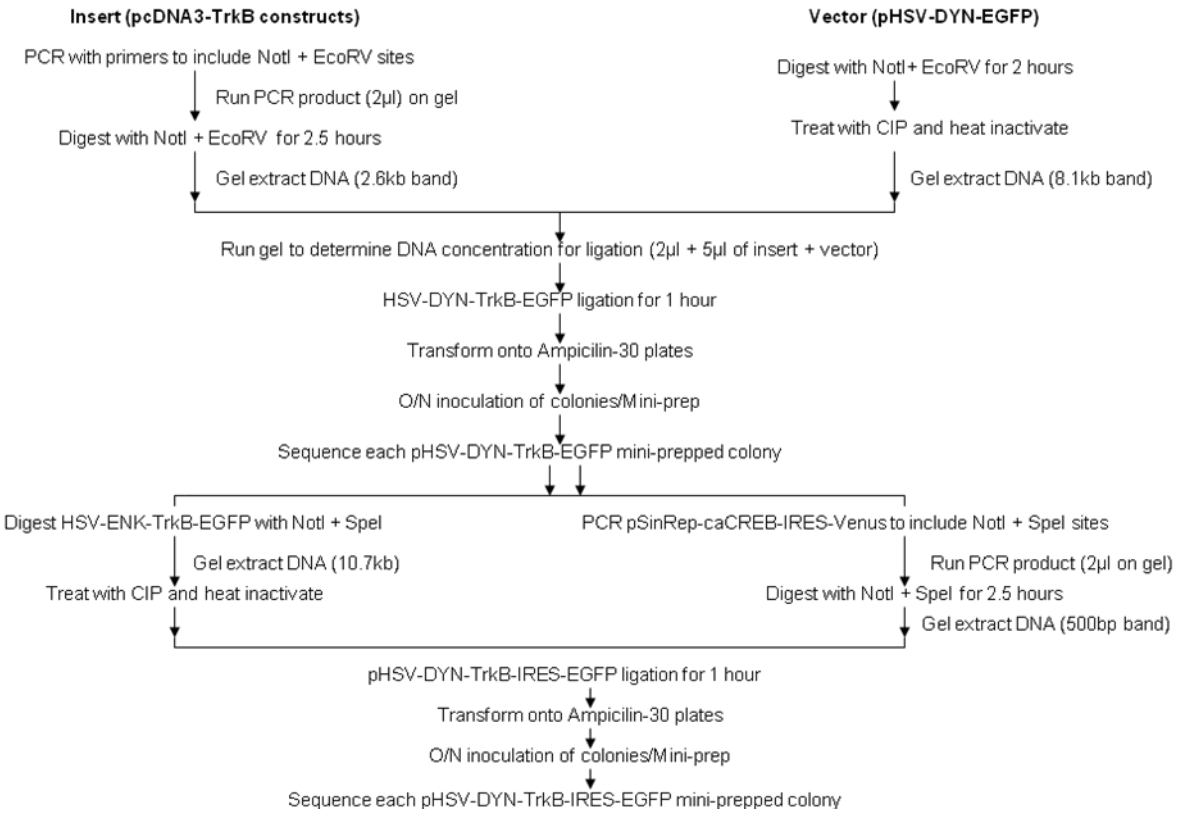

Figure 3.1 Cloning strategy for creating pDyn-TrkB-IRES2-eGFP. TrkB inserts were PCR cloned to include NotI and EcoRV restriction sites and gel purified. Simultaneously, pDyn-GFP (obtained Dr. John Neumaier) was digested with NotI and EcoRV, as well. pDyn-GFP and TrkB inserts were ligated together. In addition, an internal ribosomal entry site (IRES) was ligated into the pDyn-TrkB-GFP construct in order to transcribe GFP.

Figure 3.2 Construct map of the completed pHSV-Dyn-TrkB-IRES-GFP prior to packaging.

Preparation of TrkB insert and pDyn-eGFP vector

In order to construct a herpes simplex virus (HSV) vector that expresses either a TrkB WT, TrkB K571N, TrkB Y490F, or TrkB Y816F (2471 Kb), a flag-tagged TrkB gene was PCR cloned from pcDNA3.1 contructs containing TrkB WT and its signaling mutants as described previously (Hale et al., 2011). Briefly, full-length and mutant rat TrkB constructs were generated using PCR-based cloning strategies. TrkB constructs were subcloned into pcDNA3.1 vectors and contained an N-terminal Flag tag starting after the signal sequence cleavage site (Hale et al., 2011).

Using the sequences for these TrkB constructs, an upstream primer (5'-TAATGATATCACCATGAACTTTATCCCAGTCGACATTCC-3') and downstream primer (5'– AATGCGGCCGCCTAGCCTAGGATGTCCAGGTAGACGGG –3') were used to introduce EcoRV and NotI (New England Biolabs, Ipswich, MA) cloning sites. PCR amplification became problematic when TrkB product yields were consistently low. In order to overcome insufficient amounts of TrkB DNA, an annealing temperature gradient was run and 57.2 ºC was determined to be the annealing temperature necessary to produce a sufficient PCR product to proceed with subsequent cloning steps (Figure 3.3A). The PCR product was digested with BamHI and NotI restriction enzymes and gel purified (Figure 3.3B and 3.3C). Gel purification also proved difficult when TrkB DNA recovery from the purification columns yielded low levels of DNA (Figure 3.3D). After many tests using the kit reagents, it was determined that the columns were defective and the DNA was not binding to the column, but instead eluting during a wash step. By replacing the

columns, the DNA yield increased (Figure 3.3E).

Subcloning of TrkB and IRES inserts into pDyn-GFP

Purified TrkB DNA and pDyn-GFP were ligated during a 1 h room temperature incubation, using T4 DNA Ligase (New England Biolabs, Ipswich, MA) and a 1:3 vector to insert ratio. The ligation product was transformed into DH5α cells (Invitrogen, Grand Island, NY) on ampicilin-100 plates. Following an overnight inoculation, colonies found on the plate were mini-prepped, using a QIAGEN Plasmid Mini Kit (Qiagen, Valencia, CA), and restriction mapping was used to identify successfully ligated clones. Sequences of the clones were determined by the DNA Sanger Sequencing Core (UT Southwestern Medical Center, Dallas, TX).

In order to transcribe GFP in the completed virus, an IRES was PCR cloned from pSinRep-caCREB-IRES-Venus, using an upstream primer (5'- TAATGCGG CCGCGATCCGCCCCTCTCCCTCCCCCCCCCC -3') and downstream primer (5'- TAATGCGGCCGCCTAGCCTAGGATGTCCAGGAAGACGGG -3') that introduced NotI and SpeI (New England Biolabs, Ipswich, MA) restriction sites. The PCR product was digested with NotI and SpeI restriction enzymes and gel purified. Purified IRES DNA and the pDyn-TrkB-GFP construct were ligated during a 1 h room temperature incubation, using T4 DNA ligase, and a 1:3 vector to insert ratio. The ligation product was transformed into DH5α cells on ampicilin-100 plates. Following an overnight inoculation, colonies found on the plate were mini-prepped and restriction mapping was used to identify successfully ligated clones. Sequences of the clones were determined by the DNA Sanger Sequencing Core. The result

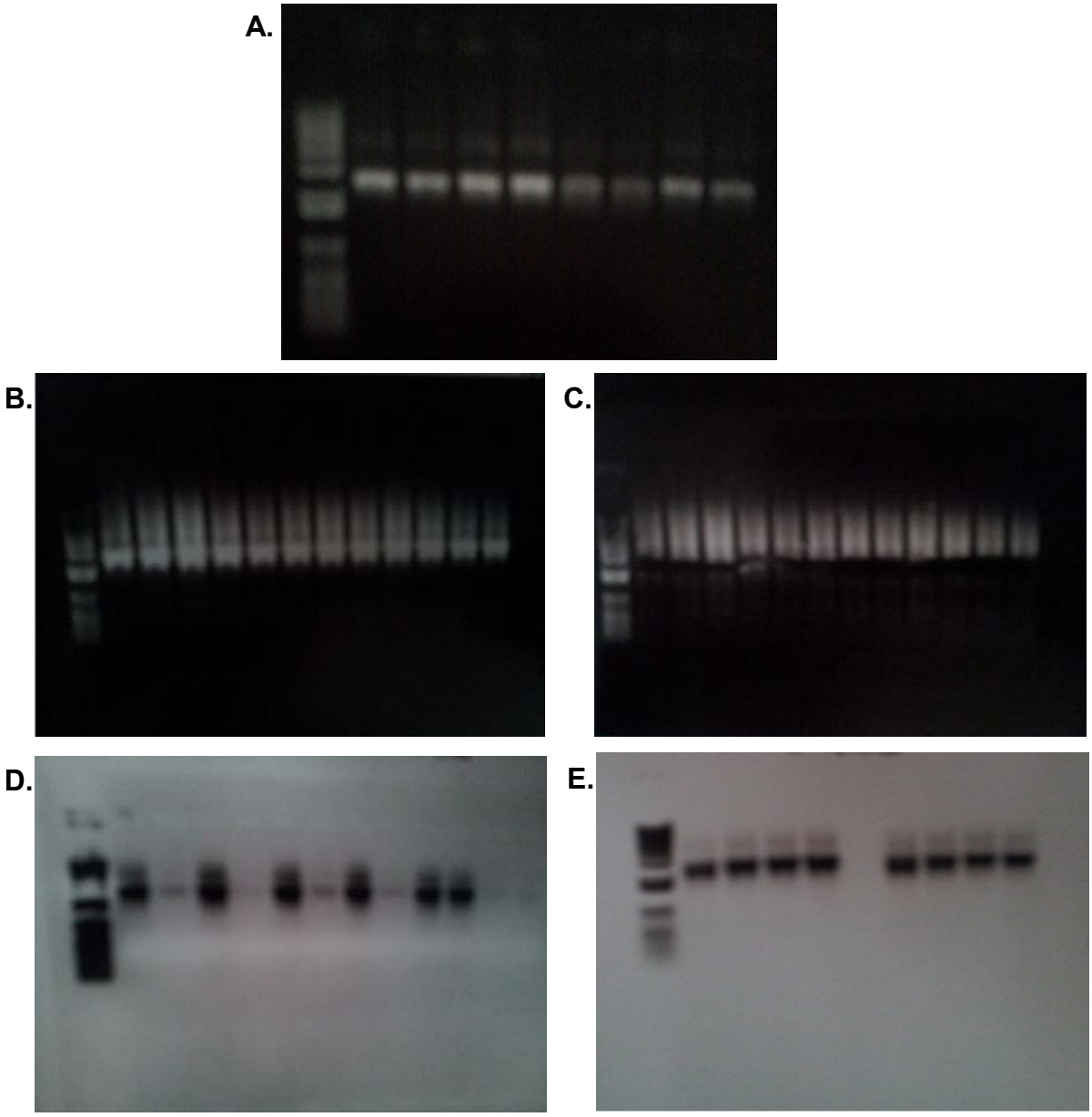

Figure 3.3 Agarose gel confirmation of cloning steps for pDyn-TrkB-eGFP. A) PCR annealing temperature gradient for wildtype TrkB. Lane 4 shows the selected annealing temperature (57 ºC) producing the greatest concentration of PCR product. B and C) Following digestion with NotI and EcoRV, TrkB PCR products were gel purified and the DNA was extracted. D) TrkB PCR products for wildtype TrkB, TrkB K571N, TrkB Y490F, and TrkB Y816F before and after column purification. Significant reduction in DNA concentration following column purification. E) TrkB DNA concentrations increased following replacement of the purification columns.

was a pDyn-TrkB-IRES-GFP constructed verified by restriction mapping and sequencing.

Packaging of pDyn-TrkB-IRES-GFP into virus

The pDyn-TrkB-IRES-GFP construct was packaged using a 5dl1.2 helper virus. HSV-PrpUC amplicons containing TrkB, a TrkB signaling mutant, or GFP were packaged into a bicistronic herpes simplex viral vector, as previously described (Neve et al., 1997). Viruses were purified on a 10% sucrose gradient. Packaged virus was suspended in 10% sucrose and 25 mM HEPES (7.3) in PBS.

Results

Validation of Dyn-TrkB-IRES-GFP viral expression in striatal cultures

Since behavioral experiments would require infusion of the virus into the NAc, striatal cultures were initially used to verify viral expression in *in vitro*. Dissociated striatal neurons (E18/19) were transfected with 2 µL of Dyn-GFP, HSV-GFP, or Dyn-TrkB WT-IRES-GFP and harvested 6 d post-transfection. As demonstrated in Figure 3.4A, the Dyn-GFP (provided by John Neumaier, University of Washington) and Dyn-TrkB-IRES-GFP had very weak to no expression of GFP or Flag (the N-terminal TrkB tag); however, an HSV-GFP under the control of the standard IE4 promoter showed high levels of expression in striatal cultures and served as a positive control. These data suggest that the Dyn-GFP and Dyn-IRES-GFP viruses may not express sufficiently.

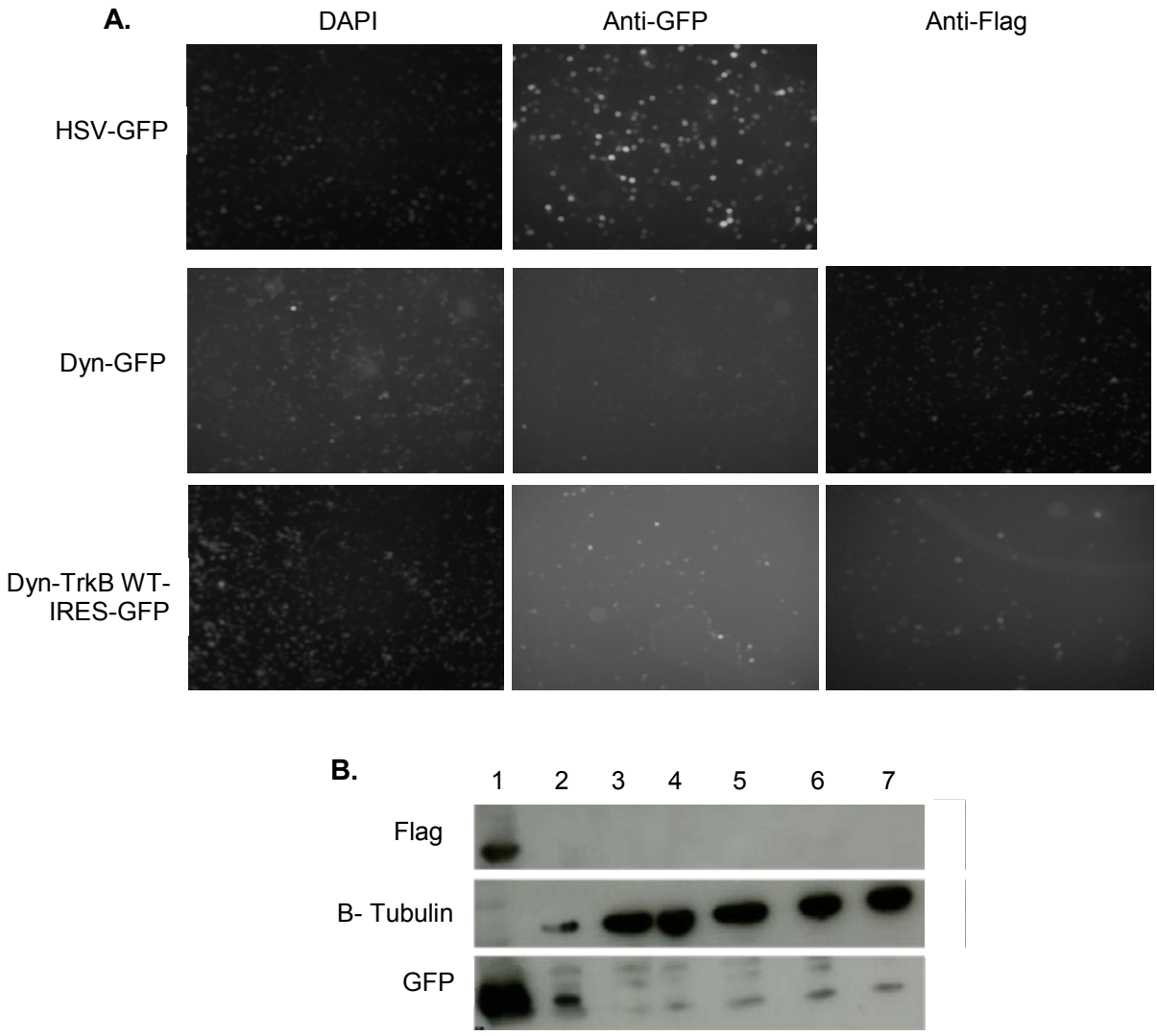

Figure 3.4 Validation of Dyn-TrkB-IRES-eGFP in striatal cultures. A) Striatal cultures were transfected with HSV-GFP, Dyn-GFP, or Dyn-TrkB WT-IRES-GFP and stained for DAPI (a nuclear stain), anti-GFP, or anti-Flag six days post-transfection. B) Striatal cultures transfected with pcDNA3-TrkB (Lane 1), HSV-GFP (Lane 2), Dyn-GFP (Lane 3), Dyn-TrkB WT-IRES-GFP (Lane 4), Dyn-TrkB Y490F-IRES-GFP (Lane 5), Dyn-TrkB Y816F-IRES-GFP (Lane 6), or Dyn-TrkB K571N-IRES-GFP (Lane 7), magnified at 10x.

To determine if viral expression was not seen due to a non-optimized immunocytochemistry protocol, striatal cultures were harvested 6 d post-infusion and a qualitative western blot was run. As presented in Figure 3.4B, HSV-GFP and a control pFlag-TrkB construct co-expressing GFP in HEK cells produced GFP bands. Both Dyn-TrkB, Dyn-TrkBY490F-IRES-GFP, and Dyn-TrkB WT-IRES-GFP, however, failed to produce Flag or GFP bands (Figure 3.4B). These data further confirmed that none of the HSV-Dyn-GFP viruses were expressing in striatal cultures,

Validation of HSV-Dyn-TrkB-IRES-GFP *in vivo*

To determine whether the lack of viral expression was due to the use of striatal cultures rather than an inherent problem with viral production, Sprague Dawley rats were infused with 2 µl per side of HSV-GFP, Dyn-GFP, or Dyn-TrkB Y490F-IRES-GFP and sacrificed 7, 14, or 21 days post-infusion. The 7- and 14-day time points were chosen since Ferguson et al. (2011) showed viral expression between 7 and 10 days post infusion. Figure 3.5 shows only the Dyn-TrkB Y490F-IRES-GFP virus infected a few cells 14 days post-infusion, while Dyn-GFP (not shown) did not visibly infect any cells. Data for 7 and 21 days are not presented, because there was no visualization of infectivity with any Dyn-GFP or Dyn-TrkB-IRES-GFP virus.

TrkB signaling-induced changes in cocaine self-administration

Previous studies demonstrated that a loss of TrkB in NAc neurons reduced the reinforcing effects of cocaine (Graham et al., 2009) and that D1 cell-specific

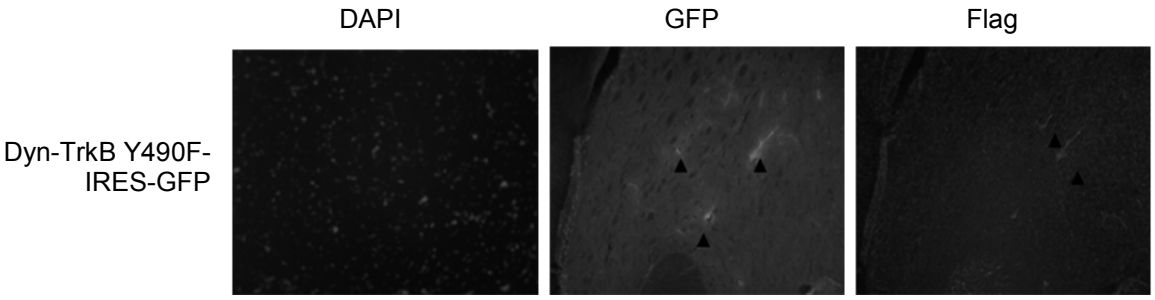

Figure 3.5 Validation of Dyn-TrkB Y490F-IRES-eGFP *in vivo*. Rats were infused with Dyn-TrkB Y490F-IRES-GFP and perfused 14 days post-infusion. Brains were sliced at 40 μm on the microtome and stained for DAPI (a nuclear stain), anti-GFP, and anti-Flag. Arrows indicate individual neurons expressing virus.

deletions of TrkB increased cocaine-induced locomotor activity (Lobo et al., 2010). In order to determine the contribution of TrkB signaling in D1 receptor-containing neurons, rats were infused with Dyn-GFP, Dyn-TrkB Y490F-IRES-GFP,Dyn-TrkB Y816F-IRES-GFP, or Dyn-TrkB K571N-IRES-GFP and trained to self-administer cocaine on an FR5 schedule (Figure 3.6A). There was no significant difference between groups on their ability to acquire cocaine self-administration behavior (Figure 3.6B). Following training, changes in cocaine intake were tested using a between-session dose response with doses given in a randomized order. As presented in Figure 3.6C, there was no significant difference in cocaine intake between groups; however, animals infused with the virus containing the PLCγ signaling mutant (TrkB Y816F) showed a trend for reduced intake at the 300 μg/kg dose. In order to prevent extinction-like effects in animals that received saline as their last dose during dose response testing, animals were re-stabilized on the training dose. Following re-stabilization, animals were tested on a PR schedule to determine changes in motivation for cocaine. Animals receiving infusions of Dyn-TrkB Y490F-IRES-GFP had a greater breakpoint compared to the other viral groups (Figure 3.6D). These data suggest that a loss ERK/PI3K signaling increased the effort an animal was willing to work for cocaine. Animals were subjected to one week of home cage withdrawal prior to reinstatement (relapse) testing. Cocaine-seeking after withdrawal was tested using an extinction paradigm. There was no significant difference between viral groups in their ability to extinguish lever-pressing

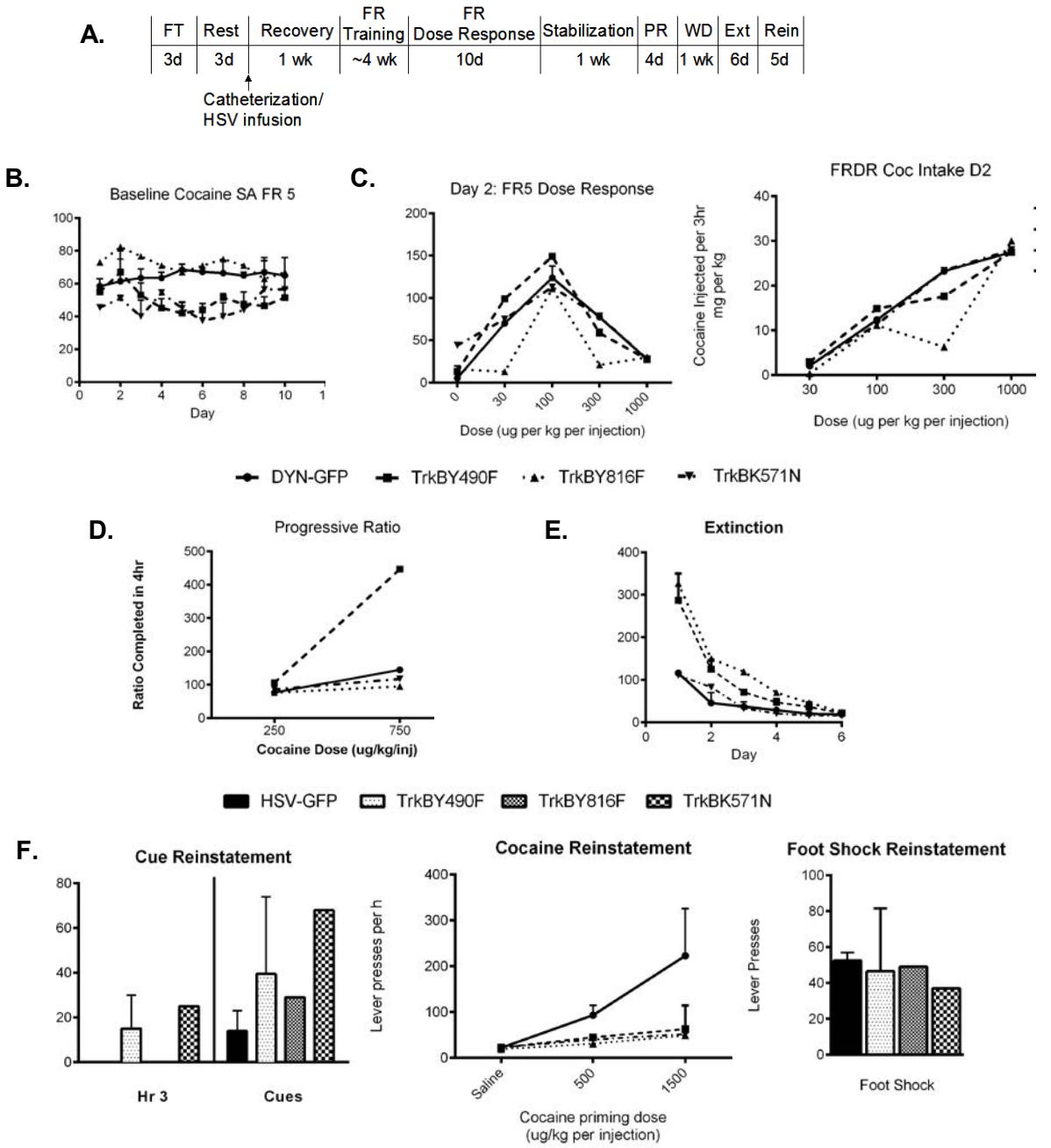

Figure 3.6 Dyn-TrkB-IRES-eGFP mediated regulation of cocaine SA behaviors. A) Experimental timeline for the self-administration (SA) behavioral paradigm. B) Infusions of wildtype or TrkB signaling mutants produced no effects on acquisition of SA (n = 3-4/ viral group). C) Animals receiving Dyn-TrkB Y816F-IRES-GFP infusions showed a decrease in cocaine intake at the 300 µg/kg cocaine dose (n = 3-4/ viral group). D) Dyn-TrkB Y490F infusions produced an increase in breakpoints on the progressive ratio schedule (n = 3-4/ viral group). E) All animals extinguished cocaine seeking over six days (n=3-4/ viral group). F) Animals receiving Dyn-TrkB K571N-IRESS-GFP infusions showed increases in cocaine seeking during cue reinstatement testing (n = 3-4/ viral group). TrkB Dyn-GFP control animals showed increases in cocaine seeking during cocaine reinstatement. There were no differences between TrkB wildtype and mutant groups during foot shock reinstatement testing.

(Figure 3.6E). Animals were subsequently tested for reinstatement of cocaine-seeking following re-exposure to cues, re-exposure to cocaine itself, and exposure to stress. D1-specific virus containing kinase dead TrkB (K571N) showed increased responding to cues, while GFP controls showed increased responding to the highest dose of cocaine priming in reinstatement tests. Additionally, there was no significant difference between groups on cocaine-seeking to footshock stress. Results are presented in Figure 3.6F.

Discussion

This study attempted to create D1 receptor cell-type specific viruses using TrkB signaling mutants. Although restriction mapping and sequencing prior to viral packaging confirmed the presence of the dynorphin promoter, TrkB insert, and GFP, validation test following packaging proved inconsistent. Expression of Dyn-GFP and Dyn-TrkB-IRES-GFP viruses was weak when visualized using immunocytochemical techniques. Additionally, harvested and lysed striatal neurons transfected with Dyn-GFP or a Dyn-TrkB-IRES-GFP virus were analyzed by western blot and produced no Flag bands (representing the presence of TrkB) and very weak GFP bands. Together these data suggest either the striatal cell culture system was not sufficient to express these viruses or that the viruses do not express in general.

To determine whether the lack of viral expression was due to the *in vitro* system used or the viruses themselves, rats were infused with HSV-Dyn-GFP or HSV-Dyn-TrkB-IRES-GFP and sacrificed 7, 14, or 21 days post-infusion. Similarly

to the striatal culture results, *in vivo* infusion of the viruses under the dynorphin promoter produced very weak expression. Furthermore, while validating the viruses, animals were infused with HSV-Dyn-GFP, and HSV-Dyn-TrkB signaling mutants (TrkB Y490F, Y816F, or K571N) and were trained to self-administer cocaine. Interestingly, behavioral trends were observed for several of the tests. Selective TrkB signaling through PLCγ increased the amount of effort exerted by the animal to obtain cocaine, animals infused with the kinase dead TrkB mutant (K571N) showed increased cocaine-seeking behaviors when re-exposed to cues associated with the drug, and animals infused with all TrkB vectors (WT and mutants) showed reduced cocaine seeking compared to the control Dyn-GFP virus in response to stress (footshocks). Although trends did occur in the behavioral data, since there was no validation of viral expression *in vivo* or *in vitro*, a preponderance of the data suggests the viruses do not express sufficiently to produce behavioral responses.

The use of a dynorphin promoter inserted in the reverse orientation of the endogenous HSV promoter (Ferguson et al., 2011) enabled the subcloning of TrkB (2.7 Kb), due to the HSV construct's large packaging capacity. When functioning appropriately, the reverse orientation prevents protein expression in non-targeted neuronal populations. Ferguson et al. (2011) demonstrates that the dynorphin promoter is capable of transcribing small genes (approximately 1 Kb); however, the TrkB gene is double the size, which could create transcriptional problems. To create a dynorphin promoter driven construct, Ferguson et al. (2011) PCR cloned a 2 Kb fragment upstream of the dynorphin gene. Choosing this particular fragment as the

dynorphin promoter may underlie the lack of protein expression from the Dyn-TrkB-IRES-GFP viruses, as determined by western blot and immunohistochemical techniques. It is possible that the dynorphin promoter fragment may be missing part of the promoter or an enhancer region, which would lead to reduced transcription of the TrkB gene and subsequently the TrkB protein. Although this potential problem was not evaluated in this chapter, determining mRNA levels by qPCR would address this issue. Additionally, the secondary structure of the mRNA can be critical to protein translation. To prevent annealing of the ribosome binding site with the first codons of the message, the 5'-end of the gene could be reconstructed to maximize its adenosine and thymidine content, while preserving the protein sequence (DeLamarter et al., 1985). Increases and decreases in the stability of short guanine-cytosine rich RNA stems can affect virus and RNA replication (Kulasegaran-Shylini et al., 2009), which could account for the lack of expression.

In addition to problems with the dynorphin promoter itself, protein expression levels for GFP in the Dyn-TrkB-IRES-GFP viruses were most likely weakened compared to HSV-GFP due to the use of the IRES. An IRES was used in the pDyn-TrkB constructs in order to allow the co-expression of GFP. Co-expressing GFP provided an alternative means to determine if viral expression occurred, since antibodies to TrkB are inconsistent in their ability to detect TrkB proteins. The efficiency of IRES-controlled expression can vary greatly between vectors, which may be due to restriction site insertion in the place of the initiating AUG codon

(Martin et al., 2006). Thus, this restriction site would interfere with polymerase binding and subsequent transcription.

In conclusion, the work presented in this chapter suggests that the Dyn-TrkB-IRES-GFP viruses are not suitable for TrkB expression both *in vitro* and *in vivo.* Although immunohistochemical and -cytochemical techniques indicate very weak expression, western blot analysis did not confirm these results. Thus, it is probable that the staining observed is a background signal. The self-administration data are inconclusive and inconsistent across tests, most likely due to individual animal behavioral responses and a low number of animals tested. The inability of the Dyn-TrkB-IRES-GFP viruses to infect striatal neurons prevents the evaluation of cell-type specific alterations to cocaine addiction. Thus, in order to study the role of TrkB and its signaling pathways in addiction, a global over-expression system that lacks D1- or D2-containing cell specificity would be necessary. Such an approach was adopted in the following chapter.

CHAPTER FOUR
Results

GLOBAL OVEREXPRESSION OF TRKB AND DISRUPTION OF SIGNALING PATHWAYS USING ADENO-ASSOCIATED VIRAL VECTORS

Introduction

Addiction is defined by an escalation in drug intake and compulsive drug-taking despite adverse economic, physiological, psychological and social consequences. Cocaine addiction results in neuroadaptations and drug-induced neuroplasticity that promote changes in protein expression and neuron morphology. Cocaine induces these protein changes by inhibiting the dopamine transporter and blocking reuptake of dopamine into presynaptic terminals; thereby, increasing the concentration of extracellular dopamine (Nestler & Malenka, 2004; Ron & Jurd, 2005; Self, 1998). These cocaine-induced increases in dopamine ultimately alter dopamine signaling in brain regions modulating reward and motivation.

One brain region of particular interest in cocaine addiction is the nucleus accumbens (NAc). The NAc consists of MSNs which comprise approximately 90% of the total neuronal population (Meredith et al., 1999) within the brain region and acts as the interface between adaptive and goal-directed behaviors by integrating memory, motivation, and emotion. The NAc can be sub-divided into two regions: the core and the shell. While the cocaine-induced increases in dopamine in the core modulates locomotor responses to initiate goal-directed behavior and drug-seeking, dopamine in the shell modulates the rewarding and reinforcing effects of cocaine.

Following cocaine treatment, there are increases in the synapse to neuron ratio in the shell of cocaine-treated (49.1%) rats (Alcantara et al., 2011). In addition, rats will self-administer cocaine directly into the NAc shell (Carlezon & Wise, 1996). Furthermore, dopamine D1- and D2-receptor antagonists decrease the incentive motivational effects of cocaine (Bachtell et al., 2005; Bari & Pierce, 2005). These alterations in morphology and behavior result from downstream effectors of dopamine signaling.

In addition to altering dopamine signaling, cocaine also affects the expression of proteins further downstream, which may account for many of the neuroplasticity- and morphology-related neuroadaptations occurring in the NAc. One protein of particular interest is BDNF, a neurotrophic factor that modulates cell survivability, plasticity, dendritic outgrowth, and cell death. Cocaine has been shown to increase BDNF mRNA (Filip et al., 2006) and protein levels in the NAc shell (Graham et al., 2007). In addition, intra-NAc infusions of BDNF have been demonstrated to increase cocaine intake (Graham et al., 2007; Horger et al., 1999) and motivation for cocaine (Graham et al., 2007) using a self-administration paradigm, and protein levels of BDNF increase during cocaine withdrawal (Corominas et al., 2007; Graham et al., 2007; Grimm et al., 2003), suggesting BDNF also modulates relapse behaviors.

These increases in BDNF also lead to activation of its receptor, TrkB. Cocaine self-administration increases BDNF-induced phosphorylation of PLCγ in the

NAc shell, as evidenced by the blockade of this phosphorylation with intra-NAc shell infusions of anti-BDNF (Graham et al., 2007). Since these alterations in phosphorylation are BDNF-mediated, the data implicate activation of TrkB. Additional studies indicate that the loss of TrkB specifically in the NAc shell reduced cocaine place conditioning and the reinforcing effects of cocaine using a self-administration paradigm (Graham et al., 2009). More recently, research suggests that D2 receptor-specific deletions of TrkB reduced locomotor activity, but increased neuronal firing in response to current injections (Lobo et al., 2010).

In order to understand the contribution of BDNF-induced molecular changes to the escalation in drug intake and the increase in the propensity for relapse, this project examined the TrkB receptor and its distinct signaling pathways (PLCγ and ERK/PI3K) in cocaine addiction. An adeno-associated viral (AAV) vector system was created to over-express wildtype TrkB or its dominant negative docking mutants to prevent the activation of either PLCγ or ERK/PI3K pathways downstream of TrkB. The ability to over-express TrkB and its signaling mutants was examined *in vivo* and *in* vitro expressing constructs in human embryonic kidney (HEK) cell cultures. The ultimate goal of this project was to study the effect of specific TrkB signaling pathways in the NAc shell on cocaine addiction and relapse behaviors. Furthermore, the use of an AAV viral vector system would extend viral expression, enabling the assessment of multiple cocaine-related behavioral tests in each animal. Although preliminary tissue staining and self-administration data suggested moderate expression and behavioral effects of AAV-TrkB and its signaling mutants,

sufficient viral expression was not confirmed in subsequent behavioral assessments or in HEK cell expression.

Materials and Methods

Animals

Male Sprague-Dawley rats (weighing 250-300 grams) were ordered from Charles-River (Kingston, RI, USA) and individually housed in wire cages for self-administration studies and housed in pairs for *in vivo* and locomotor sensitization studies. Animals were under a 12:12 hour light:dark cycle (6:00 am to 6:00 pm) and fed *ad libitum.* Animal care was provided according to the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals and* IAACUC.

AAV-TrkB viral vector cloning and packaging

The creation of the AAV vector that expresses either a wildtype TrkB, TrkBY490F, TrkBY785F, or TrkBK571N (2471 bp), required the flag-tagged TrkB gene to be PCR cloned from pcDNA3.1, as described previously (Hale et al., 2011). The TrkB PCR products were cloned into a pAAV construct, containing a multiple cloning site, using the NheI and XbaI restriction sites. The 5' beta-globin intron upstream of the CMV promoter in the pAAV construct was excised. This excision enabled the sufficient packaging of the TrkB cDNA insert. Following ligation of the TrkB cDNA and pAAV amplicon, the constructs were sent to the University of North

Carolina at Chapel Hill Gene Therapy Center. Packaged virus was dialysed in 350 mM NaCl and 5% D-Sorbitol in PBS.

HEK293T cell transfections

HEK 293 cells (American Type Culture Collection) were plated in 6-well plates at a concentration of 500,000/well in medium consisting of 1X DMEM solution (Invitrogen; Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (50 µg/mL; Sigma Aldrich; St. Louis, MO)-streptomycin (50 units/mL; Sigma), and L-glutamine (4mM; Sigma Aldrich), warmed to 37ºC. Cells were transfected using a calcium phosphate technique, as previously described (Hale et al., 2011). HEK293T cells transfected with either the pAAV-TrkBs or pAAV-GFP were incubated for 24 hours at 37ºC. Following incubation, cells were harvested in Laemmli sample buffer (BioRad) with 2-mercaptoethanol (BioRad), sonicated, and boiled for 10 minutes.

Immunohistochemistry

Rats were acutely infused with AAV-GFP, -TrkB WT, or –TrkB K571N, -TrkB Y490F, or -TrkB Y816F and returned to their homecage for 14, 21, or 90 days. Animals were euthanized with chloral hydrate and transcardially perfused (8 ml/ m) with ice-cold 1X PBS (BioRad) for 7 m followed by 4% paraformaldehyde for 15 m. Brains were dissected and stored in 4% paraformaldehyde overnight at 4 ºC. Brains were then cryoprotected in 30% sucrose in PBS for 2 - 3 days at 4 ºC. Brain sections were cut at 40 µm on a microtome and washed in 1X PBS. Slices were blocked in 0.3%

Triton X (BioRad), 3% Normal Goat Serum (NGS; Jackson Labs, Bar Harbor, Maine), and 1X PBS for 2 hours at room temperature.

For HEK cell experiments, cells were washed in ice-cold PBS, and fixed in a solution of 4% formaldehyde, 2% sucrose in 1X PBS. Dissociated neurons were incubated in with the fixation solution at room temperature for 20 minutes, and then washed with PBS. To permeabilize the neurons, PBS with 0.4% (v/v) Triton X-100 (Biorad) was added to each well. Cells were incubated for 1 hour at room temperature and then washed with PBS.

Free-floating sections or plated dissociated neurons were incubated with chicken primary anti-GFP (1:5000; Aves Labs, Inc., Tigard, OR), mouse primary anti-Flag (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or rabbit anti-TrkB (1:5000; Millipore, Billerica, MA) overnight at 4 ºC. Sections and slides were washed in 1X PBS and labeled with Cy2-Fluroescein-labeled-goat-anti-chicken (1:250; Aves Labs, Inc.), Cy2-Goat-anti-rabbit (1:250; EMD Millipore, Billerica, MA), or Cy3-conjugated AffiniPure sheep-anti-mouse (JacksonLabs). Following washes with 1X PBS, sections and slides were incubated with DAPI (1:250; Roche Applied Science, Indianapolis, IN) for 30 min at room temperature. Sections and slides were mounted onto electrostatically-charged slides, dehydrated, and coverslipped using DPX mountant.

Western Blot Analysis

Animals were infused with their respective virus (AAV-GFP or one of the AAV-TrkB viruses) and 14 d post-infusion received either an i.p. injection of saline or 20 mg/kg cocaine. Thirty minutes after the saline or cocaine injection, animals underwent live decapitation and the NAc shell was micro-dissected using a 16-gauge tissue punch. Tissue samples were homogenized in homogenization buffer: sucrose, 5 nM Hepes buffer, 500 mM NaF, 10% SDS solution, Phosphatase Inhibitor Cocktail I (Sigma, P-2850), Phosphastas Inhibitor Cocktail II (Sigma, P-5726), and Protease Inhibitor Cocktail (Sigma, P-8340) with a pH of 7.4. Samples analyzed for changes in protein regulation were loaded into individual wells of 4-20% pre-cast, acrylamide gels (BioRad). Protein separation on the gels occurred in a running buffer: 10X Tris-Glycine-SDS (BioRad) diluted with 1X sterile water for a final volume of 1 L. Gels ran at 175v at room temperature for approximately 40 min. While the gel ran, transfer buffer was prepared: 200 ml Tris-Glycine (BioRad), 400 ml methanol, and 1400 ml sterile water (total volume of 2 L). Freshly prepared transfer buffer was cooled at 4ºC prior to use. Polyvinylidene fluoride (PVDF) membranes were soaked in 100% methanol for approximately 1 min, before being submerged in cooled transfer buffer for 15 min (or until the membrane sunk). In a separate container, blotting pads and filter paper were equilibrated to the transfer buffer. Acrylamide gels were transferred to PVDF members at 300 mA in an ice bath for 1.5 h. Following transfer, membranes were blocked in 10% milk in TTBS at room temperature for 45 min. Membranes were then incubated overnight at 4ºC in

primary antibody: anti-Flag (1:2,000, Millipore, Billerica, MA) or anti-p44/42 (pErk, 1:1000, Cell Signalling, Technology, Danvers, MA). The following day, membranes were washed with TTBS at room temperature, and then were incubated for 1 h at room temperature with the respective secondary antibody: Goat anti-Rabbit (1:50,000, Biorad, Hercules, CA) for the anti-GFP primary antibodies and Goat anti-Mouse (1:25,000, Biorad, Hercules, CA) for the anti-Flag and anti-p44/42 primary antibodies. Membranes received another round of TTBS washes and were then incubated in enhanced chemiluminesence (ECL, Amersham) for 10 minutes at room temperature. ECL aids in the visualization of protein bands. X-ray films of protein blots validated the infectivity of each virus and confirmed expression of DNA plasmids.

Intracranial viral infusions

Animals were acutely infused with either AAV-TrkB (containing either wildtype TrkB, TrkB K571N, TrkB Y490F,TrkB Y816F) or AAV-GFP, using a 5 µL Hamilton syringe with a 30 gauge injector tip directed at the NAc shell (+1.7 mm anterior to bregma, ±0.8 mm lateral, -6.7 mm ventral to dura). All viruses were infused at a rate of 0.1 µl/side/30 s, and injectors were held in place for an additional 2 min to allow for local diffusion into the NAc shell prior to injector removal. The total volume of virus injected was 1.0 µl/side. Following surgery, rats were given penicillin (60,000 IU/ 0.2mL, s.c.) to prevent infection, and ketoprofen analgesia was provided (5 mg/kg, s.c., twice a day for 3 d).

Locomotor Sensitization Assay

The locomotor sensitization assay used a circular chamber, consisting of 4 infrared beam detection sensors that measured horizontal locomotion. Following the 10 d recovery period from stereotaxic surgery that acutely infused adeno-associated viruses into the NAc shell, animals were habituated to daily i.p. of saline in their home cages for 3 d. Animals (n = 12 per viral group) were tested in the locomotor chambers for 4 h sessions for the subsequent 5 d. Each session consisted of a 2 h habituation period, followed by an injection of saline or cocaine, and a 2 h test period. On day one, all animals received a saline injection. For each subsequent day animals received either saline or cocaine (10, 20, 20, 20 mg/kg, ip, respectively). Animals then remained in their home cages for 7 d of withdrawal. Following withdrawal, animals were tested in the locomotor chambers, as described above, and received a 10 mg/kg ip challenge or acute cocaine injection (for the saline group). For the remaining 2 d, the animals received a subcutaneous (*sc*) injection of either a D1 (SKF81297, Sigma, St. Louis, MO) or D2 (Quinpirole, Sigma, St. Louis, MO) agonist. For these tests, animals were placed in the locomotor chambers for 2 h of habituation, followed by hourly injections of one of the agonists given in ascending concentration order (0, 0.1, 0.3, 1.0 mg/kg, *sc*). On the subsequent day, the experimental test was completed for the remaining agonist. Data were plotted as measures of beam breaks in 10 m bins and 1 h bar graphs.

Cocaine self-administration training

Cocaine self-administration studies conducted in this thesis involved procedures adapted from previous studies (Graham et al., 2007; Graham et al., 2009). Briefly, prior to and during food training, adult, male Sprague Dawley rats (n = 8-12 per group) were food deprived. Animals were trained to self-administer sucrose pellets (45 mg) on a fixed ratio 1 (FR1) in operant chambers (Med Associates). Rats self-administered 100 pellets each day for 3 consecutive days in order to progress to cocaine self-administration training. Food training ensured each animal had established an association between lever pressing and the delivery of a reward. Following successful completion of food training, animals were returned to their homecages for 3 days with *ad libitum* access to food and water. All animals were surgically implanted with indwelling, intravenous (i.v.) catheters into their right jugular vein, with Sialastic tubing, inserted to end prior to reaching the right atrium of the heart. Rats received at least 1 week of recovery from catheter surgeries.

Following surgical recovery, animals were placed in operant chambers for cocaine self-administration training. At the start of each session, a house-light illuminated the chamber, indicating the availability of the drug. When an animal pressed the drug-paired lever, the house light turned off for a time-out period of 15 s and a drug injection of cocaine (0.5 mg/kg/50 µl infusion over a 2.5 s duration) occurred while a cue light illuminated above the drug-paired lever. The cue-light remained illuminated for the duration of the drug injection. Following the time-out period, the house light became illuminated, indicating that drug was again available.

Lever pressing on the inactive lever resulted in no consequence for the animals, and reflected a measure of non-specific activity.

Animals acquired cocaine self-administration by lever pressing cocaine (0.5mg/kg/infusion), during 3 h daily sessions 5 d a week on an FR1 reinforcement schedule; whereby, one lever press resulted in one injection of cocaine. Once animals stabilized on an FR1, their training progressed to an FR3 schedule where each infusion required 3 lever-press responses. Animals stabilized on an FR3, meaning their mean cocaine intake did not differ by more than 10% for 3 consecutive days. Following FR3 stabilization, animals trained on an FR5 schedule under the same conditions as for FR3 training. In order to progress to behavioral testing, animals stabilized on an FR5, as previously defined for FR3 training. The training time period from acquisition on FR1 to stabilization on FR5 lasted approximately 4 weeks.

Cocaine self-administration testing

Once animals stabilized on an FR5 schedule, a between-session FR dose-response test was conducted. Rats had access to a total of 5 injection doses (0, 30, 100, 300, 1000 µg/kg). Each dose was given for 2 consecutive days, in a counterbalanced order, during 3-h sessions. Animals were stabilized on the training dose (0.5 mg/kg/infusion) prior to the start of the progressive ratio (PR) schedule. For the PR dose-response testing, animals self-administered 1 of 2 injection doses (250 or 750 µg/kg), where the response requirement for successive injections increased by progressive increments (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, etc.) (Graham et

al., 2007). Each injection dose was counterbalanced and presented for 2 consecutive days during 3-hour sessions. The break point was defined as the highest ratio of lever-presses/injection achieved before a 60 min period of no earned reinforcements.

Following PR schedule testing, animals were returned to their homecages for 7 days of withdrawal. Extinction testing occurred in 5 daily, 3-h sessions in the absence of response-contingent cocaine and injection cues, during which non-reinforced drug-paired lever presses were recorded. The following week, stimuli-induced reinstatement of cocaine seeking was tested in 5 daily, 3-h sessions similar to extinction testing. During these reinstatement sessions, the stimuli was presented during the last hour of each session using the following sequence of stimuli: cue presentation (every 2 min for 1 h), immediately after an intraperitoneal (i.p.) cocaine priming injection (saline, 500, and 1,500 µg/kg, in counterbalanced order), and after 30 min of intermittent footshock stress (1.0 mA in 0.5 s with random intervals averaging 30 s) (Graham et al., 2007). Non-reinforced responding at both drug-paired and unpaired levers was analyzed for each 1-h reinstatement test.

Results

Preliminary validation of AAV-TrkB in vivo and HEK cells (cohorts 1 and 2)

Prior to viral packaging, the pAAV constructs were transfected into HEK cells and analyzed qualitatively for TrkB expression. HEK cells were lysed and cell lysate was run on a western blot. Figure 4.1A illustrates that pAAV-TrkB WT and pAAV-TrkB

K571N express in HEK cells at similar levels. Following viral packaging, rats were infused with AVV-GFP or AAV-TrkB WT and sacrificed 14 or 21 days post-infusion. Images presented in Figure 4.1B are representative of n = 4 per group. Staining suggested that both viruses expressed *in vivo* at similar levels. Based on these results, animals were infused with AAV-GFP, AAV-TrkB WT, or AAV-TrkB K571N and sacrificed 90 days later. Results for this experiment are detailed in the "Validation of AAV-TrkB *in vivo*" section. These preliminary staining data provided sufficient evidence that the viruses were expressing, permitting the start of behavioral testing.

TrkB-signaling induced changes in locomotor sensitization

Previous work has demonstrated that the loss of TrkB specifically in the D1 receptor-containing neurons in the NAc increases locomotor responses to cocaine. To determine the effect of global expression of the TrkB receptor in the NAc shell on locomotor sensitization, a separate group of rats from those animals undergoing the self-administration experiments were infused with AAV-GFP, AAV-TrkB WT, or AAV-TrkB K571N. Following habituation to i.p. injections and the locomotor chambers, rats received 10, 20, 20, and 20 mg/kg i.p. injections over the course of 4 d, as detailed in Figure 4.2A. After the last day of locomotor sensitization, animals received a week of homecage withdrawal. A 10 mg/kg cocaine challenge was given following withdrawal. AAV-GFP-infused animals showed enhanced locomotor responses to cocaine after repeated treatments consistent with behavioral sensitization. Animals expressing the kinase dead AAV-TrkBK571N showed a trend

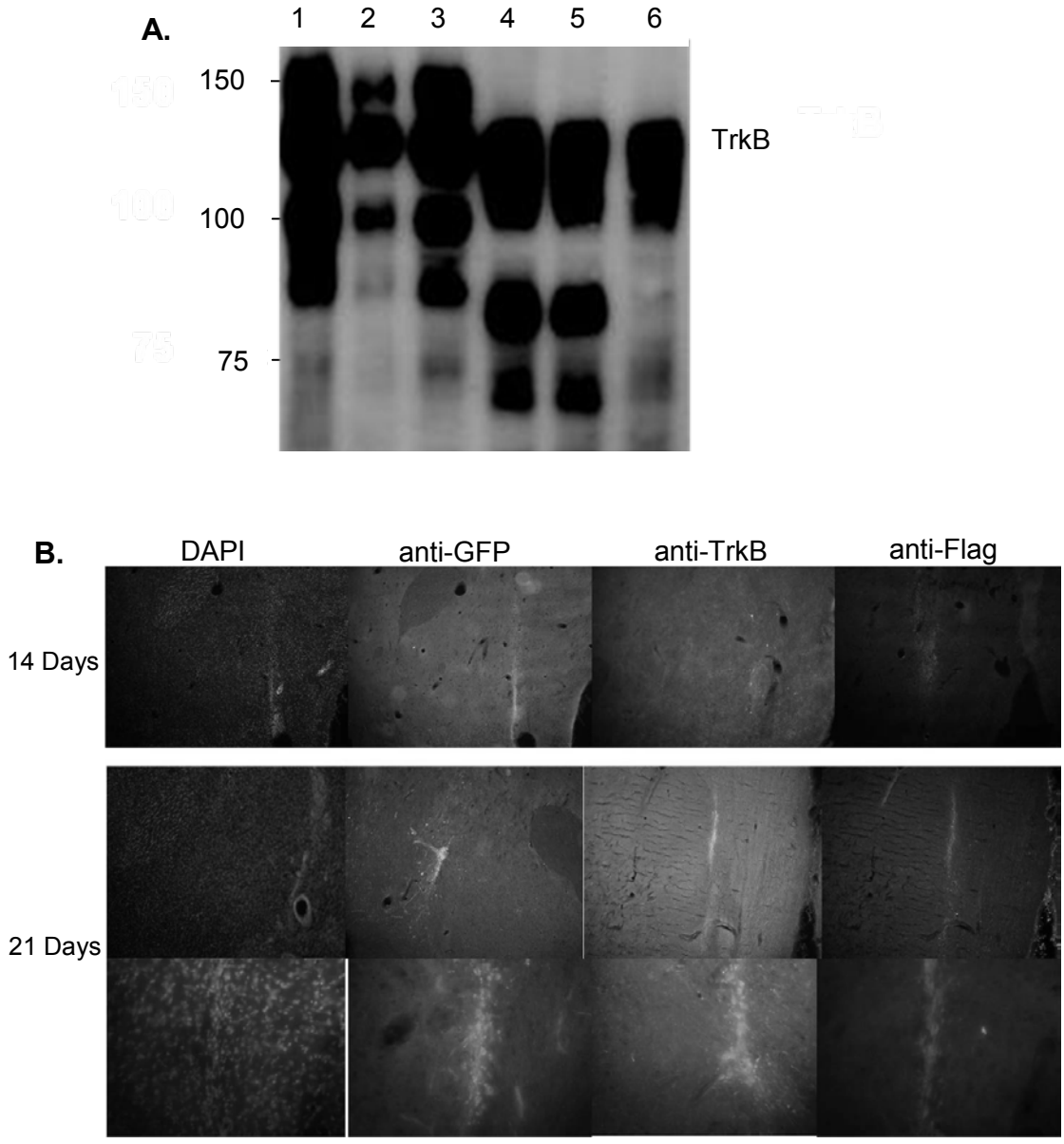

Figure 4.1 Preliminary validation of pAAV-TrkB and AAV-TrkB in HEK cells and *in vivo*. A.) pc3DNA-TrkB (lane 1 and 2), pAAV-TrkB WT (lane 3 and 4), and pAAV-TrkB K571N (lane 5 and 6) expression in HEK cells. pAAV-TrkB WT and pAAV-TrkB K571N show similar levels of protein expression *in vitro.* B.) *In vivo* expression of AAV-GFP, AAV-TrkB WT, and AAV-TrkB K571N, 14 and 21 days post-infusion. Expression levels at 21 days were better than at 14 days; however, both AAV-TrkB WT and AAV-TrkB K571N viruses expressed similarly.

for increased locomotor responses when compared to GFP-expressing controls, while animals expressing TrkB WT showed a trend for decreased sensitization (Figure 4.2B). Since D1 and D2 receptor agonists increase dopamine signaling, mimicking the effects of cocaine, in a pathway-specific manner, SKF81297 (D1 receptor agonist) and quinpirole (D2 receptor agonist) were used to determine if locomotor responses could be enhanced. Animals expressing TrkB WT and TrkB K571N receptors in the NAc demonstrated increased locomotor responses to subsequent injections of SKF81297 or quinpirole with no evidence for differential responsiveness compared to GFP controls (Figure 4.2C).

Preliminary evidence for AAV-TrkB-induced changes in cocaine SA (cohorts 1-2)

Previous studies demonstrated that a loss of TrkB reduced the reinforcing effects of cocaine (Graham et al., 2009). In order to determine the contribution of TrkB signaling in cocaine addiction, rats were infused with AAV-GFP, AAV-TrkB WT, or AAV-TrkB K571N and trained to self-administer cocaine on an FR5 schedule (Figure 4.3A). There was no significant difference between groups on their ability to acquire cocaine self-administration (Figure 4.3B). Following training, changes in cocaine intake were tested using a between-session dose-response with doses given in descending order. As presented in Figure 4.3C and Figure 4.3D, TrkB K571N produced a significant rightward shift in the threshold dose necessary for maintaining cocaine self-administration from 100 to 300 µg/kg/injection [$F_{(2, 10)}$= 0.8720 ($p < 0.01$)]. The TrkB K571N-expressing animals exhibited extinction-like

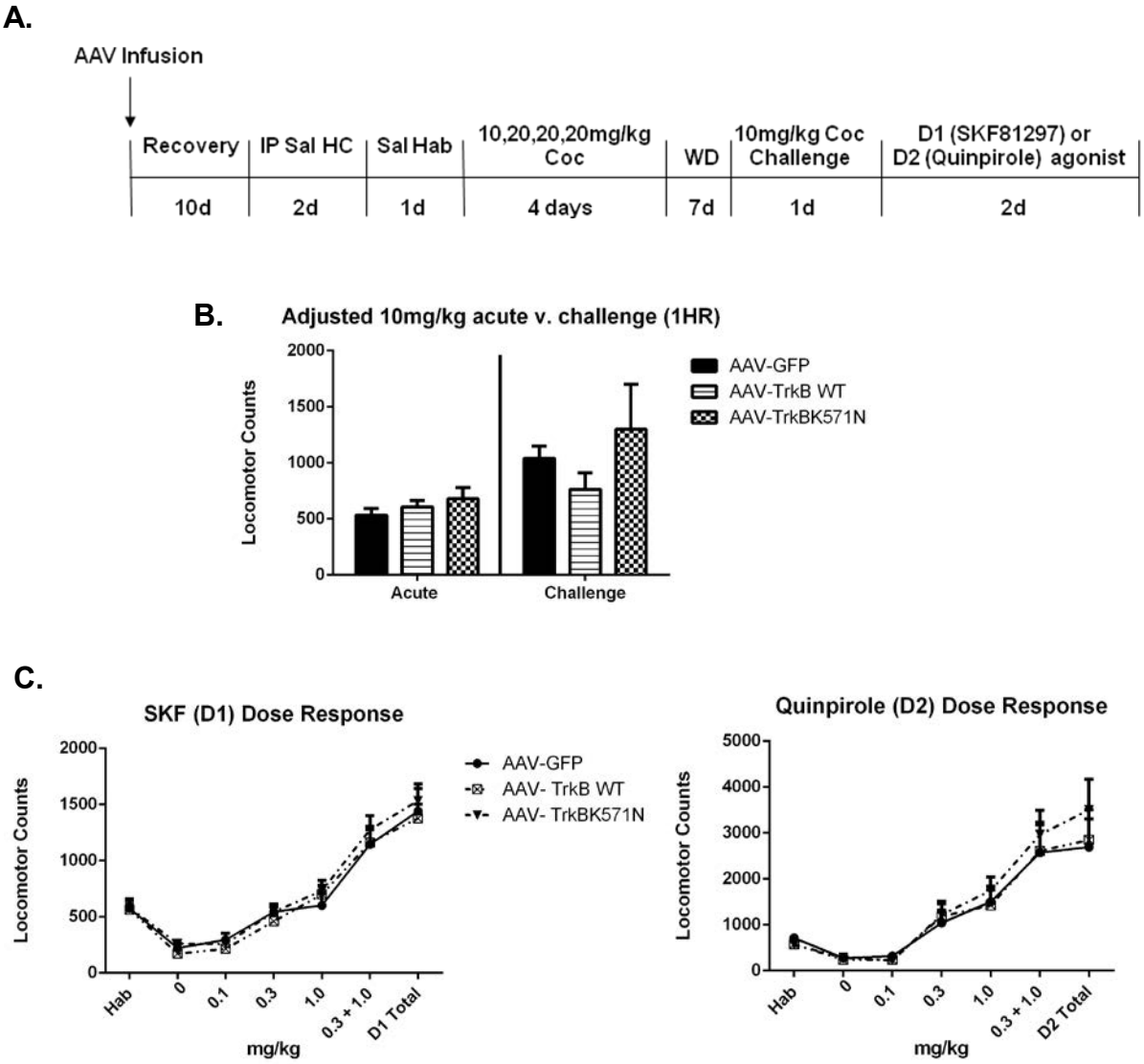

Figure 4.2 Alterations in locomotor sensitization induced by AAV-TrkB. A.) Timeline of locomotor sensitization experiment. B.) Changes in locomotor counts following an acute 10 mg/kg injection of cocaine versus a challenge 10 mg/kg cocaine injection. While there is no difference between viral groups from the acute 10 mg/kg cocaine injection, there is a trend for an increase in locomotor activity in animals expressing AAV-TrkB K571N (n = 10-12/group). C.) Responses to D1- or D2-receptor agonists on locomotor activity. There is no significant difference between viral groups on locomotor activity following increases doses of a D1-receptor agonist (SKF) or a D2-receptor agonist (quinpirole) (n = 10-12/group).

responding at the typical threshold dose of 100 ug/kg, comparable to GFP-expressing controls that extinguished at a lower 30 µg/kg/injection dose (Figure 4.3E). Following restabilization on the training dose, all study groups were tested on a progressive ratio schedule to measure changes in the motivation exerted for cocaine. TrkB K571N–expressing animals showed reduced break points for cocaine self-administration (750 µg/kg/injection) on the progressive ratio schedule, averaging about 50 responses/injection before voluntarily ceasing self-administration behavior, while GFP controls achieved a final ratio of about 150 responses/injection. This trend, however, was not significant [$F_{(2, 26)} = 1.449$ ($p = 0.2533$)] (Figure 4.3F and Figure 4.3G). Animals were subjected to one week of home cage withdrawal prior to reinstatement (relapse) testing. Cocaine-seeking after withdrawal was tested using an extinction paradigm. There was no significant difference between viral groups in their ability to extinguish lever-pressing (Figure 4.4A); however, these extinction responses were primarily specific to the previously drug-paired active lever (Figure 4.4B). Animals were subsequently tested for reinstatement of cocaine-seeking following re-exposure to cues, re-exposure to cocaine itself, and exposure to stress. Both AAV-TrkB WT- and AAV-TrkB K571N-expressing animals showed a trend for reduced responding to cue presentation compared to AAV-GFP-expressing animals. AAV-TrkB K571N showed a trend for increased responding to the 1500 µg/kg/inj priming dose of cocaine, although there was a large amount of error within the group, compared to the other viral groups. Finally, the AAV-TrkB K571N-expressing animals showed a trend for increased responding to footshock stress,

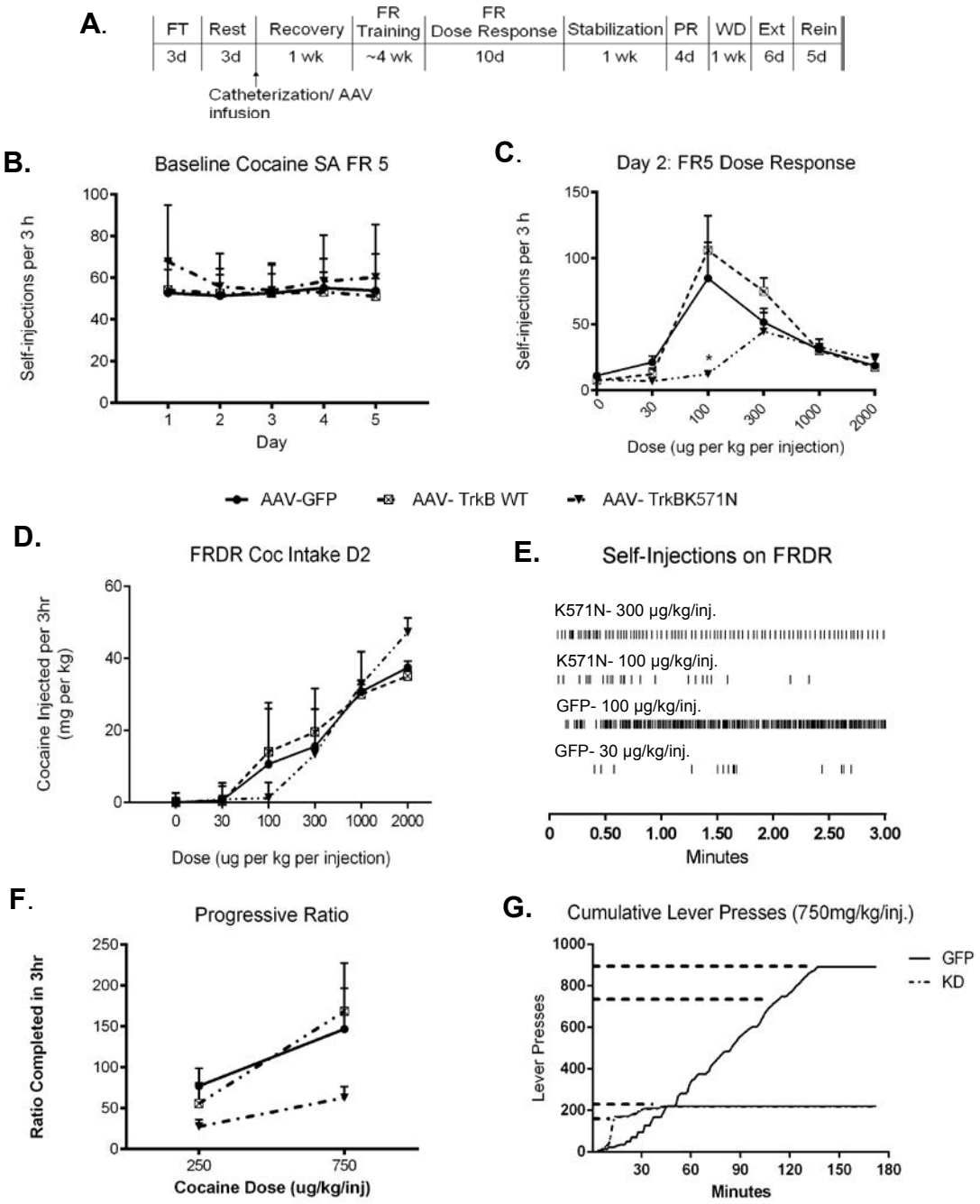

Figure 4.3 Preliminary alterations in cocaine SA by TrkB signaling. A.) Experimental timeline for cocaine self-administration (n = 1-3/ group). B.) There was no difference on between viral groups on acquisition of SA (n = 1-3/ group). C-D.) TrkB K571N produced a significant rightward shift in the threshold dose necessary for maintaining cocaine self-administration from 100 to 300 µg/kg/injection (n = 5-8/group). E.) TrkB K571N-expressing animals exhibited extinction-like responding at the typical threshold dose of 100 ug/kg, comparable to GFP-expressing controls that extinguished at a lower 30 ug/kg/injection dose (n = 5-8/group). F.) TrkB K571N–expressing animals showed reduced break points for cocaine SA (750 ug/kg/injection) on the progressive ratio schedule (n = 5-8/group). G.) Cumulative lever presses comparing AAV-GFP to AAV-TrkB K571N at the 750 µg/kg/inj. dose.

compared to the other viral groups. Results for reinstatement testing are presented in Figure 4.4C.

Validation of AAV-TrkB viral expression (cohorts 3-4)

Since preliminary data suggested sufficient expression of the viruses, additional validation experiments were undertaken. As described in the "Preliminary Validation *in vivo*" section, animals were infused with AAV-GFP, AAV-TrkB WT, or AAV-TrkB K571N and sacrificed 90 days post-infusion. Images for these staining results are not presented, because neither of the AAV-TrkB viruses showed expression. To determine whether the lack of expression was due to problems with the staining protocol or reagents, animals (n = 6) were infused with either no virus, AAV-TrkB Y490F, or AAV-TrkB K571N and left in their homecage for 60 d. Animals were then injected with saline or 20 mg/kg, i.p. cocaine and sacrificed 30 min later for dissection of NAc shell for western blot analysis. Results presented in Figure 4.5A suggest that although a plasmid known to express the Flag-tag for TrkB (pc3DNA-TrkB) showed a band for Flag, suggesting the antibody could detect the protein, no bands were visualized for Flag for any of the AAV-TrkB infused animals. In addition, the levels of pERK (p44/42) were not different between the TrkB Y490F virus, the ERK signaling mutant, and TrkB K571N. These data suggested that the viruses were not expressing for the duration of the behavioral study. Further investigation of viral expression using new batches of viruses at the 14 and 21 d time points that previously showed immunoreactivity for TrkB and flag tag (Figure 4.1B) also failed to show staining (data not shown). While staining brain slices to determine expression

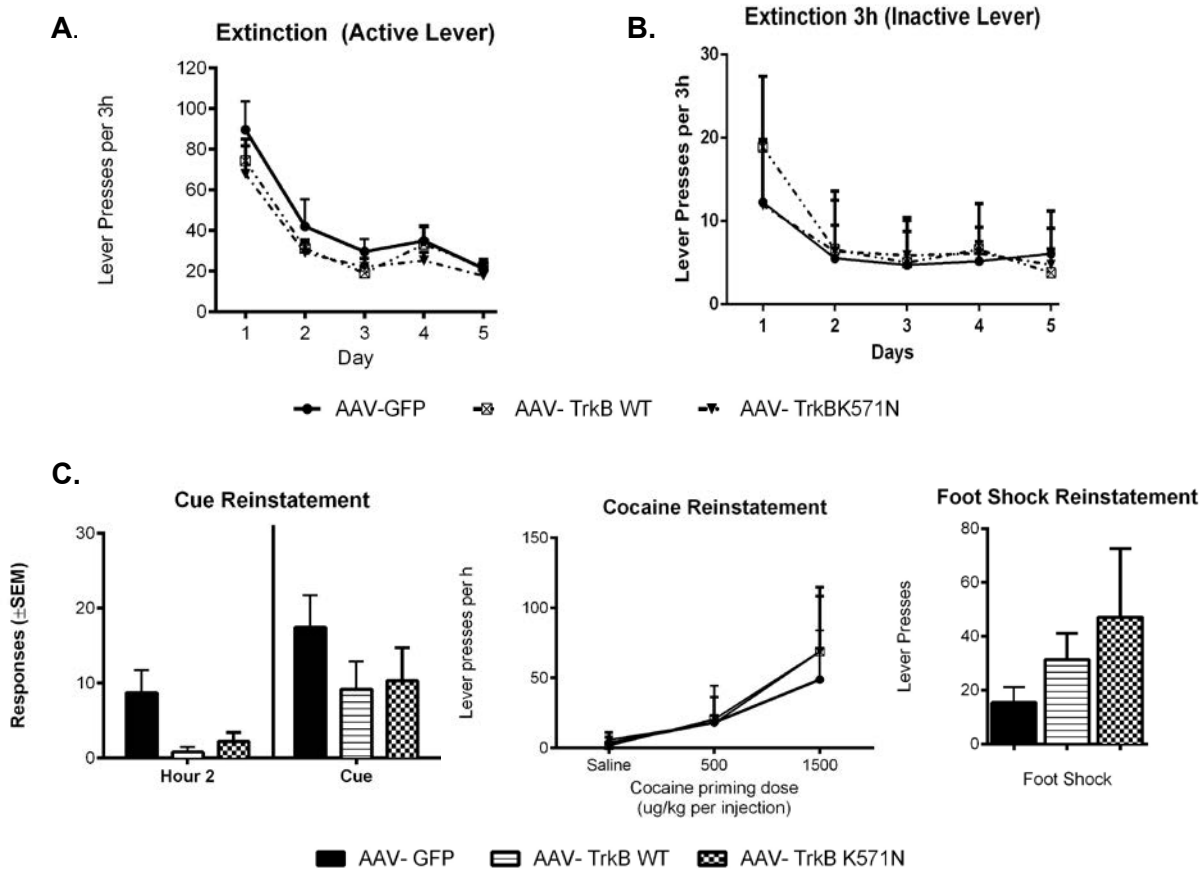

Figure 4.4 AAV-TrkB-induced changes in extinction and reinstatement. A.) There was no difference between groups on extinction responding at the drug-paired, previously active lever (n =5-8/group). B.) The extinction responding was lower at the inactive lever. C.) TrkB WT and TrkB K571N reduced cocaine-seeking to cue presentation. There was no difference between viral groups on cocaine reinstatement. TrkB K571N increased cocaine-seeking to stress (n =5-8/group).

in vivo, HEK cells also were transfected with AAV-GFP, AAV- TrkB WT, AAV-TrkB K571N, AAV-Y490F, or AAV-TrkB Y816F and were stained 4 and 7 d post-transfection. As illustrated in Figure 4.5B, HEK cells transfected with AAV-GFP infected a majority of the cells; however, the AAV-TrkB Y816F, as well as the other TrkB viruses (not shown), infected a minimal number of cells. Staining after 7 d showed no AAV-TrkB infectivity (data not shown). These data provide further evidence that the AAV-TrkB viruses were not expressing at levels observed in the initial cohorts of animals.

AAV-TrkB-induced changes in cocaine self-administration (cohorts 3 and 4)

Due to the behavioral results observed in cohorts 1 and 2, additional animals were run using the behavioral paradigm described above. Although all viral groups acquired self-administration at similar levels (Figure 4.6A), the AAV-TrkB K571N-expressing animals showed a trend for increased responding at the 100 µg/kg dose on the dose-response test compared to all other viral groups (Figure 4.6B). These data were opposite of the preliminary dose-response results. Initially, the AAV-TrkB K571N virus significantly reduced cocaine intake at the 300 µg/kg/inj. dose compared to AAV-TrkB WT; however, in cohorts 3 and 4 the cocaine intake of the AAV-TrkB K571N virus was the same as AAV-TrkB WT. Following re-stabilization on the training dose, all study groups were tested on a progressive ratio schedule. TrkB K571N-expressing animals showed an increase in breakpoints compared to all other viruses at both the 250 and 750 µg/kg doses, averaging 109 responses/injection at the 250 µg/kg and 189 responses/injection at the 750 µg/kg dose (Figure 4.6C). In

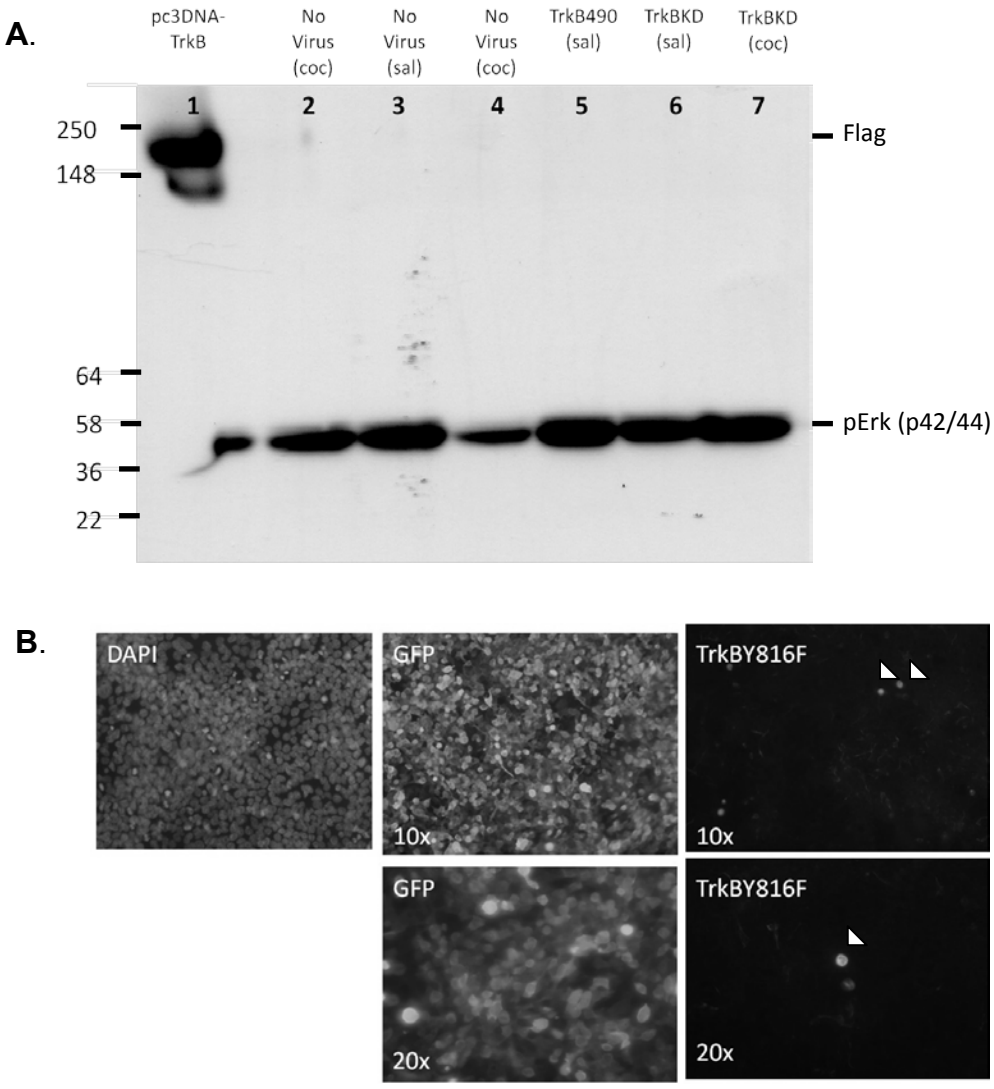

Figure 4.5 Validation of AAV-TrkB expression in cohorts 3 and 4. A.) Animals infused with AAV-TrkB K571N and AAV-TrkB Y490F and injected with saline or 20 mg/kg cocaine did not express the Flag tag, while the control (pc3DNA-TrkB) produced a band. Additionally, there were no consistent cocaine- or viral-specific effects on the phosphorylation of ERK. B.) AAV-TrkB or AAV-GFP viruses were transfected into HEK cells and harvested 4 days post-transfection. AAV-GFP infected the majority of plated cells (center panels), while AAV-TrkB (as illustrated by AAV- TrkB Y816F) infected very few cells (marked by arrows).

the original cohorts, however, TrkB K571N–expressing animals showed reduced break points for cocaine self-administration (750 µg/kg/injection). Finally, all groups showed no significant differences on extinction (Figure 4.6D). Animals receiving AAV-GFP and AAV-TrkB Y490F viruses showed decreased cocaine seeking during cue reinstatement (Figure 4.6E); however, there were no differences viral groups during cocaine reinstatement testing (Figure 4.6F). The alterations in responding on the dose-response test and the progressive ratio schedule, suggest that the viruses may no longer express at a level that induces the behavioral effects observed in cohorts 1 and 2

Discussion

This study attempted to determine the contribution of TrkB signaling in cocaine addiction using an AAV viral transfer method. Although initial cohorts of self-administration testing suggested that overexpression of kinase dead TrkB K571N in the NAc shell increased the threshold dose required to maintain self-administration on the dose-response test and reduced motivation for cocaine, subsequent validation experiments and behavioral testing did not confirm these results. Additionally, preliminary tissue staining comparing AAV-GFP and AAV-TrkB WT viral expression showed similar levels of viral infectivity. These data, however, were contradicted in subsequent behavioral cohorts where overexpression of TrkB K571N. produced increases in cocaine intake at the 100 µg/kg and increases in motivation for cocaine. These opposing behavioral data were further confirmed

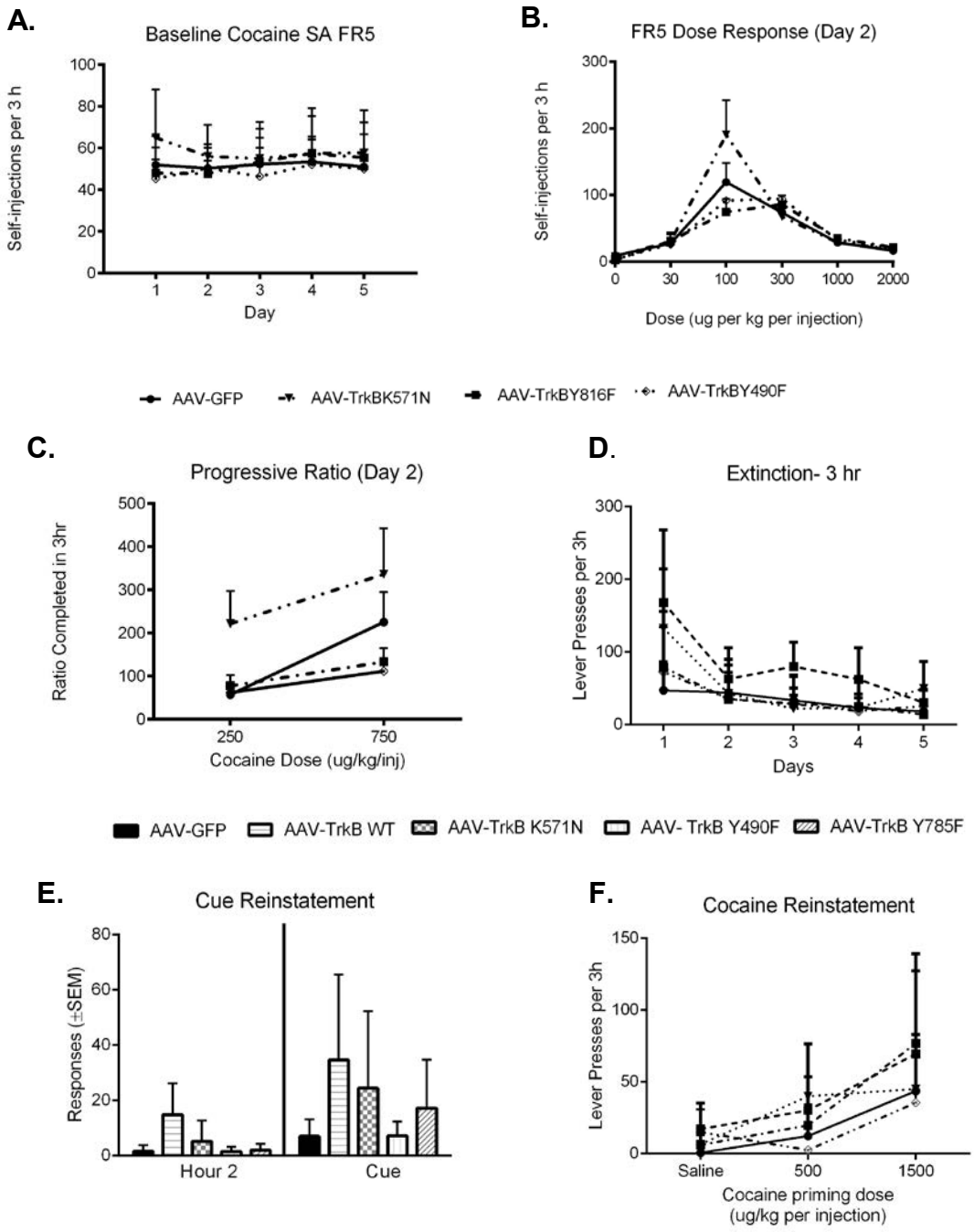

Figure 4.6 AAV-TrkB induced changes in cocaine SA in cohorts 3 and 4. A.) There was no significant difference between groups in their ability to acquire self-administration (n = 8-13/group). B.) TrkB K571N increased the rewarding effects of cocaine (n = 8-13/group). C.) TrkB K571N increased the motivation for cocaine at the highest dose (750 µg/mL/inj) (n = 5-8/group). D.) All animals extinguished cocaine seeking over five days (n = 8-13/group). E) Animals receiving AAV-GFP and AAV-TrkB Y490F viruses showed decreased cocaine seeking during cue reinstatement (n = 8-13/group). F) There were no differences viral groups during cocaine reinstatement testing (n = 8-13/group).

by HEK cell expression and tissue staining, which showed very weak to no viral expression. Together these data suggest that the viruses do not express sufficiently and consistently.

To determine whether the lack of viral expression was due to the viruses becoming inactivated because of temperature changes in the cryofreezer, original batches of AAV-GFP, AAV-TrkB WT, and AAV-TrkB K571N were compared to newly received batches of AAV-TrkB Y490F and AAV-TrkB Y816F in HEK cells expression tests. This experiment revealed that all TrkB viruses responded very weakly regardless of their duration in the cryofreezer, while the AAV-GFP infected the majority of plated cells. Thus, the change in viral expression was not due to temperature changes or storage time in the freezer, since all viruses would be affected. Similarly to the HEK cell culture results, *in vivo* infusion of the viruses produced weak expression at 14 and 21 days, time points where expression was visualized originally. Furthermore, while validating the viruses, animals were infused with AAV-GFP, AAV-TrkB WT, AAV-TrkB K571N, AAV-TrkB Y490F, and AAV-TrkB Y816F and were trained to self-administer cocaine. Unlike the preliminary findings, the AAV-TrkB K571N virus showed increases in the reinforcing effects of cocaine and motivation for the drug, but no differences were observed between viral groups during extinction and reinstatement testing. In contrast, over-expression of TrkB WT receptor in cohorts 1 and 2 failed to significantly alter cocaine self-administration compared to GFP controls, suggesting that endogenous TrkB levels convey maximal signaling in response to BDNF elevations during cocaine SA. Although trends did

occur in the behavioral data for cohorts 3 and 4, and there was no validation of viral expression *in vivo* or *in vitro*, a preponderance of the data suggests the viruses do not express sufficiently to produce behavioral responses. Thus, no behavioral data from any cohorts can be substantiated.

The use of an adeno-associated virus (AAV2 strain) provided the preferential infection of neurons and prevented retrograde infectivity, leading to localized NAc shell expression (Davidson & Breakefield, 2003). To take advantage of these characteristics, a truncated CMV promoter, with an excised beta-globin intron, was used to accommodate the relatively large size of the TrkB cDNA, since AAV has a 4-5 kb transgene capacity (Davidson & Breakefield, 2003). Introns aid in the regulation of gene expression by affecting transcription, polyadenylation, nuclear mRNA export, translational efficiency and mRNA decay (Haddad-Mashadrizeh et al., 2009; Nott et al., 2003). Since the beta-globin intron is located between the origin of replication and the multiple cloning site, it would help to ensure the mRNA will be spliced. It is possible that TrkB expression would have been improved with the presence of the intron, due to splicing having been previously shown to increase gene expression (Haddad-Mashadrizeh et al., 2009; Nott et al., 2003). This possibility, however, does not explain why the virus expressed in the initial cohorts, but failed to express in later cohorts.

Another potential explanation for why viral expression changed relates to virus-induced cell death. The AAV-TrkB viruses may be so efficient that they are leading to cellular toxicity. To test for cell death, brain slices from animals receiving

infusions of AAV-GFP or AAV-TrkB would be stained for markers of glial activation, such as glial fibrillary acidic protein (GFAP) a protein marker for astroglia that are present and activated during brain injury or damage. Additionally, research related to immunological responses to damage or injury relies on a panel of several markers related to glial activation, since immune cells have different functions and activities. Although it is possible that the TrkB viruses were causing cytotoxicity, these findings would fail to explain the 7 and 14 d expression time points that were not reproduced in the later cohorts.

In conclusion, the work presented in this chapter suggests that the AAV-TrkB viruses are not suitable for TrkB expression both *in vitro* and *in vivo.* Although immunohistochemical and -cytochemical techniques initially indicated expression, unfortunately, these techniques did not confirm the previous results in subsequent cohorts. The self-administration data are inconclusive and inconsistent between cohorts 1 and 2 and cohorts 3 and 4, most likely due to a combination of individual animal behavioral responses and a loss of viral expression. Since the truncation of the CMV promoter caused the excision of the beta-globin intron, it is possible that there is dysfunction in mRNA splicing and, subsequently, gene expression for TrkB. It is, however, possible that AAV-TrkB-induced cytotoxicity prevented visualization of viral expression at the 90 d time point. Thus, in order to study the role of TrkB and its signaling pathways in addiction, a global over-expression system using HSV, which previously has been described as having a bigger packaging capacity and stronger promoter, would be necessary. Such an approach was adopted in the next chapter.

CHAPTER FIVE
Results

GLOBAL OVEREXPRESSION OF TRKB AND DISRUPTION OF ITS SIGNALING PATHWAYS USING HERPES SIMPLEX VIRAL VECTORS

Introduction

Due to the economic, physiological, psychological, and social consequences of cocaine addiction, the transition from a recreational drug user to an addict is widely investigated. This transition is defined by an escalation in the amount of drug used and cravings during periods of withdrawal, leading to an increase in relapse (Berridge & Robinson, 1998; Wise, 2004). Cocaine addiction results in neuroadaptations and drug-induced neuroplasticity that promote changes in protein expression and neuron morphology. To induce these changes, cocaine inhibits the dopamine transporter and blocks the reuptake of dopamine into presynaptic terminals; thereby, increasing the concentration of extracellular dopamine (Nestler & Malenka, 2004; Ron & Jurd, 2005; Self, 1998). These cocaine-induced increases in dopamine ultimately alter dopamine signaling in brain regions modulating reward and motivation.

One brain region of particular interest in cocaine addiction is the nucleus accumbens. The NAc consists of MSNs which comprise approximately 90% of the total neuronal population (Meredith et al., 1999) within the brain region and acts as the interface between adaptive and goal-directed behaviors by integrating memory, motivation, and emotion. The nucleus accumbens can be sub-divided into two

regions: the core and the shell. While the cocaine-induced increases in dopamine in the core modulates locomotor responses to initiate goal-directed behavior (Canales & Iversen, 2000; Delfs et al., 1990) and drug-seeking, dopamine in the shell modulates the rewarding and reinforcing effects of cocaine (Bachtell et al., 2005; Bari & Pierce, 2005; Carlezon & Wise, 1996). Following cocaine treatment, there are increases in the synapse to neuron ratio in the shell of cocaine-treated (49.1%) rats (Alcantara et al., 2011). In addition, rats self-administer cocaine directly into the NAc shell (Carlezon & Wise, 1996). Furthermore, dopamine D1- and D2-receptor antagonists decrease the reinforcing effects of cocaine (Bachtell et al., 2005; Bari & Pierce, 2005). These alterations in morphology and behavior result from downstream effectors of dopamine signaling.

In addition to altering dopamine signaling, cocaine also affects the expression of proteins further downstream, which may account for many of the neuroplasticity- and morphology-related neuroadaptations occurring in the NAc. One protein of particular interest is BDNF, a neurotrophic factor that modulates cell survivability, plasticity, dendritic outgrowth, and cell death. Cocaine has been shown to increase BDNF mRNA (Filip et al., 2006) and protein levels in the NAc shell (Graham et al., 2007). In addition, intra-NAc infusions of BDNF have been demonstrated to increase cocaine intake (Graham et al., 2007; Horger et al., 1999) and motivation for cocaine (Graham et al., 2007) using a self-administration paradigm. Cocaine self-administration also increases protein levels of BDNF during withdrawal (Corominas

et al., 2007; Graham et al., 2007; Grimm et al., 2003), suggesting that BDNF may modulate relapse behaviors.

These increases in BDNF lead to activation of its receptor, TrkB. Chronic self-administration increases BDNF-TrkB-induced phosphorylation of PLCγ in the NAc shell, as indicated by blockade of this phosphorylation with intra-NAc shell infusions of anti-BDNF (Graham et al., 2007). Additional studies indicate that the loss of TrkB specifically in the NAc shell reduces cocaine place conditioning and the reinforcing effects of cocaine using a self-administration paradigm (Graham et al., 2009). More recently, research suggests that deletions of TrkB specifically in D2 receptor-containing neurons reduces locomotor activity, but increases neuronal firing in response to current injections (Lobo et al., 2010).

In order to understand the contribution of BDNF-induced molecular changes to the escalation in drug intake and the increase in the propensity for relapse, this project examined the TrkB receptor and its distinct signaling pathways (PLCγ and ERK/PI3K) in cocaine addiction. Since the adeno-associate viral (AAV) vector studied in Chapter 4 ultimately failed to express TrkB at sufficient levels, a herpes simplex virus (HSV) vector system was created to over-express wildtype TrkB or its docking mutants to prevent the activation of either PLCγ or ERK/PI3K pathways downstream of TrkB. The ability to over-express TrkB and its signaling mutants was examined *in vivo*. The ultimate goal of this project was to study the localized effect of specific TrkB signaling pathways in the NAc shell on cocaine addiction and relapse

behaviors. By using a herpes simplex viral approach, the signaling pathway-specific, over-expression of TrkB is short term (4-5 days), enabling the examination of behavioral changes before, during, and after expression. Thus, the long-term post-expression consequences of each TrkB signaling pathway can be investigated. Preliminary data were collected to confirm tissue expression and self-administration data suggested differential behavioral effects of overexpressing TrkB and its signaling mutants.

Materials and Methods

Animals

Male Sprague-Dawley rats (weighing 250-300 grams) were ordered from Charles-River (Kingston, RI, USA) and individually housed in wire cages for self-administration studies and housed in pairs for *in vivo* expression and western blot studies. Animals were under a 12:12 hour light:dark cycle (6:00 am to 6:00 pm) and fed *ad libitum.* Animal care was provided according to the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals and* IAACUC.

HSV-TrkB viral vector cloning and packaging

Flag-tagged TrkB, TrkB Y490F, TrkB Y785F, TrkB K571N, or GFP in pcDNA3.1 constructs were obtained from the laboratory of Chris Cowan (McLean Hospital, Harvard University). TrkB cDNA was PCR cloned using an upstream primer (5’ - CC GGATCC GCCGCCACC ATGAACTTTATCCCAGTCGACATTC - 3’)

and a downstream primer (5' - CC CTCGAGTAGCCTAGGATGTCCAGGTAGACG - 3') that introduced BamHI and XhoI cloning sites. Due to the location of the Y785F mutation, an alternate reverse primer, introducing the same restriction sites, was used (5' - CC CTCGAG CCGTCTGGGAGAAGAGGGAGTCTA - 3'). The PCR product was gel purified and cloned into an HSV-PrpUC amplicon and packaged using a 5dl1.2 helper virus. HSV-PrpUC amplicons containing TrkB, a TrkB signaling mutant, or GFP were packaged into a bicistronic herpes simplex viral vector, as previously described (Neve et al., 1997). Viruses were purified on a 10% sucrose gradient. Packaged virus was suspended in 10% sucrose and 25 mM HEPES (7.3) in PBS.

Immunohistochemistry

Rats were acutely infused with HSV-GFP, -TrkB WT, or –TrkB K571N, - and returned to their homecage for 2, 4, or 7days, since peak HSV expression occurs between days 2 and 4, and expression is undetectable by 7 days (Davidson & Breakefield, 2003). Animals were euthanized with chloral hydrate and transcardially perfused (8mL/ min) with ice-cold 1X PBS (BioRad) for 7 min followed by 4% paraformaldehyde for 15 min. Brains were dissected and stored in 4% paraformaldehyde overnight at 4ºC. Brains were then cryoprotected in 30% sucrose in PBS for 2 - 3 days at 4ºC. Brain sections were cut at 40 µm on a microtome and washed in 1X PBS. Slices were blocked in 0.3% Triton X (BioRad), 3% Normal

Goat Serum (NGS; Jackson Labs, Bar Harbor, Maine), and 1X PBS for 2 h at room temperature.

Free-floating sections were incubated with chicken primary anti-GFP (1:5000; Aves Labs, Inc., Tigard, OR), mouse primary anti-Flag (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or rabbit anti-TrkB (1:5000; Millipore, Billerica, MA) overnight at 4 ºC. Sections and slides were washed in 1X PBS and labeled with CY2-Fluroescein-labeled-goat-anti-chicken (1:250; Aves Labs, Inc.), CY2-Goat-anti-rabbit (1:250; EMD Millipore, Billerica, MA), or CY3-conjugated AffiniPure sheep-anti-mouse (JacksonLabs). Following washes with 1X PBS, sections and slides were incubated with DAPI (1:250; Roche Applied Science, Indianapolis, IN) for 30 minutes at room temperature. Sections and slides were mounted onto electrostatically-charged slides, dehydrated, and coverslipped using DPX mountant.

Western Blot Analysis

Animals were infused with their respective virus (HSV-GFP or one of the HSV-TrkB viruses) and 4 d post-infusion received either an i.p. injection of saline or 20 mg/kg cocaine. Thirty minutes after the saline or cocaine injection, animals underwent live decapitation and the NAc shell was micro-dissected using a 16-gauge tissue punch. Tissue samples were homogenized in homogenization buffer: sucrose, 5nM HEPES buffer, 500mM NaF, 10% SDS solution, Phosphatase Inhibitor Cocktail I (Sigma, P-2850), Phosphastas Inhibitor Cocktail II (Sigma, P-5726), and

Protease Inhibitor Cocktail (Sigma, P-8340) with a pH of 7.4. Samples analyzed for changes in protein regulation were loaded into individual wells of 4-20% pre-cast, acrylamide gels (BioRad). Protein separation on the gels occurred in a running buffer: 10X Tris-Glycine-SDS (BioRad) diluted with 1X sterile water for a final volume of 1 L. Gels ran at 175V at room temperature for approximately 40 min. While the gel ran, transfer buffer was prepared: 200 ml Tris-Glycine (BioRad), 400 ml methanol, and 1400 ml sterile water (total volume of 2 L). Freshly prepared transfer buffer was cooled at 4ºC prior to use. Polyvinylidene fluoride (PVDF) membranes were soaked in 100% methanol for approximately 1 min, before being submerged in cooled transfer buffer for 15 min (or until the membrane sunk). In a separate container, blotting pads and filter paper were equilibrated to the transfer buffer. Acrylamide gels were transferred to PVDF members at 300 mA in an ice bath for 1.5 h. Following transfer, membranes were blocked in 10% milk in TTBS at room temperature for 45 min. Membranes were then incubated overnight at 4ºC in primary antibody: anti-TrkB (1:2,000, Millipore, Billerica, MA), anti-p44/42 (pErk, 1:1000, Cell Signalling, Technology, Danvers, MA), or anti-GFP (1:2,000, Invitrogen, Grand Island, NY). The following day, membranes were washed with TTBS at room temperature, and then were incubated for 1 h at room temperature with the respective secondary antibody: Goat anti-Rabbit (1:50,000, Biorad, Hercules, CA) for the anti-GFP and anti-TrkB primary antibodies and Goat anti-Mouse (1:25,000, Biorad, Hercules, CA) for the anti- and anti-p44/42 primary antibodies. Membranes received another round of TTBS washes and were then incubated in enhanced

chemiluminesence (ECL, Amersham) for 10 min at room temperature. ECL aids in the visualization of protein bands. X-ray films of protein blots validated the infectivity of each virus and confirmed expression of DNA plasmids.

Intracranial viral infusions

Animals were surgically implanted with an indwelling 26 gauge bilateral intracranial guide cannula directed 1 mm above the NAc shell (+1.7 mm anterior to bregma, ±0.8 mm lateral, - 5.7 mm ventral to dura). Chronic bilateral cannulae (Plastics One) were secured to the skull with machine screws and dental acrylic, and dummy cannulae (33-gauge) were placed into the guide cannula. Following surgery, rats were given penicillin (60,000 IU/ 0.2mL, s.c.) to prevent infection, and ketoprofen analgesia was provided (5 mg/kg, s.c., twice a day for 3 d). One day prior to the within-session dose-response test and the progressive ratio test, animals were infused with either HSV-TrkB (containing either wildtype TrkB or TrkB K571N) or HSV-GFP, through chronic cannulae by inserting injectors (Plastics One) that extended 1.0 mm beyond the end of the guide cannula tip. All viruses were infused at a rate of 0.1 µL/side/30 s, and injectors were held in place for an additional 2 min to allow for local diffusion into the NAc shell prior to injector removal. The total volume of virus injected was 2.0 µL/side.

Cocaine self-administration training

Cocaine self-administration studies conducted in this thesis involved procedures adapted from previous studies (Graham et al., 2007; Graham et al., 2009). Briefly, prior to and during food training, adult, male Sprague Dawley rats (n = 8-12 per group) were maintained on a food-restricted diet. Animals were trained to self-administer in operant chambers (Med Associates) using sucrose pellets (45mg) on a fixed ratio 1 (FR1) schedule. Rats administered 100 pellets each day for 3 consecutive days in order to progress to cocaine self-administration training. Food training ensured each animal had established an association between lever pressing and the delivery of a reward. Following successful completion of food training, animals were returned to their homecages for 3 days with *ad libitum* access to food and water. All animals were surgically implanted with indwelling, intravenous (i.v.) catheters into their right jugular vein, with Sialastic tubing, inserted to end prior to reaching the heart valve. Rats received at least 1 week of recovery from catheter surgeries.

Following surgical recovery, animals were placed in operant chambers for cocaine self-administration training. At the start of each session, a house-light illuminated the chamber, indicating the availability of the drug. When an animal pressed the drug-paired lever, the house light turned off for a time-out period of 15 s and a drug injection of cocaine (0.5 mg/kg/infusion over a 2.5 s duration) occurred while a cue light illuminated above the drug-paired lever. The cue-light remained illuminated for the duration of the drug injection. Following the time-out period, the

house light became illuminated, indicating that drug was again available. Lever pressing on the inactive lever resulted in no consequence for the animals, and reflected a measure of non-specific activity.

Animals acquired cocaine self-administration by lever pressing cocaine (0.5 mg/kg/infusion), during 3 h daily sessions 5 d a week on an FR 1 reinforcement schedule; whereby, one lever press resulted in one injection of cocaine. Once animals stabilized on an FR1, their training progressed to an FR3 schedule where each infusion required 3 lever-press responses. Animals stabilized on an FR3, meaning their mean cocaine intake did not differ by more than 10% for 3 consecutive days. Following FR3 stabilization, animals trained on an FR5 schedule under the same conditions as for FR3 training. In order to progress to behavioral testing, animals stabilized on an FR5, as previously defined for FR3 training. The training time period from acquisition on FR1 to stabilization on FR5 lasted approximately 4 weeks.

Cocaine self-administration testing

Once animals stabilized on an FR5 schedule, a within-session FR dose-response test was conducted. Following a 30 min loading period when rats self-administered 0.5 mg/kg/injection, rats were allowed to self-administer each of 5 injection doses (0, 30, 100, 300, 1000 µg/kg) for a 1 h period presented in descending order. Rats were tested in the within-session dose-response procedure for 1 week prior to the HSV infusions, followed by 1 week during transient HSV expression, and then for an

additional week post-HSV. For data analysis, data was taken from day 2 of the pre-HSV test week, day 4 following HSV infusion, and day 3 of the post-HSV expression week. Animals were then stabilized on the training dose (0.5mg/kg/infusion) prior to the start of the progressive ratio (PR) schedule. For the PR dose-response testing, animals received HSV infusions and then self-administered 1 of 2 injection doses (250 or 750 µg/kg), where the response requirement for successive injections increased by progressive increments (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, etc.) (Graham et al., 2007). Each injection dose was counterbalanced and presented for 2 consecutive days during 3-hour sessions. The break point was defined as the highest ratio of lever-presses/injection achieved before a 60 min period of no earned reinforcements.

Results

Preliminary validation of HSV-TrkB in vivo

Rats were infused with HSV-GFP, HSV-TrkB WT, HSV-TrkB Y490F, or HSV-TrkB Y816F and sacrificed 4 days post-infusion. Images presented in Figure 5.1A are representative of n = 2 per group. Staining suggested that all viruses expressed *in vivo* at similar levels. Tissue slices from animals infused with HSV-TrkB K571N did not produce sufficient evidence of staining (Table 5.1A, panel 2). To determine whether the expression of the HSV-TrkB viruses leads to neuronal cell death, tissue from an animal with unilateral expression of HSV-TrkB

WT and HSV-TrkB K571N was stained with GFAP. Figure 5.1B illustrates no detectable GFAP staining, suggesting no cell death.

To determine whether the visualization of viral expression led to changes in cocaine-induced modulation of GFP, TrkB, and pERK expression, animals (n = 6-8 per group) were infused with either HSV-GFP, HSV-TrkB WT, HSV-TrkB K571N, HSV-TrkB Y490F, or HSV-TrkB Y816F and left in their homecage for 4 d. Animals were then injected with saline or 20 mg/kg, i.p. cocaine and tissue punches of NAc shell were collected after 30 min. Results presented in Figure 5.2 suggest that although TrkB, ERK, and GFP protein was detected in all groups, there was a trend for a reduction in pERK in animals infused with HSV-GFP receiving cocaine injections and the HSV-TrkB viral group [$F_{(5, 30)}$ = 1.801 (p = 0.1429)]. These results suggest that under these conditions, there were no significant cocaine-induced changes in phosphorylation of ERK, and so the ability of TrkB viruses to modulate the response was inconclusive. There were also no detectable changes in expression of total TrkB, total ERK, or GFP in the NAc shell.

Preliminary HSV-TrkB-induced changes in cocaine SA

Since previous studies demonstrated that a loss of TrkB reduced the reinforcing effects of cocaine (Graham et al., 2009), the contribution of TrkB signaling in cocaine addiction was evaluated in rats infused with HSV-GFP, HSV-TrkB WT, or HSV-TrkB K571N and trained to self-administer cocaine on an FR5 schedule (Figure 5.3A).

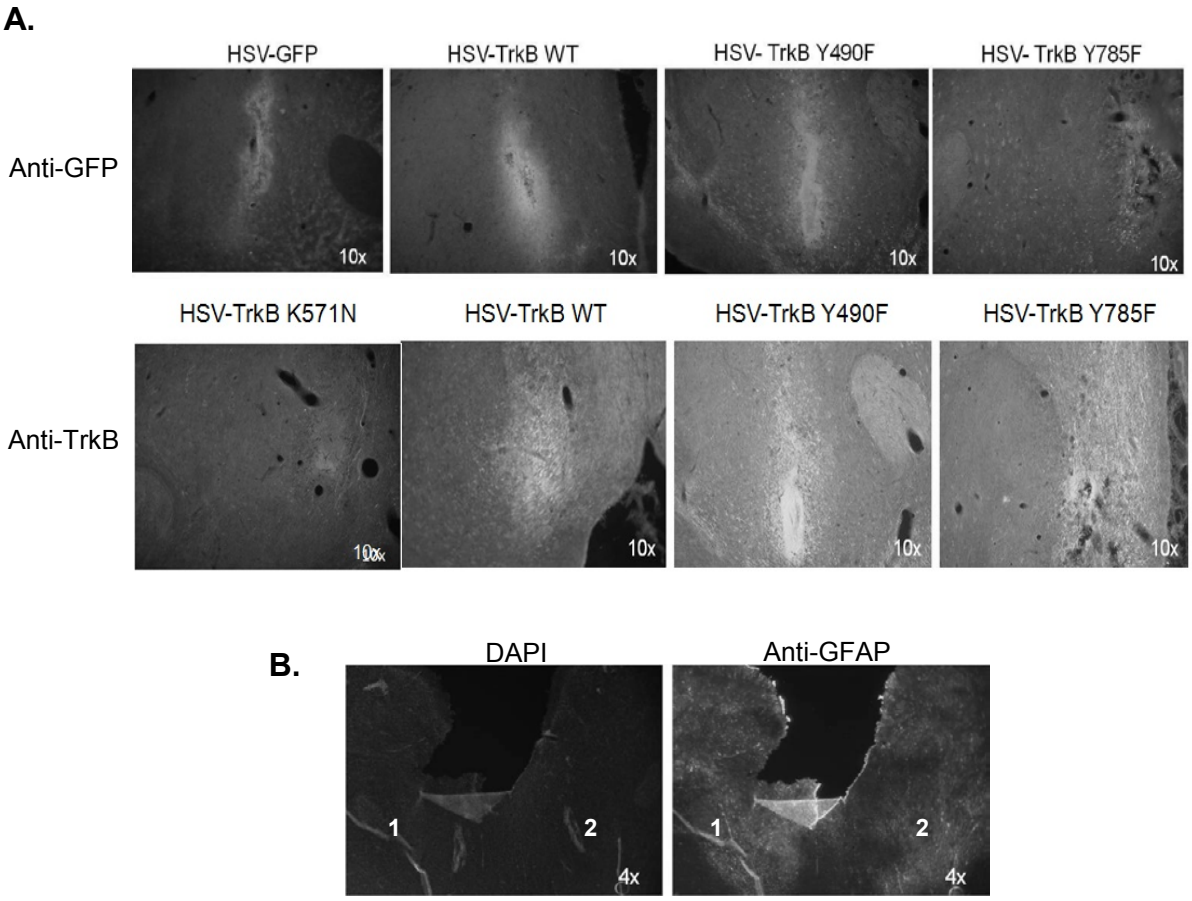

Figure 5.1 Preliminary validation of HSV-GFP and HSV-TrkB virus expression *in vivo*.
A.) Expression of HSV-GFP, HSV-TrkB WT, HSV-TrkB K571N, HSV-TrkB Y490F, and HSV-TrkB Y785F 4 days post-infusion. All viruses show similar levels of expression *in vivo.*
B.) GFAP staining, a marker of cell death, of tissue from an animal with a unilateral infusion of HSV-TrkB WT (side 2) and HSV-TrkB K571N (side 1). There was no identifiable GFAP staining 2 weeks following the infusion of either virus, suggesting cell death is not occurring.

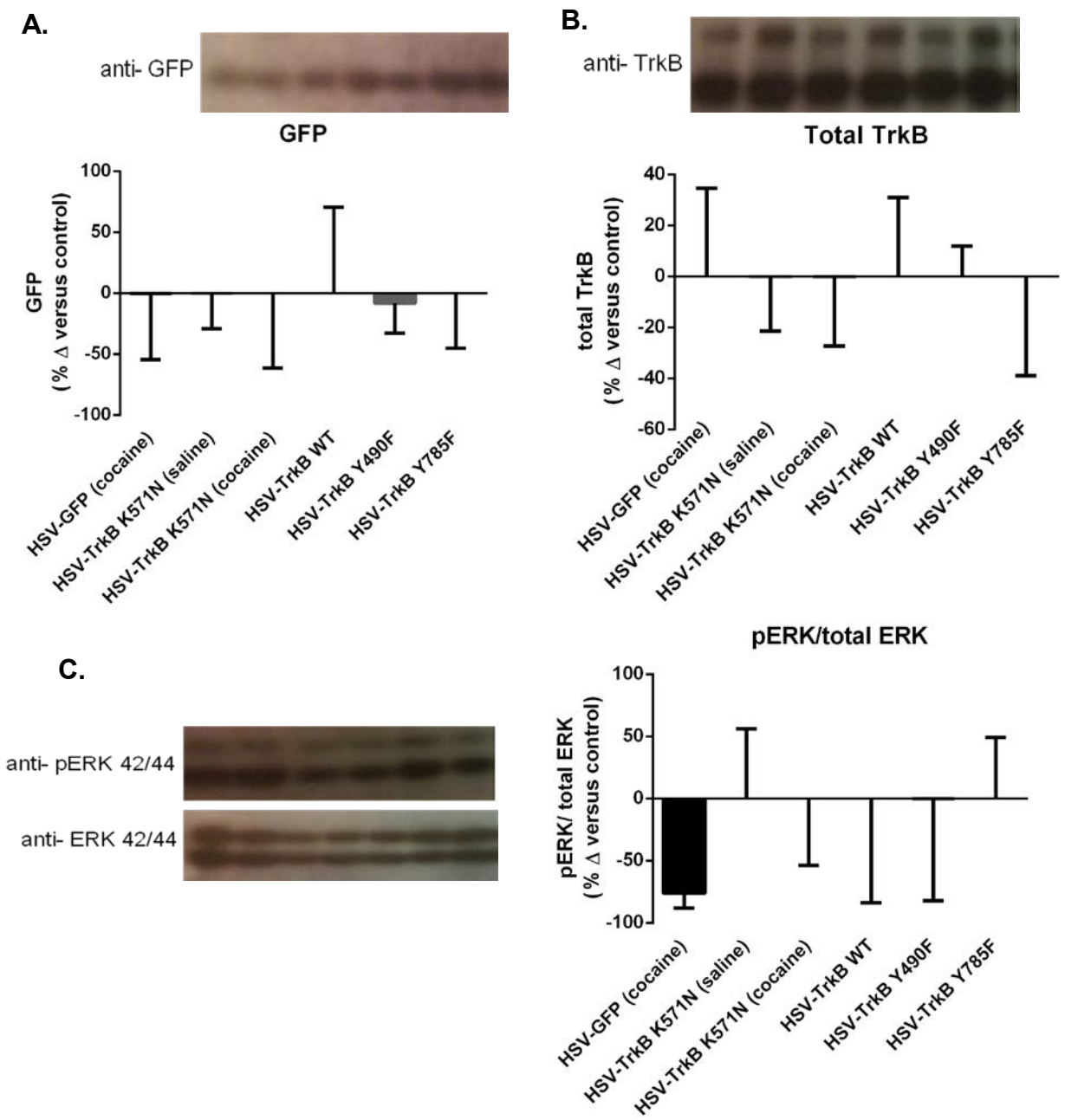

Figure 5.2 Preliminary validation of HSV-GFP and HSV-TrkB virus-induced protein expression *in vivo*. A.) Western blot of GFP protein levels in the NAc shell after infusion of the HSV vectors in the NAc shell compared to HSV-GFP controls receiving saline injections (mean ± SEM; n = 6-8/group). There was no significant difference in GFP protein levels between viral groups. B.) Western blot of TrkB protein levels in the NAc shell after infusion of the HSV vectors in the NAc shell compared to HSV-GFP controls receiving saline injections (mean ± SEM; n = 6-8/group). There was no significant difference in TrkB protein levels between viral groups. C.) Western blot of pERK protein levels in the NAc shell after infusion of the HSV vectors in the NAc shell compared to HSV-GFP controls receiving saline injections (mean ± SEM; n = 6-8/group). There was no significant difference in pERK protein levels between viral groups.

There was no significant difference between groups on their ability to acquire cocaine self-administration (Figure 5.3B). Following training, changes in cocaine intake were tested using a within-session dose response with doses given in descending order. As presented in Figure 5.3C, there was no significant difference between groups in their cocaine intake prior to the viral infusion. When changes in cocaine intake were tested during viral expression (day 3), HSV-TrkB K571N infusions produced a trend for a rightward shift in the threshold dose necessary for maintaining cocaine self-administration from 100 to 1000 µg/kg/injection (Figure 5.3C). During the post-infusion test, however, viral groups showed no significant difference in cocaine intake (Figure 5.3C). Following re-stabilization on the training dose, all study groups were tested on a progressive ratio schedule to measure changes in motivation for cocaine at 2 injection doses. TrkB K571N–expressing animals showed an increase in break points for cocaine self-administration (750 ug/kg/injection) on the progressive ratio schedule, averaging about 370 responses/injection before voluntarily ceasing self-administration behavior, while GFP controls and wildtype TrkB overexpressing animals achieved a final ratio of about 80 responses/injection. This trend, however, was not significant due to high variability and a relatively low sample size (n = 2-3/group; $p = 0.432$) (Figure 5.3D).

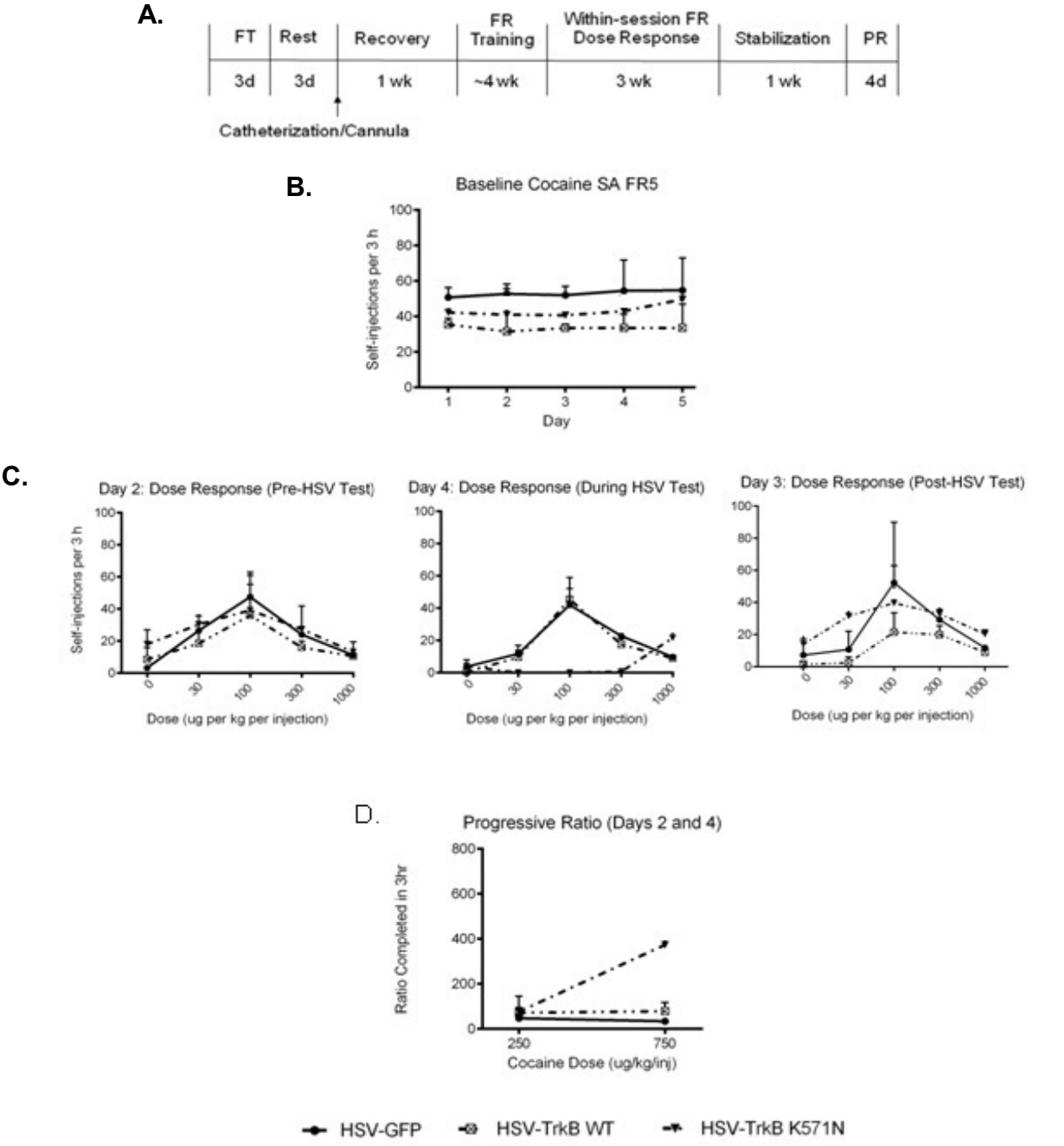

Figure 5.3 Preliminary alterations in cocaine SA by TrkB signaling. A.) Experimental timeline for cocaine self-administration. B.) There was no difference on between viral groups on acquisition of SA. C.) TrkB K571N produced a significant rightward shift in the threshold dose necessary for maintaining cocaine self-administration from 100 to 1000 µg/kg/injection during HSV expression; however, there was no significant difference between viral groups before or after HSV infusion (n = 1-3/group). D.) TrkB K571N–expressing animals showed increased break points for cocaine SA (750 ug/kg/ injection) on the progressive ratio schedule (n = 1-3/group).

Discussion

This study attempted to determine the contribution of TrkB signaling in cocaine addiction using an HSV viral transfer method. Although initial cohorts of self-administration testing suggested that overexpression of TrkB K571N in the NAc shell reduced the threshold dose of cocaine for maintaining self-administration on the fixed ratio schedule, while increasing motivation for the drug on the progressive ratio schedule, validation experiments did not confirm these results were virus-induced. To determine whether behavioral results induced by overexpression of TrkB K571N were due to cell death, tissue slices from an animal with unilateral infusions of HSV-TrkB WT and HSV-TrkB K571N, 2 weeks post-infusion, were stained for GFAP, a marker for astroglia. Staining suggested no activated astroglial presence, and, thus, no virally-induced cell death. Further tissue staining comparing HSV-GFP, HSV-TrkB WT, HSV-TrkB Y490F, and HSV-TrkB Y785F viral expression demonstrated similar levels of viral infectivity between all viruses with the exception of HSV-TrkB K571N. To confirm whether the absence of visible expression related to changes in protein expression, animals were unilaterally infused with HSV-GFP and/or one of the HSV-TrkB viruses and 4 d post-infusion received an injection of either saline or 20 mg/kg cocaine. Western blotting results indicated there was no significant difference between viral groups or saline or cocaine-injected groups on protein expression of total TrkB, total ERK, pERK, or GFP. Together these data suggest an inconsistency between behavioral results and viral expression for HSV-TrkB K571N-expressing animals, as well as for the other TrkB viral vectors.

Although overexpression of TrkB K571N produced changes in cocaine intake and motivation for the drug, the number of animals evaluated was small (n = 2-4 per viral group). Due to the inter-animal variability during self-administration tests, the results of these behavioral experiments are not conclusive, though they provide preliminary data to suggest that further experimentation is warranted. Additionally, because HSV viruses do not express for more than 4 days, verifying the efficacy of expression after testing is not possible, although guide cannula placements were generally on target. Therefore, inaccurate placement of guide cannula and virus infusion would affect behavioral responding. In this same regard, the study requires multiple virus infusions, which does not guarantee that the same population of neurons will be activated with each infusion. In order to prevent these potential problems from occurring in future cohorts, more animals per group would need to be added and each experiment should test separate groups of animals.

One consideration for the behavioral results is that the biochemical changes are not consistent. Four days post-viral infusion, animals were injected with saline or 20 mg/kg cocaine, sacrificed, and the NAc shell was microdissected. The homogenated tissue was analyzed for changes in levels of total TrkB, total ERK, pERK, and GFP. There was no significant difference between viral groups or between animals receiving saline or cocaine injections on levels of these proteins. One possible explanation for the lack of change in protein expression is that stress-induced alterations in protein levels occurred, which increased phosphorylation of targets in saline controls thus blunting cocaine and viral-induced effects on pERK.

However, animals were habituated to the injections to preclude stress-induced changes in protein expression. An additional explanation for these results is that the punches taken were not in the NAc shell, or that the viral infusion was inaccurately targeted. To avoid taking a tissue punch in the wrong brain region, the NAc shell was identified and the punch was taken around the injector tip of the cannula. It may be beneficial to perform the study on viral expression days 2 or 3, when the virus is reaching peak expression. Although stress and accuracy of viral injections and punches, and viral expression time point provide potential explanations for the biochemical results, there is still the possibility that the viruses may not enact changes at the protein level. If the viruses do not alter protein expression, then HSV-TrkB K571N-induced behavioral effects would be a result of inter-animal performance variability.

Another consideration for the self-administration results is the loss of 3-4 animals per group due to either death (overdosing) during the study (n = 3), loss of catheter patency (n = 5), and removal of animals for non-responding (n = 4). A reduction in catheter patency is a reflection of both a lengthy self-administration paradigm and a need to optimize catheter implanation surgeries. The loss of animals due to overdoses or cessation of responding can be explained by aberrant vendor batch effects. However, there is not sufficient evidence to exclude the possibility of viral effects in the brain. One potential explanation for alterations in drug taking is virus-induced cell death. The HSV-TrkB viruses may be so efficient that they are leading to cellular toxicity.

To test for cell death, brain slices from an animal receiving a unilateral infusion of HSV-TrkB WT or HSV-TrkB K571N was stained for the presence of a marker for astroglia, which is activated during brain injury or damage (GFAP). The result of this preliminary experiment showed no GFAP staining. First, due to the brevity of the experiment, no positive control was used. Thus, it is impossible to determine whether the lack of staining was due to a poor primary antibody or the lack of expression of GFAP. Second, the absence of GFAP staining is not conclusive evidence cytotoxicity did not occur. Research related to immunological responses to damage or injury relies on a panel of several markers related to glial activation, since immune cells have different functions and activities. Thus, a screening of other proteins may reveal evidence of cell death.

In conclusion, the work presented in this chapter suggests that the HSV-TrkB K571N virus may inversely affect cocaine taking and the motivation for cocaine, a somewhat strange dichotomy for these behaviors if proven correct. Although immunohistochemical techniques indicated adequate expression of HSV-TrkB viruses, protein expression associated with selective activation of TrkB signaling pathways was not demonstrated. Since the number of animals used in the self-administration experiments, as well as in the immunohistochemical studies was very low, more animals would need to be added to confirm both the behavioral and viral infectivity findings. In order to reduce animal loss in the self-administration study, it may be advantageous to use separate cohorts of animals for each test, which would prevent the use of multiple viral infusions. Unlike in the previous chapters, there is

sufficient evidence to suggest that the HSV-TrkB viruses express *in* vivo; however, it would be important to verify viral expression with different batches of virus and with each cohort infused. Thus, in order to study the role of TrkB and its signaling pathways in addiction, using HSV, it would be necessary to optimize surgical procedures and the self-administration experimental paradigm, but most importantly complete a thorough validation of virus expression *in vivo and in vitro.* Such an approach would prevent later discovery of viral ineffectiveness.

CHAPTER SIX
Conclusions and Recommendations

In order to study the role of BDNF-TrkB signaling in cocaine addiction, several approaches were used. The initial approach was to create cell-type specific viruses to express wildtype TrkB and its signaling mutants in distinct striatal output neurons. Expression of Dyn-GFP and Dyn-TrkB-IRES-GFP viruses was weak in striatal tissue and there was no evidence of receptor expression in cultured striatal neurons (Chapter 3). The self-administration data were inconclusive and inconsistent across tests, most likely due to individual animal behavioral responses and a low number of animals tested. The inability of the Dyn-TrkB-IRES-GFP viruses to infect striatal neurons prevented the evaluation of cell-type specific alterations to cocaine addiction.

One potential problem that may have led to the unsuccessful expression of the Dyn-TrkB construct related to the efficiency of its promoter. The use of a dynorphin promoter inserted in the reverse orientation of the endogenous HSV promoter (Ferguson et al., 2011) enabled the subcloning of TrkB under the control of the HSV promoter. When functioning appropriately, the reverse orientation prevented protein expression in non-targeted neuronal populations. Ferguson et al. (2011) demonstrated that the dynorphin promoter is capable of transcribing small genes (approximately 1 Kb); however, the TrkB gene is double the size, which could create transcriptional problems, although other laboratories have corroborated the low expression with GFP alone using this Dyn promoter and HSV vector. To create

a dynorphin-driven construct, Ferguson et al. (2011) PCR cloned a 2 Kb fragment upstream of the dynorphin gene. It is possible that the dynorphin promoter fragment may be missing part of the promoter or an enhancer region, which would account for the reduced transcription of the TrkB gene and subsequently the TrkB protein. Although Dyn promoter inefficiencies were not evaluated in Chapter 3, determining TrkB mRNA levels by qPCR or using a luciferase assay to determine the efficacy of the promoter would address this issue. The luciferase reporter gene would be cloned downstream of the Dyn promoter and transfected into cells. Following transfection, the cells could be lysed and a western blot run to detect the presence of the reporter or by the enzymatic activity of the reporter could be measured on a luminometer. Bioluminescent assays are more sensitive than fluorescent assays, and enable better assay performance and detection of promoter efficiency.

Additionally, the secondary structure of the mRNA can be critical to protein translation. To prevent annealing of the ribosome binding site with the first codons of the message, the 5'-end of the gene could be reconstructed to maximize its adenosine and thymidine content, while preserving the protein sequence (DeLamarter et al., 1985). Increases and decreases in the stability of short guanine-cytosine rich RNA stems can affect virus and RNA replication (Kulasegaran-Shylini et al., 2009), which could account for the lack of expression.

Due to the lack of expression of the Dyn-TrkB constructs, a global over-expression system (AAV) was used with a standard CMV promoter-driven cassette. Initial cohorts of self-administration testing suggested that overexpression of TrkB

K571N in the NAc shell increased the dose required to maintain self-administration on the dose-response test and reduced motivation for cocaine (Chapter 4). Subsequent behavioral testing, however, produced opposite effects. In addition, initial staining suggested weak expression of AAV-TrkB and its signaling mutants in striatal tissue; however, and later cohorts of animals showed no evidence of viral expression.

Similarly to the Dyn promoter, a truncated CMV promoter, with an excised beta-globin intron, was used to accommodate the size of the TrkB cDNA, since AAV only has a 4-5 kb transgene capacity (Davidson & Breakefield, 2003). Introns aid in the regulation of gene expression by affecting transcription, polyadenylation, nuclear mRNA export, translational efficiency and mRNA decay (Haddad-Mashadrizeh et al., 2009; Nott et al., 2003). Since the beta-globin intron is located between the *ori* and the multiple cloning site, the mRNA may not be spliced. It is possible that TrkB expression would have been improved with the presence of the intron, since splicing has been shown to increase gene expression (Haddad-Mashadrizeh et al., 2009; Nott et al., 2003). One way to test this hypothesis would be to insert the beta-globin intron in the standard location between the *ori* and multiple cloning site in one pAAV-TrkB construct and compare it with the pAAV-TrkB construct lacking the beta-globin intron used in Chapter 4. Constructs would be transfected into HEK cells and qPCR would detect and quantify TrkB DNA produced by both constructs to determine if the lack of the beta-globin intron reduces expression of TrkB. If the beta-globin intron is

necessary for sufficient expression of TrkB, this explanation would not account for the loss of viral expression across cohorts.

Another potential explanation for the change in viral expression relates to virus-induced cell death. The AAV-TrkB viruses may be so efficient that they are leading to cellular toxicity. Research related to immunological responses to damage or injury relies on a panel of markers of cell death, since immune cells have different functions. To test for cell death, brain slices from animals receiving infusions of AAV-GFP or AAV-TrkB could be stained for markers of glial activation, such as glial fibrillary acidic protein (GFAP) a protein marker for astroglia that are present and activated during brain injury or damage. Additionally, apoptosis can be assessed by caspase-3 immunohistochemisty. Furthermore, slice viability can also be determined through the use of trypan blue staining, a stain that is absorbed selectively by dead cells. Following staining, the number of apoptotic cells can be counted. Although it is possible that the TrkB viruses were causing cell death, these findings would fail to explain the 7 and 14 d expression time points that were not reproduced in the later cohorts.

Since the AAV viruses also proved inconsistent with regards to expression and behavioral effects, a global over-expression system using HSV with a larger packaging capacity and stronger promoter was used as previously described. Although initial cohorts of self-administration testing suggested that overexpression of kinase dead TrkB K571N in the NAc shell reduced the reinforcing effects of threshold cocaine doses but increased motivation for the drug at higher doses,

validation experiments did not confirm these results were virus-induced (Chapter 5). To determine whether behavioral results induced by overexpression of TrkB K571N were due to cell death, tissue slices from an animal 2 weeks post-infusion, were stained for GFAP. Staining suggested no astroglial presence, and, thus, no evidence for virally-induced cell death. In addition, similar levels of viral expression were demonstrated between HSV-TrkB viruses and the signaling mutants, with the exception of HSV-TrkB K571N. To confirm that the absence of visible expression related to changes in protein expression, animals were unilaterally infused with HSV-GFP and/or one of the HSV-TrkB viruses and 4 d post-infusion received an injection of either saline or 20 mg/kg cocaine. Western blotting results indicated there was no significant difference between viral groups or saline or cocaine-injected groups on protein expression of total TrkB, total ERK, pERK, or GFP. Together these data suggested a discrepancy between behavioral results and viral expression for HSV-TrkB K571N-expressing animals, as well as for the other TrkB viral vectors.

Although overexpression of TrkB K571N produced changes in cocaine intake and motivation for the drug, the number of animals evaluated was small (n = 2-4 per viral group). Due to the inter-animal variability during self-administration tests, the results of these behavioral experiments are not conclusive, though they provide preliminary data to suggest that further experimentation is warranted. Additionally, since HSV viruses do not express for more than 4 days, expression levels could not be confirmed, although guide cannulae placements suggest most infusion sites were on target. Therefore, inaccurate placement of guide cannula and virus infusion

would affect behavioral responding. In this same regard, the study requires multiple virus infusions, which does not guarantee that the same population of neurons will be activated with each infusion. In order to prevent these potential problems from occurring in future cohorts, more animals per group would need to be added and each experiment should be tested in separate groups of animals.

One consideration for the HSV-TrkB behavioral results is that the biochemical changes, seen in the western blot experiment, were inconsistent and inconclusive. For example, animals receiving cocaine challenges were habituated to the injections to preclude stress-induced changes in protein expression, but cocaine-induced elevations in ERK phosphorylation were not evident. Thus, it is possible that these stress-induced alterations obscured the effects of both cocaine and the viruses. An additional explanation for these results is that the punches taken were not in the NAc shell or that the viral infusion was inaccurately targeted. To avoid taking a tissue punch in the wrong brain region, the NAc shell was identified and the punch was taken around the injector tip of the cannula. It may also be beneficial to perform the study on viral expression days 2 or 3, when the virus reaches peak expression, instead of days 4-5 that were tested in Chapter 5. In addition, it is possible that live decapitation may not optimally preserve protein phosphorylation. Thus, it may be necessary to use microwave fixation in non-cannulated tissue. Although stress, accuracy of viral injections and punches, and viral expression time point provide potential explanations for the biochemical results, there is still the possibility that the viruses may not enact detectable changes in protein levels. If the viruses do not alter

protein expression, then HSV-TrkB K571N-induced behavioral effects would be a result of inter-animal performance variability.

Another consideration for the HSV-TrkB self-administration results is the loss of animals due to either death (overdosing) during the study, loss of catheter patency, and removal of animals for non-responding. A reduction in catheter patency is more problematic for lengthy self-administration paradigms. The loss of animals due to overdoses or cessation of responding can be explained by batch effects. However, there is not sufficient evidence to exclude the possibility of viral effects in the brain. As with the AAV-TrkB viruses, virus-induced cell death could lead to alterations in drug taking. The HSV-TrkB viruses may be so efficient that they are leading to cellular toxicity. Although preliminary results demonstrate a lack of GFAP staining, suggesting the behavioral results are unrelated to cell death, there were a few confounds to the experiment. First, no positive control was used. Thus, it is difficult to determine whether the lack of staining was due to a poor primary antibody or a lack of expression of GFAP. Second, the absence of GFAP staining does not provide conclusive evidence that cytotoxicity did not occur. As stated previously, research related to immunological responses to damage or injury relies on a panel of several markers related to glial activation, since immune cells have different functions and activities. Thus, a screening of other proteins and different techniques as mentioned above may reveal evidence of cell death.

Overall, the data suggest that loss of TrkB function with dominant negative HSV-TrkB K571N expression may inversely enhance cocaine taking and the

motivation for cocaine. Although immunohistochemical techniques indicated expression of HSV-TrkB viruses, protein expression associated with selective activation of TrkB signaling pathways was not demonstrated. Since the number of animals used in the self-administration experiments, as well as in the immunohistochemical studies was very low, more animals would need to be added to confirm both the behavioral and viral infectivity findings. In order to reduce animal loss in the self-administration study, it may be advantageous to use separate cohorts of animals for each test, which would prevent the use of multiple viral infusions. Unlike with the cell-specific HSV vectors and the AAV vectors, there is sufficient evidence to suggest that the HSV-TrkB viruses do express *in* vivo; however, it would be important to verify viral expression with different batches of virus and with each cohort infused. Thus, in order to study the role of TrkB and its signaling pathways in addiction using HSV, it would be necessary to optimize surgical procedures and the self-administration experimental paradigm, but most importantly complete a thorough validation of virus expression *in vivo and in vitro.*

Cocaine increases dopamine release that affects brain regions comprising the reward pathway, a network of brain regions implicated in mediating molecular and cellular changes and modulating behavioral responses to drugs. Chronic cocaine enhances cAMP response element binding protein (CREB)-regulated gene expression in the NAc enhances cocaine reinforcement of self-administration behavior on fixed ratio dose–response testing and motivation for cocaine on progressive ratio schedules (Larson et al., 2011). These cocaine-induced increases

in CREB have been correlated with increases in BDNF mRNA (Filip et al., 2006) and protein levels (Graham et al., 2007) in the NAc. Previous studies have shown that supplementing these daily increases with intra-NAc BDNF infusions increase lever pressing for conditioned rewards, leading to a delayed increase in daily cocaine intake (Graham et al., 2007; Horger et al., 1999), and are sufficient to increase the motivation for cocaine (Graham et al., 2007). These cocaine-induced increases in BDNF lead to a BDNF-mediated upregulation in TrkB activation of, as demonstrated by increases in PLCγ phosphorylation (Graham et al., 2009). In addition, attenuating BDNF-TrkB signaling using a TrkBsiRNA lentivirus before cocaine self-administration training suppressed cocaine seeking at 90 days of withdrawal (Li et al., 2013). These findings correlate well with the preliminary behavioral results that suggest a non-functional (kinase dead) TrkB receptor mutant reduces cocaine reinforcement (Chapter 4). Because reducing TrkB functionality decreases a component of the addictive phenotype, it would be expected that overexpressing wildtype TrkB increases these behaviors. The preliminary results, however, do not support this hypothesis, which is potentially due to BDNF being the rate limiting factor. Therefore, increasing levels of the TrkB receptor would cause a ceiling effect of the TrkB-induced behavioral effects. These data would suggest that TrkB is necessary to produce these addictive behaviors and that there is sufficient endogenous TrkB for BDNF to exact its downstream effects. As explained previously, activation of the TrkB receptor leads to phosphorylation of tyrosine residues that activate PLCγ or ERK/P13K signaling pathways. These pathways can

be dissociated, as demonstrated by opposing effects on learning and memory tasks in mice that overexpress mutations in the tyrosine residues that activate each pathway (Gruart et al., 2007; Minichiello et al., 2002).

Although the HSV-TrkB Y490F (ERK/PI3K mutant) and HSV-TrkB Y785F (PLCγ mutant) have not been tested, it is expected that each pathway will affect the addictive phenotype differently. Figure 6.1 depicts a hypothetical model of BDNF-TrkB signaling in the NAc shell during cocaine self-administration. The phosphorylation of residue Y785 leads to PLCγ phosphorylation, protein kinase C activation, increases in intracellular Ca^{2+}, and the subsequent phosphorylation of CREB, which mediates gene transcription and can target BDNF expression (Huang & Reichardt, 2003). The increases in intracellular Ca^{2+} through IP_3 stores or interactions with TRPC channels enhance the release of neurotransmitters (Amaral & Pozzo-Miller, 2012). The increased Ca^{2+} enhances the fusion of glutamate vesicles and increases the release of glutamate (Amaral and Pozzo-Miller, 2012). Thus, PLCγ can increase the release of presynaptic glutamate and depolarize and cause an action potential postsynaptically. Additionally, PLCγ can modulate α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression and trafficking (Song & Huganir, 2002), as well as inhibit GABAergic responses (Tanaka et al., 1997).

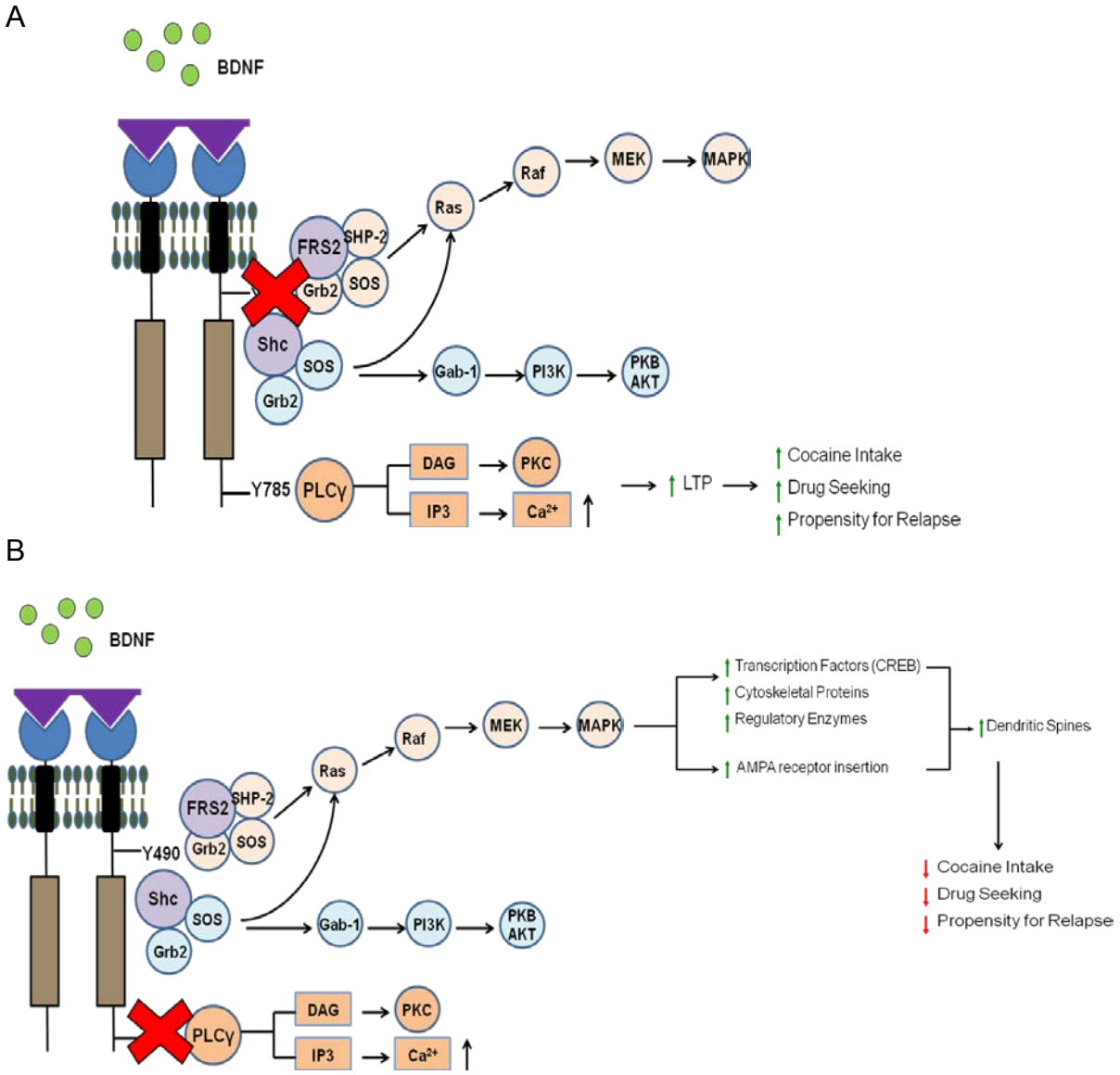

Figure 6.1 Hypothetical model of BDNF-TrkB signaling in the NAc shell during cocaine self-administration. A.) The phosphorylation of residue Y785 leads to PLCγ phosphorylation, protein kinase C activation, and increases in intracellular Ca^{2+} through IP_3 stores or interactions with TRPC channels to enhance the release of neurotransmitters, such as glutamate. These increases in presynaptic glutamate can depolarize a neuron and cause an action potential postsynaptically. These alterations may enhance cocaine reinforcement, motivation, and the propensity for relapse. B.) The phosphorylation of residue Y490 leads to the recruitment of proteins that activate different intracellular signaling cascades: Ras-Raf-Erk and PI3K -Akt. ERK targets transcription factors, through the activation of MNK, and can activate CREB and subsequently BDNF to amplify BDNF-TrkB effects in a vicious positive feedback loop. Alternatively, ERK activation could lead to activation of translation factors that initiate the translation of scaffolding and cytoskeletal proteins that stabilize dendrite formation. This ERK-related activity has been linked to increased AMPA receptor insertion. The increases in dendritic spines and potential insertion of AMPA receptors may ultimately oppose the addictive phenotype.

In particular, overexpression of the ERK/PI3K mutant, which preserves PLCγ signaling, may enhance cocaine reinforcement, motivation, and the propensity for relapse, since PLCγ is important for the acquisition and recall of memory tasks and the induction of LTP (Gruart et al., 2007; Minichiello, 2009; Minichiello et al., 2002). In addition, cocaine-induced TrkB activation has been demonstrated through increases in PLCγ phosphorylation specifically. Since Graham et al. (2009), has demonstrated that a knockdown of TrkB produces a similar reduction in cocaine reinforcement to the results presented in Chapter 4, the results suggest that TrkB-mediated PLCγ signaling is important for cocaine-related behaviors, specifically increased cocaine reinforcement and relapse behaviors.

Alternatively, the phosphorylation of residue Y490 leads to the recruitment of proteins that activate different intracellular signaling cascades: Ras-Raf-Erk and PI3K -Akt (Huang & Reichardt, 2003). ERK targets transcription factors, cytoskeletal proteins, and regulatory enzymes, as well as other kinases to induce plasticity (Thomas & Huganir, 2004). For example, activation of ERK can lead to activation of MNK, which phosphorylates CREB. Chronic cocaine increases CREB activity in the NAc (Nestler, 2004), leading to CREB-induced increases in BDNF (Larson et al., 2011). Thus, activation of BDNF-TrkB-ERK signaling may lead to increases in CREB activation and subsequent increases in BDNF, which would amplify BDNF-TrkB effects in a vicious positive feedback loop. Alternatively, ERK activation could lead to activation of translation factors that initiate the translation of scaffolding and cytoskeletal proteins the stabilize dendrite formation. ERK activity has been linked

to increased AMPA receptor insertion through its interactions with calcium/calmodulin-dependent protein kinase II (CAMKII) (Lisman et al., 2002). Increases in AMPA receptor function in the NAc shell are sufficient to reduce sensitization and drug seeking (Bachtell et al., 2008). Thus, the increases in dendritic spines and potential insertion of AMPA receptors may oppose the addictive phenotype.

TrkB-mediated activation of ERK/PI3K signaling, however, has not yet been demonstrated. Thus, it would be important to determine whether cocaine-induced TrkB activation alters ERK protein and/or phosphorylation levels. Assuming cocaine-induced TrkB activation leads to increases in ERK/PI3K signaling, further studies relating to the changes in dendritic spine count and structure would be interesting. Previous studies have shown that 4 weeks of daily cocaine injections is sufficient to increase total dendritic spine growth in the NAc shell and core (Kolb et al., 2003; Robinson & Kolb, 1999). Since activation of the ERK/PI3K pathway induces dendritic spine formation and neuronal survival in other brain regions (Bibel & Barde, 2000), respectively, it is important to investigate the effect of these dendritic spines in the NAc on cocaine addiction. Studies suggest that the growth of these spines opposes behavioral sensitization (Pulipparacharuvil et al., 2008) and hippocampal-dependent learning and memory (Barbosa et al., 2008). Thus, BDNF-TrkB mediated increases in dendritic spines may oppose the addictive phenotype in animals that chronically self-administered cocaine.

If TrkB activated ERK/PI3K does not alter neuron morphology, these data would suggest that ERK-mediated alterations in morphology may be NMDA-dependent. D1 receptors co-localize with NMDA receptors on dendrites (Dumartin et al., 2007) and inhibition of NMDA or ERK in the NAc shell has been shown to attenuate changes in dendritic branching and spine density of MSNs (Ren et al., 2010).

Additional future studies would also include the use of a virus with cell-type specificity. A recent study demonstrated that D1 receptor-specific deletions of TrkB increased locomotor activity in rats, while D2 receptor-specific deletions of TrkB reduced locomotor activity (Lobo et al., 2010). However, the D1-specific deletion of TrkB showed a slight non-significant increase in the excitability of MSN neurons, and the D2-specific deletion of TrkB showed a strong increase in neuronal firing in response to current injections (Lobo et al., 2010). These data suggest that BDNF-TrkB signaling selectively exerts differential effects on behavior and neuronal functionality based on the neuronal population activated. Although the Dyn promoter has proven useful for the expression of small proteins, further work to optimize the Dyn promoter's ability to express the TrkB receptor, as stated previously, to express is necessary. Of particular interest is how the behavioral and dendritic spine analysis results from a global overexpression of TrkB and its signaling mutants translates to cell-type specific changes within the NAc.

In addition to using cell-type specific viruses for rat self-administration, floxed-TrkB mice infused with each virus would provide the most straightforward approach

to this project. The floxed-TrkB animals would ensure little to no endogenous TrkB activity, since the viruses used in the rat model require homodimerization of TrkB subunits to prevent activation of the desired pathway. However, when heteromeric dimerization occurs between wildtype and mutant TrkB subunites, the TrkB receptors become non-functional (Luikart et al., 2008). Thus, overexpression of TrkB signaling mutants can inactivate the endogenous wildtype TrkB and leave a pathway specific TrkB receptor. The floxed-TrkB mouse model would significantly reduce the presence of non-functional TrkB receptors due to heterodimerization.

The viral approaches and preliminary data obtained from this thesis work will lead to studies that increase the understanding of the transition from recreational drug use to addiction. As demonstrated, BDNF plays an important role in cocaine reinforcement, motivation for cocaine, and relapse, and initial findings in this and other work suggest that these changes occur through its interactions with the TrkB receptor. Insight gained about the involvement of TrkB and its signaling pathways may prove helpful when designing treatments targeting relapse or disrupting molecular changes that promote this transition. By decreasing drug-taking and relapse behaviors, the detriment to societal health and the financial burden of addiction will abate.

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