GSK3: A NEUROMODULATOR OF COCAINE-INDUCED BEHAVIORAL RESPONSES

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ABSTRACT

GSK3: A NEUROMODULATOR OF COCAINE-INDUCED BEHAVIORAL RESPONSES

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Cocaine is a highly abused psychostimulant with repeated use potential culminating in addiction, a disease associated with compulsive drug seeking, use and high rates of relapse despite adverse consequences. It is well established that cocaine acts by binding to and blocking monoamine transporters therefore increasing synaptic extracellular monoamine concentrations. Cocaine also increases extracellular levels of the excitatory amino acid glutamate within the neural circuitry comprising the ascending dopamine system. Cocaine induces a number of behavioral and neurochemical manifestations following acute and repeated administration. As such, elucidating the molecular mechanisms involved in the behavioral and neuromodulatory effects of cocaine are critical to the development of effective pharmacotherapies for cocaine addiction.

The overall aim of this research was to identify a novel kinase that may be involved in the behavioral effects of cocaine. Thus, we chose to investigate glycogen synthase kinase-3 (GSK3), which has recently gained attention as being critical in dopaminergic and glutamatergic signal transduction. GSK3 is a critical mediator of many intracellular signaling systems. The activity of GSK3 is regulated by several kinases

including Akt, with inactivation occurring via phosphorylation of the inhibitory serine-21 (α-isoform) and serine-9 (β-isoform) residues. It is well established that acute cocaine administration causes hyper-locomotion in animal models and that repeated cocaine administration elicits a sensitized or increased response to the locomotor-stimulating properties of the drug. The studies outlined herein sought to determine whether non-selective and selective inhibition of GSK3 would regulate acute cocaine-induced hyper-locomotion. Further, we investigated the role of GSK3 in the development of cocaine-induced locomotor sensitization. Results of the research outlined herein demonstrate that pharmacological inhibition of GSK3 reduced both the acute behavioral responses to cocaine and the long-term neuroadaptations produced by repeated cocaine, therefore suggesting a role for GSK3 in the behavioral manifestations associated with cocaine exposure.

Previous studies have assessed the role of the dopamine D1 receptor in locomotor behaviors. As cocaine indirectly activates dopamine D1 receptors, we investigated whether activation of GSK3 was necessary for the expression of dopamine D1 receptor-mediated behaviors. To assess the role of GSK3 in dopamine D1 receptor-induced hyperactivity, GSK3 was inhibited prior to administration of the selective dopamine D1 receptor agonist SKF-82958. Selective inhibition of GSK3 reduced ambulatory and stereotypic activity produced by SKF-82958. These data implicate a role for GSK3 in the behavioral manifestations associated with dopamine D1 receptor activation.

To further assess the importance of GSK3 in cocaine-induced behaviors we investigated the role of GSK3 in various facets of cocaine-conditioned reward. We show that selective inhibition of GSK3 prevented the development of cocaine-conditioned reward using a conditioned place preference paradigm, indicating a reduction in the rewarding properties of cocaine. Relapse to drug-seeking can be precipitated by certain stimuli including the drug itself, drug-paired contextual cues and stress. Memory of drug-paired cues is highly resistant to extinction and the molecular mechanisms underlying relapse have not been clearly defined. Our results demonstrate that inhibition of GSK3 interfered with the reconsolidation of cocaine-associated contextual memories by preventing the retrieval of cocaine conditioned place preference. Inhibition of GSK3 in a neutral environment 24 hours prior to the test for reinstatement, however, did not prevent reinstatement of cocaine place preference following a cocaine priming injection. Thus, our results indicate that GSK3 serves an important role in cocaine-conditioned reward and is a critical intracellular signaling protein for the development of cocaine place preference. GSK3 is also essential to the reconsolidation and subsequent retrieval of cocaine-associated contextual cues.

In addition to studying the role of GSK3 in cocaine-induced behaviors, we assessed the neuromodulatory effects of cocaine on GSK3 activity. As stated previously, the activity of GSK3 is regulated by a number of kinases including Akt (protein kinase B). Recent evidence suggests that psychostimulants regulate the activity of Akt and subsequently GSK3 in various brain regions. Here, the ability of cocaine to regulate the activity of Akt and GSK3 was investigated. Enzymatic activity was assessed by determining protein phosphorylation in the brain. Mice administered acute injections of cocaine showed a significant decrease in phosphorylated Akt (Thr. 308) and GSK3β in the caudate putamen as determined by Western blot analysis. Cocaine did not alter pAkt (Thr. 308) or pGSK3β in the nucleus accumbens or frontal cortex. The role of dopaminergic and glutamatergic receptors on cocaine-induced attenuation of pAkt (Thr. 308) and pGSK3β was also assessed. Blockade of the dopamine D1, D2 or glutamatergic NMDA receptor prevented cocaine-induced attenuation of pGSK3β in the caudate putamen. Only blockade of the dopamine D2 receptor prevented the effect of cocaine on pAkt (Thr. 308) levels in the caudate putamen. The results of the present study indicate that the activity of Akt and GSK3 is selectively regulated in the brain following acute cocaine, an effect that is contingent upon both dopaminergic and glutamatergic receptor regulation.

In summary, the experiments described in this dissertation tested the initial hypothesis that GSK3 mediates acute cocaine-induced hyperactivity and locomotor sensitization. Acute cocaine administration increased the phosphorylation of GSK3 in the caudate putamen, therefore enhancing kinase activity. Further, the increase in GSK3 activity following cocaine administration is contingent upon activation of the dopamine D1 and D2 receptors and the glutamatergic NMDA receptor. Results presented herein also demonstrate a role for GSK3 in cocaine-conditioned reward. Selective inhibition of GSK3 prevented the development of cocaine conditioned place preference. Inhibition of GSK3 also prevented the retrieval of cocaine contextual memories, therefore playing an important role in reconsolidation. Thus, the results presented in this dissertation indicate that GSK3 is a neuromodulator of cocaine-induced behaviors and may be an important factor underlying cocaine addiction.

DEDICATION

I am dedicating my work to a number of people…

--To the memory of my father Elwood. My only hope is that he would be happy and proud of my educational success and that somehow, somewhere, and in some way he can see this.

--To my mother, Diane, for her love and support throughout every educational endeavor I have completed. Her strength throughout the years has been nothing short of inspirational and her love and devotion to my brother and I is truly unconditional. She is the epitome of understanding and her joy means the world to me. She will have excellent accommodations at a spectacular “retirement community” in the future (that was a joke).

--To my brother, Adam, for his love and support during this final stage of my academic career. I am blessed to have him as my older yet not as devastatingly handsome brother. He has allowed me to “sponge” off of him for several years without complaint. I can only hope that one day in our old age I can return the favor.

--To my fiancée, Dr. Jennifer Albano. Her happiness is the reason for my motivation. She has provided me with an open mind and certainly an open ear throughout this entire process. She is my mood stabilizer and the very thought of her makes me smile. I become a better person everyday that I know her and am lucky to be marrying her.

--To my future in-laws, Monica and Peter Albano. They have welcomed me into their home and have shown me love and support like a son. I am truly lucky to have them in my life. They too will be provided with a vacation-like atmosphere at a spectacular “retirement community” along with my mother (that too was a joke).

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Akt	protein kinase B
ANOVA	analysis of variance
cAMP	cyclic adenosine 5’ monophosphate
CREB	cyclic AMP-response element binding protein
GSK3	glycogen synthase kinase-3
i.p.	intraperitoneal
MOR	mu opioid receptor
NMDA	N-methyl-D-aspartate
PDK-1	phosphoinositide-dependent kinase 1
PH	pleckstrin homology
PKA	protein kinase A
PKC	protein kinase C
Ser	serine
Thr	threonine

CHAPTER 1

GENERAL INTRODUCTION

Scientific Rationale

Cocaine is a highly abused psychostimulant with repeated use potentially culminating in drug addiction, a brain disease with complex psychological and social factors (Leshner, 1997; Gawin, 1991). Cocaine addiction is characterized by compulsive drug use despite adverse consequences and high relapse rates during drug abstinence (Mendelson and Mello, 1996). In 2005, an estimated 22.2 million persons (9.1 percent of the population aged 12 or older) were classified with substance dependence or abuse, based on criteria specified in the *Diagnostic and Statistical Manual of Mental Disorders*, 4^{th} edition (DSM-IV), (SAMHSA, 2005). Of these persons, 3.6 million were classified as being dependent on illicit drugs but not alcohol, with nearly 2.4 million persons being current users of cocaine (SAMHSA, 2005). There are a number of factors underlying addiction including socio-economic status, environmental factors, cues and triggers, genetics, and life stressors (Volkow et al., 2004). In addition, recent findings indicate an increase in the rate of cocaine use starting at the age of 12, thus reaffirming studies previously published suggesting that most cocaine users had their first contact with cocaine during adolescence (Johanson and Fischman, 1989). As such, early exposure to drugs of abuse may be a strong predictor of later drug use and dependence (Johanson and Fischman, 1989; Kandel and Davies, 1992). Investigations to identify novel pharmacotherapies to combat cocaine addiction are underway, yet currently, there are no effective therapeutics available for the treatment of cocaine addiction (O'Brien, 2005; Sofuoglu and Kosten 2006). Given the increasing and continued usage of cocaine, it is essential to identify and investigate potential mechanisms by which cocaine use may culminate in addiction.

The overall aim of this research was to identify a novel kinase that may modulate cocaine-induced behavioral responses. Glycogen synthase kinase-3 (GSK3) is a protein kinase that was originally isolated from skeletal muscle and identified as a key regulatory enzyme for glycogen metabolism (Embi et al., 1980; Rylatt et al., 1980). This enzyme is widely expressed in all tissues with abundant levels in the brain (Woodgett, 1990). A study by Leroy and Brion (1999) shows a widespread expression of GSK3 in the adult brain, suggesting a fundamental role for this kinase in neuronal signaling pathways. There are two isoforms of GSK3 (α-isoform) and (β-isoform) with inhibition of GSK3 occurring via phosphorylation of the N-terminal serine-21 (GSK3α) or serine-9 (GSK3β), catalyzed by protein kinase B (Akt) (Cross et al., 1995). GSK3 has recently gained attention as a kinase essential to the hyperactivity associated with increased extracellular dopamine (Beaulieu et al., 2004). As such, we chose to investigate the role of GSK3 as a potential modulator of cocaine-induced behaviors.

To assess the role of GSK3 on the behavioral effects associated with cocaine the experiments presented herein determined:

1. The role of GSK3 in acute cocaine-induced hyperactivity as well as cocaine-induced locomotor sensitization. The role of GSK3 on such behavioral manifestations was determined using non-selective (valproate) and selective (SB 216763) inhibitors of GSK3.

2. If GSK3 mediated dopamine D1 receptor agonist-induced hyperactivity using the selective inhibitor of GSK3 SB 216763 and the dopamine D1 receptor agonist SKF-82958.
3. The role of GSK3 was also assessed in cocaine-conditioned reward. The importance of GSK3 in the development, retrieval, and reinstatement of cocaine-conditioned reward was investigated using a conditioned place preference paradigm.
4. Whether acute cocaine regulated GSK3 activity as determined by measuring the phosphorylation of GSK3 serine-21 (α-isoform) and serine-9 (β-isoform) in the caudate putamen, nucleus accumbens and frontal cortex. The activity of the kinase upstream of GSK3 (Akt) was also assessed in these brain regions. Further, we determined the involvement of dopaminergic and glutamatergic receptors in cocaine-induced regulation of GSK3.

Pharmacology of Cocaine

Cocaine is a psychostimulant that produces a number of behavioral effects and neurochemical adaptations. Further, continued use of cocaine may lead to drug addiction, which is characterized by drug craving and relapse into compulsive drug-seeking behavior (Jaffe et al., 1989). Numerous studies have been conducted to determine the neural circuitry, receptor types and intracellular signaling mechanisms underlying cocaine addiction and of relapse, yet there are currently no effective pharmacotherapies for cocaine addiction (O'Brien, 2005; Sofuoglu and Kosten 2006). In the central nervous system, cocaine increases synaptic neurotransmitter concentration by blocking the reuptake of dopamine, norepinephrine and serotonin by acting on monoamine transporters (Heikkila et al., 1975; Ritz et al., 1987). For example, cocaine increases extracellular dopamine in the brain by binding to and blocking the dopamine transporter (Heikkila et al., 1975; Gatley et al., 1997) (Figure 1.1). Increased dopaminergic transmission has been linked to the euphoric effects of cocaine (Volkow et al., 1999) and the dopamine transporter is a key substrate involved in the behavioral and neurochemical effects associated with cocaine (Giros et al., 1996).

The pharmacokinetics of cocaine in the brain is contingent upon a number of factors including physical/chemical form and route of administration of the drug. For example, smoked cocaine induces a faster self-reported "high" (1.4 ± 0.5 min) than intranasal cocaine (14.6 ± 8 min) (Fowler et al., 2001). Cocaine also shows differential distribution in the brain with very high and rapid uptake in the striatum and a clearance half-time of approximately 20 minutes with behaviorally active doses occupying approximately 60-77% of the dopamine transporters in this region (Fowler et al., 2001).

Following administration, cocaine is metabolized with a half-life of approximately 0.7-1.5 hours (Jeffcoat et al., 1989). Approximately half of the absorbed dose of cocaine is hydrolyzed in the liver by carboxylesterase to benzoylecgonine, ecgonine, and norcocaine (Jindal and Lutz, 1986; Fleming et al., 1990; Benowitz, 1993). In addition to its actions at dopamine transporters, cocaine exhibits local anesthetic properties by blocking voltage-gated sodium channels and causes vasoconstriction by inhibiting the local reuptake of norephinephrine (Catterall and Mackie, 2006).

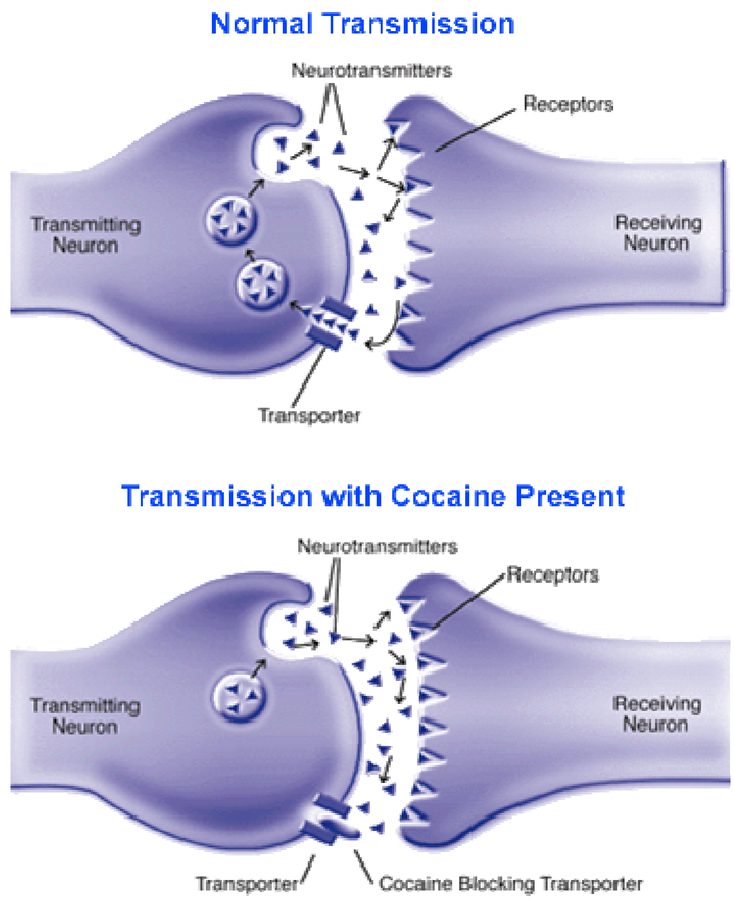

Figure 1. 1: Cocaine binds to monoamine transporters. Under normal physiological conditions, the transmitting (pre-synaptic) neuron releases dopamine that binds to receptors on the receiving (post-synaptic) neuron. The dopamine transporter removes the released dopamine from the synapse and transports it back in to the transmitting neuron. Cocaine acts by blocking the dopamine transporter, resulting in an increase in extracellular dopamine in the synapse by decreasing dopamine reuptake into the nerve terminal and breakdown by monoamine oxidases. (Modified from nida.nih.gov).

In the periphery, an evaluation of the distribution and kinetics of cocaine shows uptake in the heart, kidneys, liver, gastrointestinal tract, bladder and adrenals, but not the lungs (Volkow et al., 1992). The distribution to and accumulation of cocaine in the heart may enhance cardiac toxicity by preventing the reuptake of norepinephrine (Fowler et al., 1994) and therefore damaging the myocardium (Seifen et al., 1989).

Cocaine and Dopaminergic Transmission

The neurobiological mechanisms underlying the behavioral and neuromodulatory effects of cocaine are associated with the ascending dopamine system (Wise and Rompre, 1989). Anatomically, dopaminergic transmission is mediated by the mesocorticolimbic dopamine system with cell bodies originating in the ventral tegmental area and projecting to the nucleus accumbens, olfactory tubercle, prefrontal cortex and amygdala or the nigrostriatal system which projects from the substantia nigra to the caudate putamen (Koob et al., 1998) (Figure 1.2). The nucleus accumbens and caudate putamen (together called the striatum) are critical structures within the ascending dopamine system and are involved in the behavioral responses associated with cocaine administration (Caine, 1998). As such, perturbations of mesocorticolimbic dopamine function following cocaine administration results in enhanced dopamine in the nucleus accumbens at the synaptic level (Weiss, et al., 1992) and subsequently increased locomotor activity and stereotypic behavior in animal models (Kelley and Iversen, 1976; Kalivas et al., 1988). Likewise, lesions of the nucleus accumbens block or attenuate the rewarding effects of intravenous cocaine (Roberts et al., 1977, 1980). The caudate putamen (dorsal striatum) is also critical to the behavioral effects of cocaine. Previous investigations suggest that the caudate putamen is critical to habitual response behaviors associated with cocaine such as cue-induced cocaine-seeking (Garavan et al., 2000; Vanderschuren et al., 2005). In addition to behavioral responses associated with compulsive drug-seeking, the caudate putamen is also critical to acute cocaine-induced locomotor responses. Acute cocaine administration induces an increased locomotor response in animals and increases extracellular dopamine in the caudate putamen (Kuczenski et al., 1991). Further, acute injection of the dopamine D2 receptor antagonist sulpride into the caudate putamen prior to cocaine prevents acute cocaine-induced locomotion (Baker et al., 1996). A recent study using Pitx3 mutant mice (Semina et al., 2000; Rieger et al., 2001) further characterized the role of the caudate putamen in acute cocaine-induced locomotion. Pitx3 mutant mice display a near complete loss of A9 substantia nigra dopamine neurons, a 90% reduction in dorsal striatal dopamine levels (Hwang et al., 2003; Nunes et al., 2003; Smits et al., 2005; van den Munckhoff et al., 2003) and an attenuated locomotor response to acute cocaine administration as compared to heterozygote controls (Beeler et al., 2009). The vast majority of neurons in the striatum are GABAergic medium spiny neurons that differ depending upon connectivity within the basal ganglia (Freund et al., 1984; Borgkvist and Fisone, 2007) and are involved in motor function via thalamic projections to a number of cortical areas (Albin et al., 1989). GABAergic medium spiny neurons activate the striatonigral pathway via direct innervation of the substantia nigra pars reticulata and the internal globus pallidus or the striatopallidal pathway via indirect innervation of the internal globus pallidus via projections for the subthalamic nuclei and external globus pallidus (Borgkivist and Fisone, 2007).

At the receptor level, the behavioral and neuromodulatory effects of dopamine in the ascending dopamine system are mediated by five dopamine receptor subtypes; members of the G-protein coupled receptor family. These receptors are subdivided into two categories D1-like (D1 and D5) or D2-like (D2, D3, and D4) based on sequence homology and pharmacology (Kebabian et al., 1972; Sibley et al., 1993). Dopamine D1 and D2 receptors are highly expressed in the rat brain in regions receiving dopaminergic innervation (Sibley et al., 1993; Meador-Woodruff, 1994) and these two classes of receptors exert their function within the mesolimbic and mesocortical systems by coupling to specific G-proteins. For example, D1 receptors are coupled to stimulatory G-proteins (G_s/G_{olf}) resulting in the activation of adenylate cyclase, increase in cAMP (Kebabian et al., 1972; Stoof and Kebabian, 1984; Sibley et al., 1993), and subsequent activation of cAMP-dependent protein kinase (PKA) (Edelman et al., 1987; Mellon et al., 1989). Dopamine D1 receptors can also influence calcium-dependent signal transduction by coupling to the Gq protein and releasing calcium from intracellular stores (Bergson et al., 2003). In contrast, dopamine D2 receptors are coupled to inhibitory G-proteins (Gi/Go) therefore resulting in the inhibition of adenylate cyclase and cAMP production (Kebabian et al., 1972; Sibley et al., 1993). Dopamine D2 receptors are also coupled to phospholipase C with activation of the receptor leading to the activation of calcineurin, a Ca2+/calmodulin-dependent protein phosphatase (Nishi et al., 1997). Dopamine D1 receptors are located postsynaptically and primarily expressed in striatonigral neurons, while dopamine D2 receptors are located both pre and postsynaptically and expressed primarily in striatopallidal neurons (Gerfen et al., 1990). Evidence shows, however, that in the dorsal and ventral striatum subpopulations of neurons exhibit receptor colocalization (Surmeier et al., 1992; Aizman et al., 2000). A recent investigation using drd1a- and drd2-enhanced green fluorescent protein transgenic mice (Gong et al., 2003) has futher characterized the distribution of dopaminergic receptors in striatonigral and striatopallidal neuronal populations (Bertran-Gonzalez et al., 2008). Here, it is estimated that approximately 17% of neurons in the shell of the nucleus accumbens (ventral striatum) express both dopamine D1 and D2 receptors whereas coexpression of both receptors is found in only 5-6% of neurons in the dorsal striatum and core of the nucleus accumbens (Bertran-Gonzalez et al., 2008).

The relationship between cocaine and the dopaminergic D1 and D2 receptors has been extensively characterized both behaviorally and neurochemically. Dopamine D1 and D2 receptors are critical to the behavioral and neuromodulatory effects associated with acute and chronic cocaine administration and cocaine causes changes in the functionality of dopaminergic receptors. For example, the expression of immediate early genes such as c-fos is induced through dopamine D1 receptors by cocaine (Robertson et al., 1990; Young et al., 1991; Cole et al., 1992) and mice lacking the dopamine D1 receptor display a reduction in ΔFosB protein induction in the nucleus accumbens and caudate putamen following repeated cocaine administration (Zhang et al., 2002). Cocaine-induced regulation of dopaminergic receptors occurs following daily binge pattern administration of cocaine for 14 days which upregulates dopamine D1 receptors in the olfactory tubercle, nucleus accumbens, ventral pallidum and substantia nigra of rats (Unterwald et al., 1994). Dopamine D2 receptors, however are elevated in the olfactory tubercle, rostral nucleus accumbens and rostral caudate putamen 7 days after daily binge pattern cocaine administration and return to control levels within 14 days (Unterwald et al., 1994).

Cocaine and Glutamatergic Transmission

Glutamate is an excitatory amino acid that mediates most of the excitatory synaptic transmission in the brain (Rao and Finkbeiner, 2007). Following release from presynaptic terminals, glutamatergic transmission is mediated by ionotropic and/or metabotropic glutamate receptors. Interestingly, the neural circuitry encompassing the mesocorticolimbic dopamine system is highly interconnected with glutamatergic efferents (Tzschentke and Schmidt, 2003). Dopaminergic neurons in the ventral tegmental area receive glutamatergic input from the prefrontal cortex (Sesack and Pickel, 1992; Carr and Sesack, 2000) and amygdala (Wallace et al., 1992) and the nucleus accumbens also receives glutamatergic input from corticolimbic structures including the prefrontal cortex, amygdala and hippocampus (Kelley et al., 1982; Christie et al., 1987; Groenewegen et al., 1987; Gorelova and Yang, 1997). Activation of the glutamatergic projections from the prefrontal cortex to the ventral tegmental area increases the activity of dopaminergic cells and increases dopamine release in the nucleus accumbens (Mereu et al., 1991; Johnson et al., 1992; Tzschentke, 2001). Acute (Ungless et al., 2001) and repeated cocaine (White et al., 1995) exposure also increases glutamate-induced dopamine neuron firing in the ventral tegmental area (Figure 1.2).

Cocaine increases extracellular glutamate levels in the ventral tegmental area (Kalivas and Duffy, 1995, 1998), nucleus accumbens (Smith et al., 1995; Pierce et al., 1996; Reid and Berger, 1996) caudate putamen (McKee and Meshul, 2005) and prefrontal cortex (Reid et al., 1997). Repeated cocaine administration depresses basal extracellular glutamate levels (Baker et al., 2003; Kozell and Meshul, 2003), yet repeated cocaine enhances the amount of glutamate released as compared to a single cocaine injection (Kalivas and Duffy, 1998; McFarland et al., 2003). Cocaine also induces changes in the expression of ionotropic N-methyl-D-aspartate (NMDA) glutamate receptor mRNA and protein levels. Acute cocaine exposure causes a decrease in mRNA expression of the NR1 subunit of the NMDA receptor in the nucleus accumbens, caudate putamen and ventral tegmental area (Ghasemzadeh et al., 1999). Repeated cocaine administration increases NMDA receptor protein levels in the ventral tegmental area (Fitzgerald et al., 1996), yet investigations of receptor expression in the nucleus accumbens have been inconsistent. For example, reports show no changes (Fitzgerald et al., 1996) or decreases (Yamaguchi et al., 2002) in NMDA receptor mRNA expression following repeated cocaine administration. Decreases in NMDA receptor protein levels are observed 24 hours (Loftis and Janowsky, 2000) but not 1 week following the last cocaine injection (Zhang et al., 2007) suggesting that NMDA receptor protein and mRNA expression is spatially and temporally regulated following repeated cocaine administration.

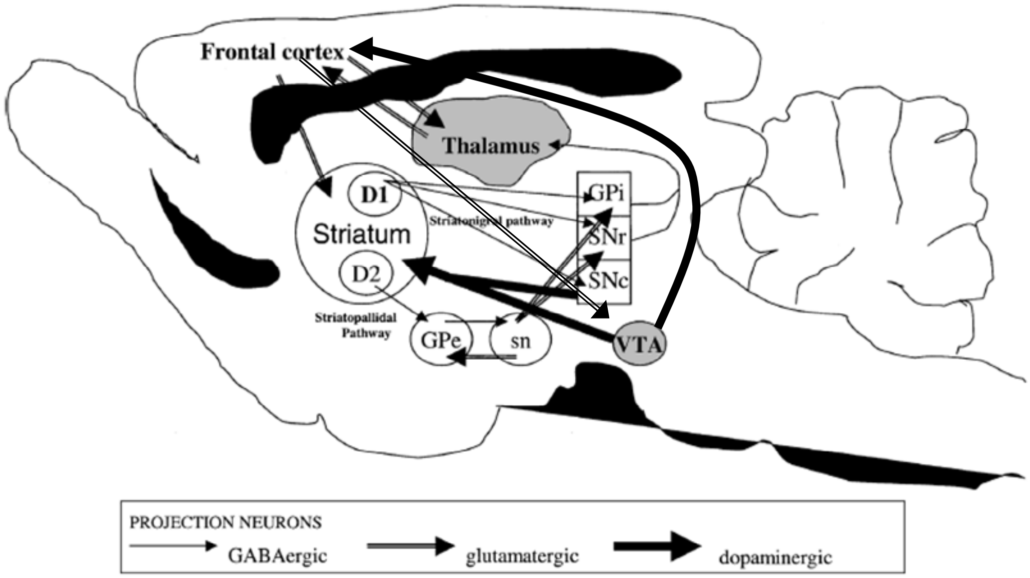

Figure 1.2: Neural circuitry involved in cocaine-induced behavior and neurochemistry. Dopaminergic, GABAergic and glutamatergic projections to critical brain regions in the ascending dopamine system are shown. Abbreviations: GPe, globus pallidus-external; GPi, globus pallidus-internal; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticula; sn, subthalamic nucleus; VTA, ventral tegmental area. (Hummel and Unterwald, 2002)

Given the effect of cocaine on glutamatergic transmission, previous investigations have highlighted the importance of glutamate and the NMDA receptor in the regulation of cocaine-induced behaviors and neurochemistry. Antagonizing NMDA receptors in the nucleus accumbens decreases the rewarding and locomotor stimulating effects of cocaine (Pulvirenti et al., 1991, 1992). Neurochemically, cocaine-induced striatal neuropeptide expression (Hanson et al., 1995) and ERK phosphorylation is contingent upon NMDA receptor activation (Valjent et al., 2000; Jenab et al., 2005).

Cocaine-Induced Hyperactivity and Sensitization

The role of dopaminergic and glutamatergic transmission in acute and sensitized cocaine-induced hyperactivity has been extensively investigated. Sensitization to cocaine/psychostimulants develops following repeated administration. Sensitization refers to an increased response to subsequent cocaine/psychostimulant dosing after previous exposure. Sensitization has been shown to develop to the locomotor stimulating effects of cocaine and other psychomotor stimulants in rodents (Post and Rose, 1976; Shuster et al., 1982; Stewart and Badiani, 1993). Evidence also indicates that sensitization develops in humans following repeated psychostimulant exposure (Angrist et al., 1974; Bartlett et al., 1997). The ability of psychostimulants to induce or express sensitization is modulated by factors including learning and the environmental context in which drug is administered (Robinson et al., 1998). As sensitization may model certain aspects of addictive behavior such as drug craving and relapse (Robinson and Berrridge, 1993) identification of new molecular targets to prevent sensitized responses to psychostimulants are warranted.

Acute cocaine causes a dose-dependent increase in locomotor activity and rearing in animals (Ushijima et al., 1995). Systemic and intraaccumbens infusions of the dopamine D1 receptor antagonist SCH-23390 attenuate acute cocaine-induced hyper-locomotion (Cabib et al., 1991; Baker et al., 1998). Similar to pharmacological inhibition, mice lacking the dopamine D1 receptor also fail to show an increase in locomotor activity following acute cocaine administration (Xu et al., 1994; Karasinska et al., 2005). Antagonism of the dopamine D2 receptor also decreases acute cocaine-induced hyper-locomotion (Ushijima et al., 1995), yet attenuation of cocaine hyper-locomotion is achieved only at doses of the antagonist that decrease locomotor activity when given alone (Chausmer and Katz, 2001).

The dopamine D1 and D2 receptors also serve an important role in the development of cocaine-induced locomotor sensitization. Administration of the dopamine D1 receptor antagonist SCH-23390 prior to daily cocaine prevents the development of sensitization (McCreary and Marsden, 1993), and dopamine D1 receptor knockout mice do not show locomotor sensitization to cocaine as compared to wildtype controls (Karlsson et al., 2008). The development of cocaine sensitization can also be blocked by the dopamine D2 receptor antagonist haloperidol (Karler et al., 1994) and repeated systemic administration of the dopamine D2 receptor agonist quinpirole with the D1 receptor agonist SKF-38393 mimics the induction of cocaine sensitization (Henry et al., 1998).

In addition to the role of dopaminergic receptors in the acute hyper-locomotor response associated with cocaine, glutamatergic receptors play a critical role in acute and sensitized cocaine-induced hyper-locomotion. For example, administration of the noncompetitive glutamatergic NMDA receptor antagonists MK-801 or ketamine attenuates acute cocaine-induced hyper-locomotion in mice (Uzbay et al., 2000). Systemic administration of MK-801 also prevents the development of behavioral sensitization to cocaine (Karler et al., 1989), apomorphine (Druhan et al., 1993) and L-DOPA (Pinheiro-Carrera et al., 1995). Microinjections of MK-801 or the competitive NMDA receptor antagonist CPP into the ventral tegmental area and amygdala, but not the nucleus accumbens also prevents the development of cocaine-induced locomotor sensitization (Kalivas and Alesdatter, 1993). Further, mice with reduced expression of the NR-1 subunit of the NMDA receptor (Mohn et al., 1999) show a reduction in acute and sensitized cocaine-induced hyper-locomotion as compared to wild-type control mice (Ramsey et al., 2008).

Cocaine Conditioned Reward, Retrieval and Reinstatement

There are a number of neural substrates, receptors and intracellular signaling mechanisms involved in cocaine conditioned reward and relapse. The development of cocaine reward has been extensively studied using the conditioned place preference paradigm. Conditioned place preference is a behavioral paradigm structured around two distinct phases termed acquisition/conditioning and expression. During the acquisition phase, animals are exposed to drug that is paired with a specific environment. The time spent and number of pairings to that environment is dependent upon factors including the drug used and behavioral end point. During the expression phase, animals are tested for their preference toward their drug-paired environment (time spent in the drug-paired side) in a drug-free state. Increased time spent in the drug-paired environment after conditioning is thought to reflect a heightened motivational relevance to drug-associated stimuli (Mueller and Stewart, 2000). The conditioned reward paradigm in rodents is thought to model compulsive-like behaviors in human cocaine addicts such as drug-seeking and drug-craving (Bardo and Bevins, 2000; Robinson and Berridge, 2001).

Dopaminergic and glutamatergic receptors have been extensively characterized in terms of their role in the development cocaine conditioned reward. Changes in extracellular dopamine levels exert a functional role in brain reward, both to natural reinforcers and addictive drugs (Wise and Bozarth, 1987; Carboni et al., 1989; Koob, 1992; Kelley and Berridge, 2002; Wise, 2002; Bonci et al., 2003). The induction of cocaine conditioned place preference is contingent upon dopamine D1 receptor stimulation as antagonism of the receptor during the cocaine-conditioning phase prevents the acquisition of cocaine place preference (Cervo and Samanin, 1995). The dopamine D1 receptor also functions as a primary reward to cocaine-naïve animals as dopamine D1 receptor agonists induce place preference (Graham et al., 2007). Interestingly, pharmacological inhibition of the dopamine D2 receptor does not affect the induction of cocaine place preference (Cervo and Samanin, 1995; Baker et al., 1996) and administration of the dopamine D2 receptor agonist quinpirole fails to produce place preference in cocaine-naïve animals (Graham et al., 2007). Glutamatergic NMDA receptors are critical to the induction of cocaine-induced place preference as pharmacological inhibition (Kim et al., 1996; Harris and Aston-Jones, 2003) or genetic (Heusner and Palmiter, 2005) deletion of the receptor prevents the development of cocaine-induced place preference.

Retrieval of cocaine-associated contextual memories involves reactivation and the potential strengthening of previously learned memories, a process known as reconsolidation (Mactutus et al., 1979; Przybyslawski and Sara, 1997). During this process, memory traces are labile and can be manipulated pharmacologically (Przybyslawski and Sara, 1997; Przybyslawski et al., 1999; Nader et al., 2000; Valjent et al., 2006). Previous investigations focusing on reconsolidation and retrieval of cocaine-associated memories have highlighted the importance of glutamatergic receptors in these processes. For example, pharmacological blockade of NMDA receptors with MK-801 during reconsolidation prevents the expression of cocaine place preference (Kelley et al., 2007; Brown et al., 2008; Itzhak, 2008). To date, studies focusing on dopaminergic receptor regulation and the reconsolidation of cocaine-associated memories are lacking. Dopamine receptors have however been implicated in self-administration studies investigating context-induced reinstatement of cocaine seeking. Here, pretreatment with the dopamine D1 receptor antagonist SCH-23390 or D2 receptor antagonist raclopride prior to re-exposure to the drug-associated context (conditioned stimulus) prevented the renewal of cocaine seeking (Crombag et al., 2002).

In addition to the development and retrieval of cocaine-conditioned place preference, reinstatement procedures have been used as a model to study relapse to cocaine seeking. Following the initial acquisition and expression of place preference, animals undergo extinction training. Extinction training consists of repeated daily testing and repeated exposure to the previously drug-paired environment (conditioned stimulus) in the absence of drug (unconditioned stimulus), therefore resulting in a decline in place preference (Mueller and Stewart, 2000). The extinction of place preference is followed by a priming injection of drug or exposure to stress that acts to renew the salience of the drug-related environmental stimuli, thus reestablishing the previously acquired place preference (Mueller and Stewart, 2000; Kreibich and Blendy, 2004). Interestingly, dopamine D1 and D2 receptors differentially effect cocaine-primed reinstatement. Following extinction of cocaine place preference, the dopamine D1 receptor agonist SKF-81297 dose-dependently reinstates place preference in cocaine-conditioned rats (Graham et al., 2007). The dopamine D2/D3 receptor agonist quinpirole fails to block cocaine-induced reinstatement as measured by place preference (Graham et al., 2007). The role of the glutamatergic NMDA receptor on cocaine-primed reinstatement is contingent upon memory reactivation to the conditioned and unconditioned stimulus. Here, re-exposure of animals to the conditioned stimulus (place preference chamber) and unconditioned stimulus (cocaine) in the presence of the NMDA receptor antagonist MK-801 prevents cocaine-primed reinstatement of place preference when tested a day later (Brown et al., 2008).

Glycogen Synthase Kinase-3 and Neuronal Signaling

Orignially identified for its regulation of glycogen metabolism (Embi et al., 1980), glycogen synthase kinase-3 (GSK3) has been since shown to be critical for a number of cellular processes including apoptosis (Cross et al., 2001) and synaptic plasticity (Peineau et al., 2008). GSK3 is highly expressed in the brain including the frontal cortex, nucleus accumbens, caudate putamen, hippocampus and amygdala (Leroy and Brion, 1999). Expression of GSK3 is also cell-specific with immunoreactivity detected in hippocampal (Leroy and Brion, 1999) and cortical pyramidal cells (Chen et al., 2007) as well as hippocampal glial cells (Peineau et al., 2007). Cell-specific localization of GSK3 within brain regions such as the nucleus accumbens and caudate putamen has yet to be characterized. Interestingly, activation of the dopamine D1 and D2 receptors activates the kinase upstream of GSK3 (Akt) in primary striatal neurons (Brami-Cherrier et al., 2002). Further, transduction of medium spiny neurons with constitutively active Akt increases the induction of DARPP-32 protein levels (Bogush et al., 2007). This suggests that Akt modulates dopaminergic intracellular signaling cascades within subpopulations of striatal neurons. Thus, it is tempting to speculate that GSK3 may also modulate dopaminergic signaling within a similar subpopulation of striatal neurons, however evidence presented herein demonstrates a differential regulation of GSK3 and Akt in the brain, an effect that is contingent upon inactivation of specific dopamine receptors (see Chapter 5).

As stated previously, GSK3 is a protein kinase that is regulated via phosphorylation of the N-terminal serine-21 (GSK3α) or serine-9 (GSK3β). It is well established that insulin regulates GSK3 activity (Welsh et al., 1993; Welsh et al., 1998) via phosphorylation of GSK3 by activation of phosphatidyl-inositide (PI) 3-kinase (Cohen et al., 1997) and subsequently Akt (Cross et al., 1995). In addition to PI3-kinase and Akt, protein kinase A (PKA) inhibits GSK3α/β activity in a cAMP-dependent manner (Fang et al., 2000) while protein kinase C (PKC) only inhibits GSK3β (Goode et al., 1992) (Figure 1.3). In addition to kinase induced regulation of GSK3, activity of the kinase is regulated by protein complex formation and intracellular localization. Binding of GSK3 to Axin increases the activity of GSK3 and enhances the phosphorylation of β-catenin (Ikeda et al., 1998), a substrate of GSK3. Further, GSK3 is spatially regulated, an effect that is contingent upon a redistribution of GSK3 from the cytosol to the nucleus (Bijur and Jope, 2001). The accumulation of GSK3 in the nucleus is mediated by apoptotic factors including Akt as inhibitors of Akt increase the accumulation of nuclear GSK3 which effects the ability of GSK3 to modulate substrates such as cyclin D1 (Bijur and Jope, 2001).

Activation of GSK3 by Akt requires recruitment of Akt to the plasma membrane by PI (3,4,5)P3 and PI (3,4) which have a high affinity for the PH domain of Akt (Burgering and Coffer, 1995; Franke et al., 1995). Recruitment of Akt to the plasma membrane facilitates the activation of Akt via phosphorylation of two regulatory (Thr. 308) and (Ser. 473) sites (Alessi et al., 1996). Akt (Thr. 308) lies within the T loop of the kinase and its phosphorylation is catalyzed by PDK-1 (Alessi et al., 1997; Stephens et al., 1998). Akt (Ser. 473) is located within a hydrophobic region near the carboxyl terminus of the kinase (Alessi et al., 1996), yet the mechanism by which this residue is phosphorylated is currently unknown.

Regulation of GSK3 activity is critical in that GSK3 phosphorylates more than 40 substrates (Jope and Johnson, 2004). Interestingly, GSK3 regulates a number of transcription factors involved in gene expression including cyclic AMP response element binding protein (CREB) which serves an important role in the addictive properties of drugs of abuse (Carlezon et al., 1998). CREB regulates many critical processes, such as formation of long-term memory, maintenance of synaptic plasticity, and apoptosis (Struthers et al., 1991; Davis et al., 1996; Deisseroth et al., 1996; Silva et al., 1998; Bevilaqua et al., 1999; Shaywitz and Greenberg, 1999). The role of CREB in addiction has been extensively investigated as repeated exposure to stimulant drugs increases activity of the cAMP-PKA pathway within the nucleus accumbens (Terwillinger et al., 1991). In addition, direct activation of PKA activity, which increases CREB phosphorylation within the nucleus accumbens, reduces the rewarding effects of cocaine, whereas PKA inhibition has the opposite effect (Self et al., 1998). Given that PKA inhibits GSK3 (Fang et al., 2000) and inhibition of GSK3 activates CREB (Grimes and Jope, 2001), GSK3 may be important in mediating cocaine-induced behaviors.

GSK3: A Target for Therapeutics and Dopaminergic Transmission

In addition to its regulation by kinases, GSK3 has recently gained attention as a potential target in the treatment of bipolar disorder and schizophrenia. Lithium and valproate, therapeutics used as mood stabilization agents to combat bipolar disorder, have been shown to decrease the activity of GSK3 (Bowden et al., 1994). Previous investigations suggest that the reduction of GSK3 activity by lithium stems from a competitive inhibition with Mg^{++}, which reduces the catalytic activity of GSK3 independent of any changes in phosphorylation (Ryves and Harwood, 2001). Lithium has since, however, been shown to decrease GSK3 activity in vivo by increasing the phosphorylation of the α and β isoforms acutely in the striatum (Beaulieu et al., 2004) and chronically in the cerebral cortex and hippocampus (DeSarno et al., 2002) of mice. The increased phosphorylation of GSK3 by lithium is contingent upon the activation of PI3K and Akt by lithium (Chalecka-Franaszek and Chuang, 1999; DeSarno et al., 2002).

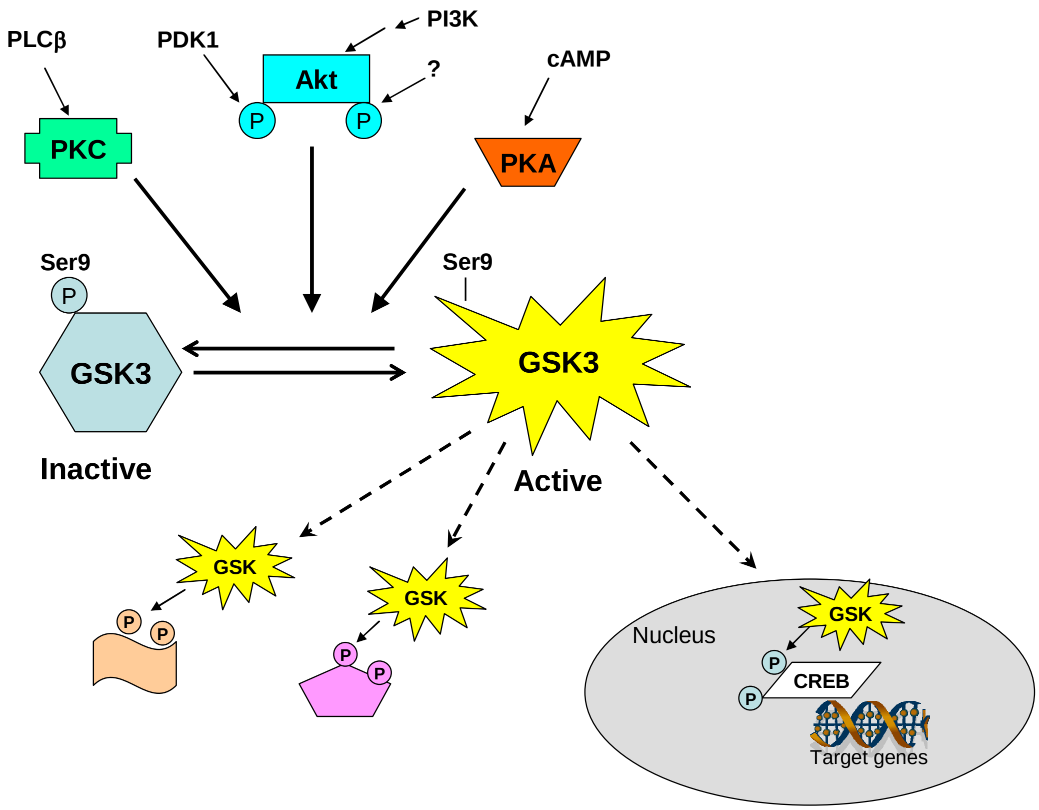

Figure 1.3: Regulation of GSK3 activity by phosphorylation by multiple kinases. A number of intracellular signaling proteins regulate the phosphorylation and subsequent activity of GSK3. Activation/inhibition of GSK3 regulates intracellular signaling proteins and transcription factors such as CREB. PKA (protein kinase A), Akt (protein kinase B), PKC (protein kinase C), GSK3 (glycogen synthase kinase-3), CREB (cyclic AMP dependent response element binding protein).

Valproate is an anticonvulsant and mood stabilization agent used to treat a number of disease states including epilepsy and mania (Jeavons and Clark, 1974; Bowden et al., 1994). Previous in vitro evidence suggests that exposure of neuroblastoma SH-SY5Y cells to valproate inhibits GSK3 by increasing the phosphorylation of the upstream kinase Akt and subsequently the phosphorylation of GSK3 (DeSarno et al., 2002). Valproate also regulates GSK3 phosphorylation in vivo as it protects against hypoxia-induced serine-9 dephosphorylation of GSK3β in the cortex, hippocampus, and striatum of mice (Roh et al., 2005).

GSK3 has also been implicated as critical to the pathophysiology of disease states including schizophrenia (Lovestone et al., 2007). In postmortem frontal cortex samples, subjects with schizophrenia exhibit approximately 40% lower GSK3β mRNA levels (Kozlovsky et al., 2004), GSK3β protein levels (Kozlovsky et al., 2000) and kinase activity (Kozlovsky et al., 2001) as compared to controls. These results are consistent with the studies showing that GSK3 and Akt protein levels in postmortem frontal cortex samples of schizophrenic patients are lower than controls (Emamian et al., 2004).

Similar to lithium and valproate, therapeutics used in the treatment of schizophrenia regulate the activity of Akt and GSK3. The antipsychotic and dopamine D2 receptor antagonist haloperidol increases the phosphorylation of pAkt (Thr. 308) without changing pAkt (Ser. 473) acutely (Emamian et al., 2004). In addition to haloperidol, atypical antipsychotics such as risperidone and clozapine that show binding affinity to both the dopamine D2 and serotinergic 5-HT2A receptors (Meltzer et al., 1989; Schotte et al., 1996) increase Akt and GSK3 phosphorylation in vitro and in vivo (Kang et al., 2004; Li et al., 2007), therefore inhibiting GSK3. Genetic manipulation of dopamine receptors also modulates the activity of GSK3 as well as Akt. Mice lacking the dopamine D2 receptor display an increase in phosphorylated Akt (Thr. 308) and GSK3 (Ser. 9) and a subsequent decrease in Akt (Ser. 473) in the striatum (Beaulieu et al., 2007). Further, mice lacking the dopamine D3 receptor show a similar increase in phosphorylated Akt (Thr. 308) and GSK3 (Ser. 9) yet display no change in Akt (Ser. 473) phosphorylation in the striatum (Beaulieu et al., 2007).

In studying GSK3 as a potential target for the treatment of disease, selectivity of inhibitors is a key issue if GSK3 inhibitors are to be used as a pharmacological tool in which to investigate the role of GSK3 in cellular processes (Meijer et al., 2004). Lithium, valproate, and antipsychotics effect the functioning of a number of receptors and intracellular signaling proteins. However, several selective pharmacological inhibitors of GSK3 have been developed. For example, the malemide derivative SB 216763 reversibily inhibits GSK3 in an ATP-competitive manner with a Ki of 9 nM (Coghlan et al., 2000; Lockhead et al., 2001). SB 216763 shows little or no inhibition of kinases upstream of GSK3 including PDK-1, PKA and Akt (Coghlan et al., 2000) and therefore is a useful pharmacological tool in which to investigate the role of GSK3 in behaviors associated with drugs of abuse and other diseases (Parkitna et al., 2006).

Psychostimulant-Induced Regulation of Akt/GSK3

Previous studies have highlighted the importance of dopamine and dopaminergic receptor manipulation on the regulation of Akt and GSK3. Interestingly, the phosphorylation of Akt/GSK3 has a distinct temporal and spatial pattern of regulation contingent upon drug and pattern of administration. For example, administration of the indirect dopaminergic agonist amphetamine results in a time-dependent attenuation of pAkt (Thr. 308) and pGSK3α/β (Ser. 21/9) (Beaulieu et al., 2004) with no change in pAkt (Ser. 473) in the striatum of wild-type mice (Beaulieu et al., 2005). Mice lacking the dopamine transporter, which exhibit an elevation in extracellular striatal dopamine (Giros et al., 1996), also show a decrease in striatal pAkt (Thr. 308) levels 60 minutes following acute administration of methylphenidate (Beaulieu et al., 2006). These studies differ from those of Svenningsson and colleagues (Svenningsson et al., 2003), who show increases in phosphorylated GSK3β in the frontal cortex and striatum 15 minutes post-amphetamine injection. Rats sensitized to the locomotor stimulating effects of amphetamine also display a unique temporal pattern of striatal pAkt (Thr. 308) regulation following an amphetamine challenge. Rats sensitized to amphetamine show an increase in striatal pAkt (Thr. 308) 15 minutes following an amphetamine challenge and a subsequent decrease in pAkt (Thr. 308) 120 minutes following amphetamine as compared to rats with no amphetamine history (Shi and McGinty, 2007). Binge pattern administration of cocaine also regulates Akt and GSK3 phosphorylation in select brain regions. Phospho-Akt (Thr. 308) and pGSK3α/β levels are significantly reduced in the amygdala of rats following 14-day binge-pattern cocaine administration (Perrine et. al., 2008). In addition, 1-day of binge-pattern cocaine administration increases pAkt (Thr. 308) levels in the amygdala but decreases pAkt (Thr. 308) in the nucleus accumbens with no change in phosphorylated GSK3 in these brain regions. This indicates that the Akt/GSK3 signaling cascade is regulated in a distinct temporal and spatial pattern.

The Role of the Akt/GSK3 Signaling Pathway in Psychostimulant-Induced Behavioral Repsonses

Recent evidence suggests that the Akt/GSK3 signaling cascade is critical to psychostimulant induced hyperactivity. Beaulieu and colleagues (2004) show that heterozygote GSK3β mice display an attenuated response to acute amphetamine. In addition, selective and non-selective inhibitors of GSK3 attenuate locomotor hyperactivity associated with elevated extracellular striatal dopamine in mice lacking the dopamine transporter (Beaulieu et al., 2004). Administration of the selective GSK3 inhibitors SB 216763 and AR-A014418 also reduce the hyperactivity in mice (Kozikowski et al., 2007) and rats (Gould et al., 2004) produced by amphetamine. Non-selective inhibitors of GSK3 such as lithium (DeSarno et al., 2002) and valproate (Chen et al., 1999) attenuate the hyper-locomotor phenotype of mice lacking the dopamine transporter (Beaulieu et al., 2004). Previous studies show that valproate can also attenuate the development but not the expression of methamphetamine- and cocaine-induced behavioral sensitization in mice (Li et al., 2005). Further, valproate prevented both the development and expression of methylphenidate sensitization (Yang et al., 2000a, b). Interestingly, intracerebroventricular administration of the PI3K inhibitor LY294002 during the initiation phase of cocaine sensitization blocks the expression of locomotor sensitization in rats (Izzo et al., 2002). To date, investigations as to the role of Akt and GSK3 in psychostimulant-induced hyperactivity and sensitization have focused primarily on non-selective inhibition of GSK3. As such, investigations focusing on the role of selective inhibition GSK3 on cocaine-induced hyper-locomotion are warranted.

General Summary of Objectives

Cocaine induces hyperactivity and conditioned reward responses through dopaminergic and glutamatergic receptors. Previous studies have also highlighted the importance of intracellular signaling proteins in modulating cocaine-induced behaviors. The protein kinase GSK3 is critical to hyperactivity as transgenic mice expressing a constitutively active mutated form of GSK3β exhibit an increase in locomotor activity in response to a novel environment as compared to wild-type controls (Prickaerts et al., 2006). GSK3 also mediates hyperactivity responses associated with increased extracellular dopamine levels. This has been investigated using genetic and pharmacologically manipulated animal models. For example, selective and non-selective inhibition of GSK3 in mice lacking the dopamine transporter that exhibit elevated striatal extracellular dopamine (Giros et al., 1996) attenuates the hyper-locomotor response exhibited in these animals (Beaulieu et al., 2004). Inhibition of GSK3 also attenuates amphetamine-induced hyper-locomotion in mice (Kozikowski et al., 2007) and rats (Gould et al., 2004). In addition to the role of GSK3 in dopamine-mediated hyperactivity, previous studies indicate that dopaminergic receptors regulate both GSK3 and the upstream kinase Akt. Mice lacking the dopamine D2 receptor display an increase in pAkt (Thr. 308) and pGSK3 (Ser. 9) and a decrease in pAkt (Ser. 473) (Beaulieu et al., 2007). Further, mice lacking the dopamine D3 receptor show a similar increase in phosphorylated Akt (Thr. 308) and GSK3 (Ser. 9) yet display no change in Akt (Ser. 473) phosphorylation in the striatum (Beaulieu et al., 2007).

We chose to investigate the role of GSK3 in cocaine-induced behaviors. We hypothesized that pharmacological inhibition of GSK3 would attenuate acute cocaine-induced hyper-locomotion and the development of locomotor sensitization. Further, we wished to characterize the importance of GSK3 in dopamine D1 receptor agonist-induced locomotion. An additional focus of this research was to characterize the acute effect of cocaine on Akt and GSK3 phosphorylation in the caudate putamen, nucleus accumbens and frontal cortex. Given the importance of dopaminergic and glutamatergic receptors in the behavioral and neuromodulatory effects of cocaine, we also investigated whether pharmacological inhibition of these receptors mediates cocaine-induced regulation of Akt and GSK3. Although numerous studies have elucidated the neural circuity and receptor types underlying cocaine conditioned reward, to date, investigations identifying the importance of GSK3 in cocaine conditioned reward are lacking. As such, an additional goal of this project was to characterize the role of GSK3 in cocaine-conditioned reward retrieval and reinstatement. Identification of GSK3 as a molecular target in which to combat the development of cocaine conditioned reward and drug-seeking behaviors will provide new insight as to the mechanisms underlying cocaine addiction.

CHAPTER 2

COCAINE-INDUCED HYPERACTIVITY AND SENSITIZATION ARE DEPENDENT ON GSK3

Introduction

Cocaine is a highly abused psychostimulant with repeated use potentially culminating in drug addiction. Elucidating the molecular mechanisms underlying acute/occasional drug use and repeated drug taking is essential for understanding addiction. As such, the aim of the present study was to investigate the role of the intracellular signaling protein, glycogen synthase kinase 3 (GSK3) on behaviors associated with acute and repeated cocaine administration.

Cocaine is a monoamine transporter inhibitor therefore blocking the reuptake of dopamine, serotonin and norepinephrine into presynaptic neurons resulting in enhanced synaptic levels of these neurotransmitters (Heikkila et al., 1975). Dopaminergic cell bodies originate in the ventral tegmental area and the substantia nigra and project to the nucleus accumbens and caudate putamen, respectively. Dopamine has a functional role in reward processes, both to natural reinforcers and addictive drugs (Koob, 1992). In addition, the importance of dopaminergic transmission in the locomotor-stimulating-effects of cocaine is well established (Kelly and Iversen, 1976; Kalivas et al., 1988), with repeated cocaine administration eliciting a sensitized or increased response to the locomotor-stimulating properties of the drug (Post and Rose, 1976; Robinson and Berrridge, 1993).

It is well established that both acute and repeated cocaine administration alter dopaminergic neurotransmission (Nestler, 2004). Thus, we chose to investigate GSK3, which has recently gained attention as a kinase that may be critical in both the behavioral and neurochemical underpinnings of dopaminergic signaling (Beaulieu et al., 2004). There is widespread expression of GSK3 in the adult brain, suggesting a fundamental role for this kinase in neuronal signaling pathways (Leroy and Brion, 1999) and its activity is regulated by a number of kinases such as Akt (protein kinase B), with inactivation of GSK3 occurring via phosphorylation at the serine-21 (α- isoform) and serine-9 (β-isoform) residues (Grimes and Jope, 2001). Interestingly, therapeutics used in the treatment of mood disorders and schizophrenia such as lithium, valproate and haloperidol affect GSK3. A therapeutically-relevant dosing regimen of lithium over 4 weeks increases the phosphorylation of the inhibitory serine-9-residue of GSK3β in mouse brain (De Sarno et al., 2002). Likewise, administration of the D2 receptor antagonist and antipsychotic agent haloperidol increases the phosphorylation of serine-9 GSK3β in the rodent brain (Emamian et al., 2004). Valproate also inhibits GSK3β via phosphorylation of the serine-9 residue in neuroblastoma SH-SY5Y cells (De Sarno et al., 2002) and protects against hypoxia-induced serine-9 dephosphorylation of GSK3β in the cortex, hippocampus, and striatum of mice (Roh et al., 2005). Moreover, valproate or specific inhibitors of GSK3 attenuate the increased horizontal activity associated with enhanced extracellular dopamine in dopamine transporter knockout mice (Beaulieu et al. 2004).

Based on previous studies indicating the importance of GSK3 in the regulation of dopamine-dependent behaviors, we investigated the role of this kinase in cocaine-induced activity and locomotor sensitization.

Methods

Animals

Male CD-1 mice (8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed five per plastic cage (28 x 18 x 14 cm) without additional enrichment objects in a temperature- and relative humidity-controlled room with a 12-hr light/dark cycle (lights on at 7:00 a.m.). Animals were housed for seven days prior to behavioral testing and were handled and weighed daily. All animals had access to standard laboratory chow and tap water *ad libitum*. All animal testing was conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and with an approved protocol from Temple University Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrochloride, generously supplied by the National Institute on Drug Abuse, and valproate (Sigma; St. Louis, MO) were dissolved in sterile saline (0.9% NaCl). SB 216763 (Tocris; Ellisville, MO) was dissolved in propylene glycol and brought up to volume in distilled water (70:30). Sterile saline (0.9% NaCl) or 70% propylene glycol were used for control injections.

Behavioral Testing/Drug Administration

All animals were placed in locomotor activity monitors for 30 minutes prior to drug administration and testing. Following the 30 minute habituation period, mice were pretreated with saline or valproate (50-300 mg/kg, i.p.) (Beaulieu et al., 2004) followed by an injection of saline or cocaine (20 mg/kg, i.p.) 45 minutes later. Separate sets of mice were pretreated with vehicle or SB 216763 (0.25-7.5 mg/kg, i.p.) (Beaulieu et al., 2004) followed by an injection of saline or cocaine (20 mg/kg, i.p.) 5 minutes later. Activity was measured for 60 minutes following the second injection using the Digiscan

DMicro (Accuscan, Inc., Columbus, OH) system. The activity monitors consist of transparent plastic boxes (45 x 20 x 20 cm) set inside metal frames that are equipped with 16 infrared light emitters and detectors. The number of photocell beam breaks are recorded by a computer interface. Ambulation was recorded as consecutive beams breaks resulting from horizontal movement, while stereotypy was recorded by repetitive beam breaks.

Behavioral Sensitization

Mice were pretreated with vehicle or SB 216763 (2.5 mg/kg, i.p.) followed five minutes later by a second injection of saline or cocaine (20 mg/kg, i.p.). This was repeated once a day for five days. After treatment day 5, animals were left drug-free for 7 days. On day 13, all animals were challenged with cocaine (20 mg/kg, i.p.) in the absence of SB 216763 and activity was recorded for 60 minutes.

Data Analysis

Behavioral data were analyzed using two-way ANOVA with pre-treatment and treatment factors followed by a Bonferroni test for multiple comparisons (GraphPad Prism 4, La Jolla, CA). EC50 values were determined using nonlinear regression as the mean effect vs. dose (Tallarida, 2000).

Results

Acute cocaine-induced activity was attenuated by valproate

Cocaine-induced ambulatory and stereotypic activity was measured following pretreatment with valproate (50-300 mg/kg, i.p.). The data displayed in Figure 2.1 represent cumulative ambulatory or stereotypy counts over 60 minutes for animals in each experimental group. Two-way ANOVA of the ambulatory data showed significant interaction, pretreatment and treatment effects (Interaction:$F(3,54)=4.348$, $p=0.0081$; Pre-treatment:$F(3,54)=4.350$, $p=0.0081$; Treatment:$F(1,54)=34.24$, $p<0.0001$). Bonferroni post-hoc analysis revealed that pretreatment with valproate at doses of 150 and 300 mg/kg significantly attenuated cocaine-induced ambulation (*$p<0.05$; ***$p<0.001$ sal/coc vs. valproate/coc). Valproate, 50 mg/kg, did not significantly attenuate cocaine-induced ambulation ($p>0.05$). Nonlinear regression analysis of the mean effect versus dose yielded an EC50 value of 186 mg/kg ± 17mg/kg for valproate in attenuating cocaine-induced ambulation.

The data displayed in Figure 2.1 also show cumulative stereotypy counts over 60 minutes. Two-way ANOVA of the stereotypy data showed significant interaction, pretreatment and treatment effects (Interaction:$F(3,54)=3.170$, $p=0.0315$; Pre-treatment:$F(3,54)=3.845$, $p=0.0144$; Treatment:$F(1,54)=24.44$, $p<0.0001$). Bonferroni post-hoc analysis revealed that pretreatment with valproate, 300 mg/kg, significantly attenuated cocaine-induced stereotypy (***$p<0.001$; sal/coc vs. valproate/coc). Acute cocaine-induced stereotypic activity was not significantly changed following pretreatment of valproate, 50 or 150 mg/kg, ($p>0.05$). Nonlinear regression analysis of the mean effect versus dose yielded an EC50 value of 186 mg/kg ± 15mg/kg for valproate in attenuating cocaine-induced stereotypy. Valproate alone had no effect on ambulation or stereotypy ($p>0.05$).

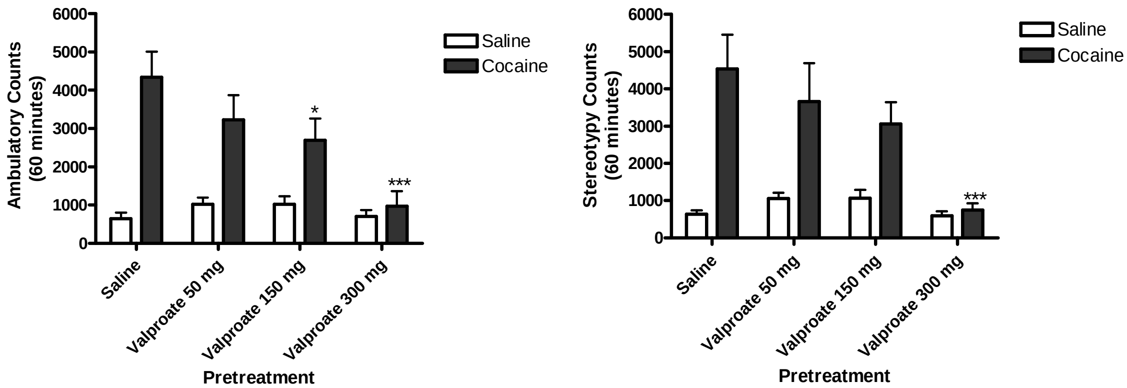

Figure 2.1: Valproate attenuated the acute behavioral stimulating effects of cocaine. Adult male CD-1 mice were pretreated with saline or valproate (50-300 mg/kg, i.p.) 45 minutes prior to an injection of cocaine (20 mg/kg, i.p.) or saline. Ambulatory and stereotypic activity was measured for 60 minutes following cocaine or saline injection. Pretreatment of mice with valproate (150 or 300 mg/kg, i.p.) significantly attenuated cocaine-induced ambulatory activity while pretreatment with valproate (300 mg/kg, i.p.) significantly attenuated cocaine-induced stereotypy. Valproate when administered with saline yielded no significant effect on ambulatory or stereotypic activity at any dose. All data were analyzed by a two-way ANOVA and Bonferroni post-hoc analysis (*$p<0.05$; ***$p<0.001$; vs. sal/coc). Data points represent mean ± SEM of cumulative ambulatory or stereotypy counts over 60 minutes (n=7-11/group).

Inhibition of GSK3 attenuated acute cocaine-induced activity

Acute cocaine-induced ambulatory and stereotypic activity was measured following pretreatment with the GSK3 inhibitor SB 216763 (0.25-7.5 mg/kg, i.p.). The data displayed in Figure 2.2 represent cumulative ambulatory or stereotypy counts over 60 minutes. Two-way ANOVA indicated significant interaction, pretreatment, and treatment effects of SB 216763 on cocaine-induced ambulatory activity (Interaction:$F(6,104)=7.621$, $p<0.0001$; Pretreatment:$F(6,104)=10.31$, $p<0.0001$; Treatment:$F(1,104)=39.78$, $p<0.0001$). Bonferroni post-hoc analyses indicated that pretreatment with SB 216763 at doses of 1.0-7.5 mg/kg significantly attenuated cocaine-induced ambulatory activity (***$p<0.001$; veh/coc vs. SB 216763/coc). Nonlinear regression analysis of the mean effect versus dose yielded an EC50 value of 1.21 mg/kg ± 0.176mg/kg for SB 216763 in attenuating cocaine-induced ambulation. Acute cocaine-induced ambulatory activity was not significantly changed following pretreatment with the lower doses of SB 216763 (0.25-0.5 mg/kg) ($p>0.05$). In addition, SB 216763 when administered with saline had no effect on ambulatory activity ($p>0.05$).

The data displayed in Figure 2.2 also show the cumulative stereotypy counts over 60 minutes. Two-way ANOVA analysis indicated significant interaction, pretreatment and treatment effects of SB 216763 (Interaction:$F(6,103)=8.980$, $p<0.0001$; Pretreatment:$F(6,103)=11.18$, $p<0.0001$; Treatment:$F(1,103)=43.48$, $p<0.0001$). Bonferroni post-hoc analysis revealed that pretreatment with SB 216763 at doses of 0.5-7.5 mg/kg significantly attenuated cocaine-induced stereotypic activity (*$p<0.05$; ***$p<0.001$; veh/coc vs. SB 216763/coc). Nonlinear regression analysis of the mean effect versus dose yielded an EC50 value of 1.28 mg/kg ± 0.210mg/kg for SB 216763 in attenuating cocaine-induced stereotypy. Acute cocaine-induced stereotypic activity was not significantly changed following pretreatment with SB 216763 (0.25 mg/kg) ($p>0.05$). Further, SB 216763 when administered with saline had no effect on stereotypic activity at any dose tested ($p>0.05$).

Inhibition of GSK3 prevented the development of cocaine-induced locomotor sensitization in mice

Adult male CD-1 mice were pretreated with vehicle or SB 216763 (2.5 mg/kg) prior to daily administration of cocaine (20 mg/kg) for 5 days. All animals were drug-free for 7 days and subsequently challenged with cocaine (20 mg/kg) on day 13 in the absence of SB 216763, and activity was monitored for 60 minutes (Figure 2.3). Two-way ANOVA analysis indicated significant interaction and pretreatment effects (Interaction:$F(1,25)=6.320$, $p=0.0187$; Pretreatment:$F(1,25)=6.978$, $p=0.0140$; Treatment:$F(1,25)=1.119$, $p=0.3002$). Bonferroni post hoc analysis indicated that animals administered daily cocaine had higher activity counts following a cocaine challenge as compared to those given daily saline, indicating the development of sensitization (*$p<0.05$; veh/sal vs. veh/coc). In addition, animals injected with SB 216763 plus cocaine for 5 days had significantly lower activity after the cocaine challenge than those pretreated with vehicle followed by cocaine (**$p<0.01$; veh/coc vs. SB/coc), indicating that SB 216763 blocked the development of behavioral sensitization.

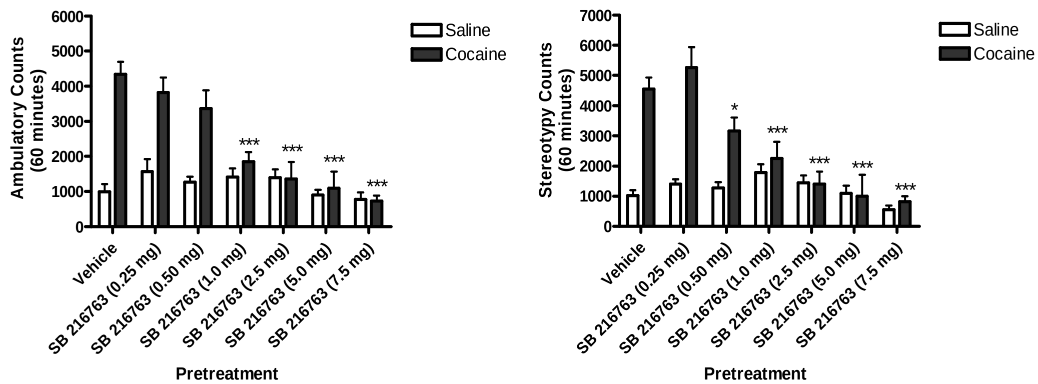

Figure 2.2: Inhibition of GSK3 attenuated the acute behavioral stimulating effects of cocaine. Adult male CD-1 mice were pretreated with vehicle or the selective GSK3 inhibitor SB 216763 (0.25-7.5 mg/kg) 5 minutes prior to injection of cocaine (20 mg/kg, i.p.) or saline. Ambulatory and stereotypic activity was measured for 60 minutes following cocaine or saline injection. Mice pretreated with SB 216763 (1.0-7.5 mg/kg) exhibited a significant attenuation of cocaine-induced ambulation. Likewise, pretreatment with SB 216763 (0.5-7.5 mg/kg) significantly attenuated stereotypic activity. SB 216763 alone had no effect on either ambulatory or stereotypic activity. Data were analyzed by a two-way ANOVA and Bonferroni post-hoc analysis (*$p<0.05$, ***$p<0.001$ vs. veh/coc). Data points are represented as the means ± SEM of cumulative ambulatory or stereotypy counts over 60 minutes (n=6-17/group).

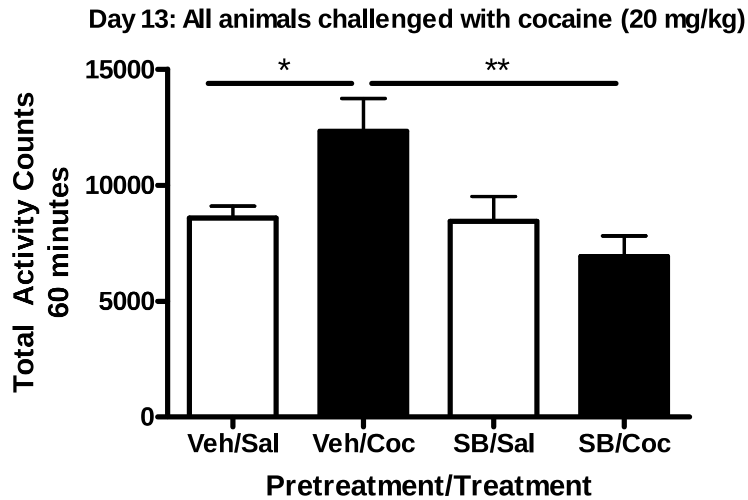

Figure 2.3: Inhibition of GSK3 prevented the development of cocaine-induced locomotor sensitization. Adult male CD-1 mice administered daily cocaine (20 mg/kg, i.p.) for five days exhibited a sensitized locomotor response to a cocaine challenge on day 13 as compared to vehicle treated controls (veh/sal vs. veh/coc, *p<0.05). Pretreatment with SB 216763 (2.5 mg/kg, i.p.) prior to daily cocaine attenuated cocaine-induced sensitization following a cocaine challenge (20 mg/kg, i.p.) on day 13 (veh/coc vs. SB/coc, **p<0.01). Data points represent mean ± SEM (n=6-8/group). Data were analyzed using a two-way ANOVA and Bonferroni post-hoc analysis.

Discussion

Our data support the hypothesis that GSK3 is critical to cocaine-induced behaviors. Here we show that valproate, which inhibits GSK3, and the selective inhibitor of GSK3 SB 216763 dose-dependently attenuated acute cocaine-induced hyperactivity. Further, GSK3 activity is necessary for the development of the sensitized locomotor response associated with repeated cocaine administration as selective inhibition of GSK3 prevented the development of cocaine-induced behavioral sensitization.

In the present study, pretreatment with valproate dose-dependently attenuated acute cocaine-induced ambulatory and stereotypic activity. Valproate is an anticonvulsant and mood stabilization agent used to treat a number of disease states including epilepsy and mania (Jeavons and Clark, 1974; Bowden et al., 1994). Valproate acts by enhancing the inhibitory actions of GABA via inhibition of GABA degradation, decreasing GABA turnover and increasing GABA synthesis (Owens and Nemeroff, 2003; Johannessen and Johannessen, 2003). Valproate is used clinically in combination with atypical and typical antipsychotics for the treatment of bipolar disorder and schizophrenia (Basan et al., 2004). Valproate is also a direct inhibitor of histone deacetylase (Gottlicher et al., 2001; Phiel et al., 2001) and has neuroprotective effects by suppressing apoptosis (Kanai et al., 2004). Valproate can also regulate the function of GSK3, as valproate has been shown to inhibit GSK3 activity in human neuroblastoma SHSY5Y cells (Chen et al., 1999). Valproate can alter behaviors induced by increases in extracellular dopamine, such as those seen in mice lacking the dopamine transporter. Dopamine transporter knockout mice have 5-fold higher levels of striatal synaptic dopamine (Giros et al., 1996). Administration of valproate to these mice attenuates their heightened ambulatory and stereotypic activity (Beaulieu et al., 2004). Although valproate can block dopamine-dependent behaviors, valproate alone does not significantly change extracellular dopamine levels in the nucleus accumbens or caudate putamen (Biggs et al., 1992). Previous studies have shown that valproate can attenuate the development but not the expression of methamphetamine- and cocaine-induced behavioral sensitization in mice (Li et al., 2005), as well as both the development and expression of methylphenidate sensitization (Yang et al., 2000a, b). While our studies demonstrating that valproate can attenuate acute cocaine-induced locomotion agree with previous studies with other psychostimulants (Li et al., 2005), it is difficult to ascertain whether the actions of valproate on such activity are the result of alterations in GABAergic functionality or perhaps inhibition of GSK3. As such, we chose to further evaluate the role of GSK3 on cocaine-induced behaviors using the specific GSK3 inhibitor SB 216763.

SB 216763 is a malemide derivative and inhibits GSK3 in an ATP-competitive manner with a Ki of 9 nM (Coghlan et al., 2000). Here, we show that pretreatment with SB 216763 dose-dependently attenuated cocaine-induced ambulatory and stereotypic activity. These results parallel previous studies highlighting the importance of the beta isoform of GSK3 in behaviors associated with psychostimulant administration or altered dopaminergic transmission. For example, SB 216763 dose-dependently attenuates the hyper-locomotion seen in dopamine transporter knockout mice (Beaulieu et al., 2004). Further, GSK3β heterozygote null mice display a decreased locomotor response to acute amphetamine as compared to wild-type controls (Beaulieu et al., 2004), whereas transgenic mice expressing a constitutively active mutated form of GSK3β exhibit an increase in locomotor activity in response to a novel environment as compared to wild-type controls (Prickaerts et al., 2006), thus giving further credence to the importance of GSK3β in dopamine-stimulated hyperactivity. These studies along with our data suggest that GSK3 is a mediator of the acute hyper-locomotor responses induced by psychostimulant administration, as selective inhibition of the kinase attenuates both acute amphetamine (Beaulieu et al., 2004) and cocaine-induced locomotion in mice (current study).

Although a number of studies indicate the importance of GSK3 in normal and acute psychostimulant-induced behaviors, molecular models encompassing how GSK3 regulates psychostimulant-induced behaviors have yet to be fully established. Previous studies suggest that GSK3 may be associated with a number of intracellular signaling cascades and dependent upon both temporal and spatial perturbations of specific neurotransmitters and receptor subtypes. For example, when focusing on the relationship between GSK3 and dopamine, acute administration of the indirect dopaminergic agonist amphetamine attenuates the Ser-9 phosphorylation of GSK3β in the mouse striatum 90 minutes post-injection, therefore activating the kinase (Beaulieu et al., 2004). In addition, striatal preparations from dopamine transporter knockout mice show a reduction in the inhibitory serine-9 phosphorylation of GSK3β (Beaulieu et al., 2004), suggesting that the kinase is regulated by extracellular dopamine. Further, immunoblotting analyses from the striatum of dopamine transporter knockout mice show a decrease in the phosphorylation of Akt (Beaulieu et al., 2004), an upstream kinase capable of regulating GSK3β phosphorylation. Intracerebroventricular injection of the cAMP analog 8-Br-cAMP to mice lacking the dopamine transporter increases the phosphorylation of DARPP-32 at Thr-34 without changing Akt or GSK3β phosphorylation (Beaulieu et al., 2004), suggesting that the relationship between dopamine and GSK3β may be independent of cAMP.

In addition to identifying a role for GSK3 in acute cocaine-induced behaviors, we also investigated the importance of GSK3 in the development of cocaine-induced behavioral sensitization. Identification of new molecular targets to prevent sensitized responses to drugs of abuse is salient in that the etiology of sensitization may model certain aspects of addictive behavior such as drug craving and potential relapse (Robinson and Berrridge, 1993). Here, we show that when daily cocaine administration is preceded by the GSK3 inhibitor SB 216763, the development of cocaine-induced locomotor sensitization is prevented. This is the first study indicating a role for GSK3 in the behavioral manifestations of repeated cocaine administration. The mechanism by which GSK3 prevents the development of cocaine-induced sensitization is currently unknown; however there are a number of neural and anatomical substrates underlying the development of cocaine-induced sensitization, one of which involves dopamine. For example, administration of the dopamine D1 receptor antagonist SCH-23390 prior to daily cocaine prevents the development of sensitization (McCreary and Marsden, 1993), and dopamine D1 receptor knockout mice do not show locomotor sensitization to cocaine as compared to wildtype controls (Karlsson et al., 2008). The development of cocaine sensitization can also be blocked by the dopamine D2 receptor antagonist haloperidol (Karler et al., 1994). These studies indicate the importance of dopamine receptors in the induction of cocaine sensitization. As administration of the D2 receptor antagonists haloperidol and raclopride inhibit GSK3 (Emamian et al., 2004; Beaulieu et al., 2004), it may be that D2 receptor antagonists interfere with cocaine sensitization by a mechanism involving GSK3 inhibition.

Another mechanism by which GSK3 may prevent the development of cocaine sensitization is by interfering with glutamatergic transmission. Animals sensitized to cocaine show an increase in extracellular glutamate in the core of the nucleus accumbens as compared to nonsensitized and saline pretreated animals (Pierce et al., 1996) and pretreatment with the NMDA receptor antagonist MK-801 prevents the development of cocaine sensitization in mice (Karler et al., 1989), therefore highlighting the importance of glutamate in cocaine-induced behavioral sensitization. Of note, stimulation of the NMDA receptor causes an activation of GSK3 via protein phosphatase-1 in the adult mouse brain (Szatmari et al., 2005), whereas inhibition of GSK3 reduces the whole cell current of the NMDA receptor in cortical pyramidal neurons and can cause NMDA receptor internalization (Chen et al., 2007). In addition, the NMDA receptor antagonist memantine can increase the inhibitory phosphorylation of the ser-9 residue of GSK3β in the cerebral cortex, striatum, and hippocampus of mice (De Sarno et al., 2006). This suggests that the cellular and behavioral manifestations associated with the development of cocaine sensitization may be due to an interaction between the NMDA receptor and GSK3β. Future studies to identify the specific intracellular signaling cascade involved in GSK3-induced regulation of cocaine sensitization and plasticity are warranted.

In summary, the results of our study indicate that activation of GSK3 is critical to the acute locomotor-stimulating effects of cocaine, as well as to the development of cocaine sensitization. Investigations determining the role of GSK3 in other facets of addiction such as drug-induced reward, relapse and reinstatement are currently underway in our laboratory. The importance of GSK3 in the behavioral and neurochemical adaptations associated with other drugs of abuse may yield important insights as to the role of GSK3 in addiction.

CHAPTER 3

INHIBITION OF GSK3 ATTENUATES DOPAMINE D1 RECEPTOR AGONIST-INDUCED LOCOMOTION IN MICE

Introduction

The behavioral and neuromodulatory effects of dopamine are mediated via D1-like and D2-like dopamine receptors, members of the G-protein coupled receptor family (Missale et al., 1998). The dopamine D1 receptor is highly expressed in striatal regions of the brain (Weiner et al., 1991; Mansour et al., 1991) and is coupled to stimulatory G-proteins (G_s/G_{olf}). Activation of dopamine D1 receptors results in the activation of adenylate cyclase and subsequent increases in cAMP (Kebabian et al., 1972; Sibley et al., 1993). Increases in cAMP following dopamine D1 receptor stimulation can regulate a number of intracellular signaling cascades via activation of protein kinase A and phosphorylation of cAMP response element binding protein (Konradi et al., 1994).

Previous studies have assessed the role of the dopamine D1 receptor in locomotor behaviors. Administration of the D1 receptor full-agonist SKF-82958 or partial agonist SKF-38393 increases locomotor activity in mice (Halberda et al., 1997; Desai et al., 2005). In addition, antagonism of the dopamine D1 receptor prevents cocaine-induced hyperactivity (Cabib et al., 1991). Thus, given the importance of dopamine D1 receptor stimulation in locomotion, the goal of the present study was to identify intracellular signaling molecules involved in locomotor activity produced by dopamine D1 receptor activation.

One kinase that has recently gained attention as being critical to the behavioral and neurochemical manifestations of dopamine and dopaminergic receptors is glycogen synthase kinase-3 (GSK3) (Beaulieu et al., 2004). GSK3 is widely expressed in the adult rat brain (Leroy and Brion, 1999) and is inactivated via phosphorylation of the serine 21 (α-isoform) or serine 9 (β-isoform) residues (Grimes and Jope, 2001). GSK3 is selectively inhibited by malemide derivatives such as SB 216763 (Coghlan et al., 2000). Inhibition of GSK3 by SB 216763 attenuates hyperactivity in mice lacking the dopamine transporter (Beaulieu et al., 2004). Inhibition of GSK3 with SB 216763 also attenuates acute cocaine-induced hyper-locomotion and prevents the development of cocaine-induced locomotor sensitization (Miller et al., 2009a). Based on previous studies indicating the importance of GSK3 in hyper-locomotor responses associated with dopamine, we investigated whether inhibition of GSK3 would alter ambulatory and stereotypy responses following direct dopamine D1 receptor stimulation.

Methods

Animals

Male CD-1 mice (8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed five per plastic cage (28 x 18 x 14 cm) without additional enrichment objects in a temperature- and relative humidity-controlled room with a 12-hr light/dark cycle (lights on at 7:00 a.m.). Animals were housed for seven days prior to behavioral testing and were handled and weighed daily. All animals had access to standard laboratory chow and tap water *ad libitum*. All animal testing was conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and with an approved protocol from Temple University Institutional Animal Care and Use Committee.

Drugs

(±)-SKF-82958 ((±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetra-hydro-1H-3-benzazepine HBr) (O'Boyle et al., 1989) (Sigma; St. Louis, MO) was dissolved in sterile saline (0.9% NaCl). SB 216763 (Tocris; Ellisville, MO) was dissolved in propylene glycol and brought up to volume in distilled water (70:30). Propylene glycol (70%) was used for control injections.

Behavioral Testing/Drug Administration

Animals were placed in locomotor activity monitors for 30 minutes prior to drug administration and testing. Following the 30 minute habituation period, mice were pretreated with vehicle or SB 216763 (0.25-7.5 mg/kg, i.p.) followed by an injection of saline or SKF-82958 (1.0 mg/kg, i.p.) 5 minutes later. Activity was measured for 30 minutes following the second injection using the Digiscan DMicro (Accuscan, Inc., Columbus, OH) system. The activity monitors consist of transparent plastic boxes (45 x 20 x 20 cm) set inside metal frames that are equipped with 16 infrared light emitters and detectors. The number of photocell beam breaks is recorded by a computer interface. Ambulation was recorded as consecutive beams breaks resulting from horizontal movement, while stereotypy was recorded by repetitive beam breaks.

Data Analysis

Behavioral data were analyzed using two-way ANOVA with pre-treatment and treatment factors followed by a Bonferroni test for multiple comparisons (GraphPad Prism 4, La Jolla, CA). EC50 values were determined using nonlinear regression as the mean effect vs. dose (Tallarida, 2000).

Results

Inhibition of GSK3 attenuated acute SKF-82958-induced ambulation

Ambulatory activity was measured following pretreatment with the GSK3 inhibitor SB 216763 (0.25-7.5 mg/kg, i.p.) followed by an injection of SKF-82958 (1.0 mg/kg, i.p.). The data displayed in Figure 3.1 represent ambulatory counts recorded over 30 minutes. Two-way ANOVA indicated significant pretreatment and treatment effects of SB 216763 on SKF-82958-induced ambulatory activity (Interaction:$F(5,84)=2.077$, $p=0.0763$; Pretreatment: $F(5,84)=2.408$, $p=0.0432$; Treatment:$F(1,84)=91.11$, $p<0.0001$). Bonferroni post-hoc analyses indicated that SKF-82958 significantly increased ambulatory activity compared with saline-injected controls ($^{\#}p<0.001$; veh/sal vs. veh/SKF). Pretreatment with SB 216763 at doses of 0.25-7.5 mg/kg significantly attenuated SKF-82958-induced ambulatory activity (*$p<0.05$; **$p<0.01$ ***$p<0.001$; veh/SKF vs. SB 216763/SKF). Nonlinear regression analysis of the mean effect versus dose yielded an EC50 value of 0.19 mg/kg ± 0.13 mg/kg for SB 216763 in attenuating SKF-82958-induced ambulation. In addition, SB 216763 when administered with saline had no effect on baseline ambulatory activity ($p>0.05$; veh/sal vs. SB/sal).

Inhibition of GSK3 attenuated acute SKF-82958 induced stereotypy

The data displayed in Figure 3.2 represent stereotypy counts recorded over 30 minutes post SKF-82958 injection. Two-way ANOVA indicated significant pretreatment and treatment effects of SB 216763 (Interaction:$F(5,87)=1.090$, $p=0.3717$; Pretreatment:$F(5,87)=2.403$, $p=0.0432$; Treatment:$F(1,87)=51.85$, $p<0.0001$). Bonferroni post-hoc analysis revealed that SKF-82958 significantly increased stereotypy as compared with saline controls ($^{\#}p< 0.001$; veh/sal vs. veh/SKF). Administration of SB

216763 at doses of 1.0-7.5 mg/kg significantly attenuated SKF-82958-induced stereotypic activity (**$p<0.01$; ***$p<0.001$; veh/SKF vs. SB 216763/SKF). Nonlinear regression analysis of the mean effect versus dose yielded an EC50 value of 0.44 mg/kg ± 0.12 mg/kg for SB 216763 in attenuating SKF 82958-induced stereotypy. SKF-82958-induced stereotypic activity was not significantly changed following pretreatment with the lower doses of SB 216763 (0.25-0.50 mg/kg) ($p>0.05$). Administration of SB 216763 had no effect on baseline stereotypic activity at any dose tested ($p>0.05$; veh/sal vs SB/sal).

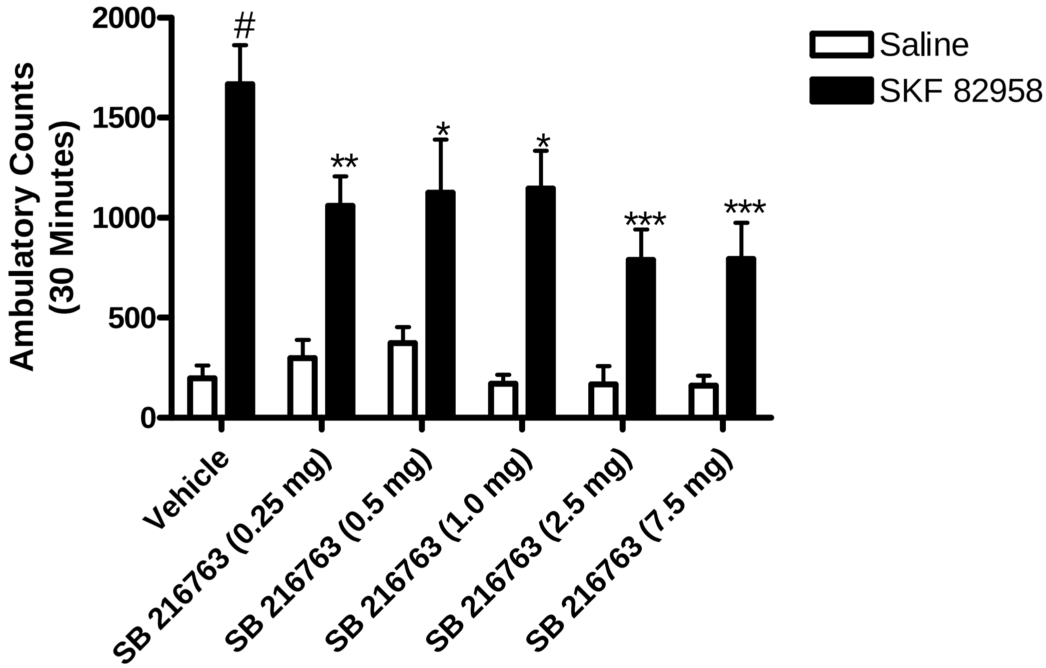

Figure 3.1: Inhibition of GSK3 attenuated dopamine D1 receptor-induced ambulation in mice. Adult male CD-1 mice were pretreated with vehicle or the selective GSK3 inhibitor SB 216763 (0.25-7.5 mg/kg, i.p.) 5 minutes prior to injection of SKF-82958 (1.0 mg/kg, i.p.) or saline. SKF-82958 significantly increased ambulatory activity as compared with saline controls ($^{\#}p < 0.001$; veh/sal vs. veh/SKF). Mice pretreated with SB 216763 (0.25-7.5 mg/kg) exhibited a significant attenuation of SKF 82958-induced ambulation. SB 216763 alone had no effect on baseline ambulatory activity. Data were analyzed by a two-way ANOVA and Bonferroni post-hoc analysis (*$p<0.05$, ***$p<0.001$ SB/SKF-82958 vs. veh/SKF- 82958). Data points represent the means ± SEM of cumulative ambulatory counts over 30 minutes (n=6-12/group).

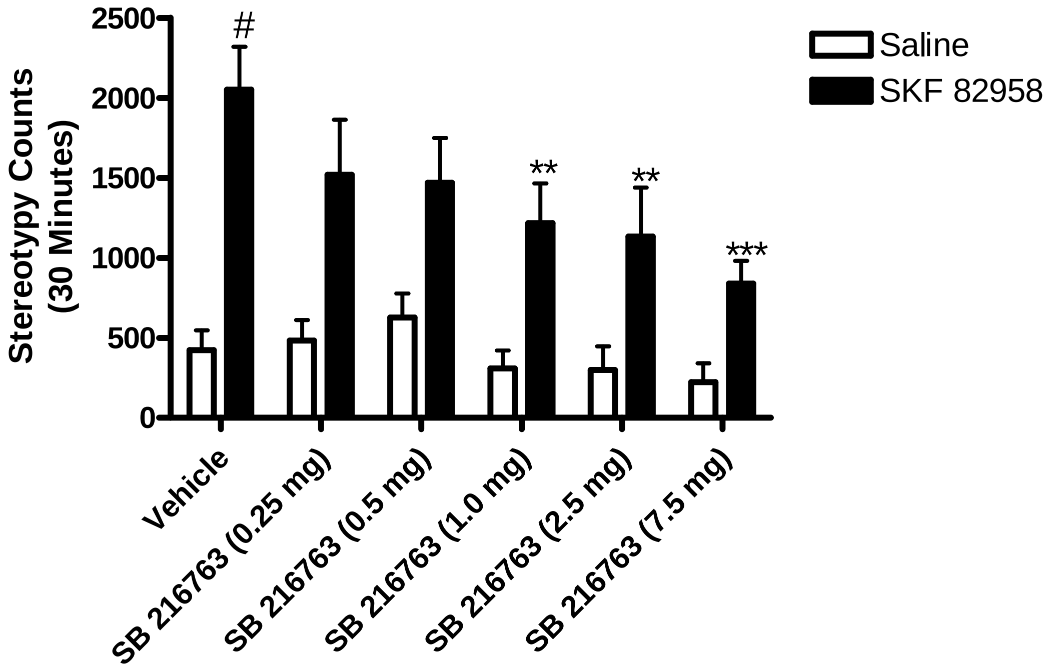

Figure 3.2: Inhibition of GSK3 attenuated dopamine D1 receptor-induced stereotypy in mice. Adult male CD-1 mice were pretreated with vehicle or the selective GSK3 inhibitor SB 216763 (0.25-7.5 mg/kg, i.p.) 5 minutes prior to injection of SKF-82958 (1.0 mg/kg, i.p.) or saline. SKF-82958 produced a significant increase in stereotypic activity as compared with saline controls ($^{\#}p < 0.001$; veh/sal vs. veh/SKF). Mice pretreated with SB 216763 (1.0-7.5 mg/kg) exhibited a significant attenuation of SKF 82958-induced stereotypy. SB 216763 alone had no effect on baseline stereotypic activity. Data were analyzed by a two-way ANOVA and Bonferroni post-hoc analysis (*$p<0.05$, ***$p<0.001$ SB/SKF-82958 vs. veh/SKF- 82958). Data points represent the means ± SEM of cumulative stereotypy counts over 30 minutes (n=6-13/group).

Discussion

Previous studies have implicated the importance of dopamine D1 receptor stimulation in animal models of hyperactivity. Our data parallel previous reports demonstrating that acute administration of the dopamine D1 receptor agonist SKF-82958 yields a hyperactive response in rodents (Desai et al., 2005; Meyer and Schults, 1993) consisting of increases in both ambulatory and stereotypic behaviors. The data presented herein are the first to demonstrate that GSK3 is critical to the increased ambulatory and stereotypy responses produced by stimulation of dopamine D1 receptors.

In the present study, pretreatment with SB 216763 attenuated dopamine D1 receptor-induced ambulation and stereotypy. SB 216763 inhibits GSK3 in an ATP-competitive manner with a Ki of 9 nM (Coghlan et al., 2000). This is the first study highlighting the importance of GSK3 in dopamine D1 receptor induced hyperactivity, although previous studies indicate the importance of GSK3 in behaviors associated with altered dopaminergic transmission. Administration of the selective GSK3 inhibitors SB 216763 and AR-A014418 reduce the hyperactivity in mice (Kozikowski et al., 2007) and rats (Gould et al., 2004) produced by the indirect dopaminergic agonist amphetamine. Further, heterozygote GSK3β knock-out mice display an attenuated response to acute administration of amphetamine as compared to wild-type controls (Beaulieu et al., 2004). Non-selective inhibitors of GSK3 such as lithium (DeSarno et al., 2002) and valproate (Chen et al., 1999) attenuate the hyper-locomotor phenotype of mice lacking the dopamine transporter (Beaulieu et al., 2004). These previous studies demonstrate the importance of GSK3 in dopamine-mediated hyperactivity, although they do not indicate which dopamine receptor(s) may be involved.

Our data indicate that inhibition of GSK3 attenuated dopamine D1 receptor-induced locomotion. Multiple lines of evidence suggest that GSK3 plays an important role in hyperactivity via dopamine receptors and the Akt/GSK3 signaling cascade in the striatum. Akt is an important intracellular kinase in that it negatively regulates GSK3 activity. Mice lacking the dopamine transporter demonstrate a persistent elevation of striatal extracellular dopamine (Giros et al., 1996) and show increases in GSK3 activity and concomitant decreases in Akt phosphorylation in the striatum (Beaulieu et al., 2004). Previous studies investigating the regulation of GSK3 activity by the dopamine D1 receptor are contradictory and suggest that regulation is contingent upon spatial and temporal perturbations of the receptor and kinases upstream of GSK3. For example, mice lacking the dopamine D1 receptor do not show changes in baseline GSK3β phosphorylation in the striatum (Beaulieu et al., 2007). Likewise, the increase in GSK3 activity seen in the striatum of mice lacking the dopamine transporter is not blocked by the dopamine D1 receptor antagonist SCH-23390 (Beaulieu et al., 2004). In contrast to those findings, it has been shown that stimulation of the dopamine D1 receptor with SKF-38393 increases Akt phosphorylation in striatal neurons, and overexpression of a dominant negative form of Akt inhibits CREB phosphorylation induced by dopamine D1 receptor stimulation (Brami-Cherrier et al., 2002). Our data provide support for the relationship between dopamine D1 receptors and the Akt/GSK3 signaling cascade as selective inhibition of GSK3 attenuated dopamine D1 receptor-induced hyperactivity.

Recent evidence suggests that dopamine D2 and D3 receptors can regulate GSK3 activity. Administration of the dopamine D2 receptor antagonist raclopride to dopamine transporter knock-out mice decreases GSK3 activity as evidenced by an increase in

GSK3 phosphorylation in the striatum (Beaulieu et al., 2004). Mice lacking the dopamine D2 receptor show higher levels of phosphorylation of Akt and GSK3β in the striatum than wild-type controls (Beaulieu et al., 2007). GSK3β and Akt phosphorylation levels are also increased in the striatum of mice lacking the dopamine D3 receptor (Beaulieu et al., 2007). This differs from studies investigating GSK3 and Akt activity in the limbic forebrain of dopamine D3 receptor mutant mice. In this case, D3 receptor mutants show an increase in basal GSK3 activity with no change in basal Akt in the limbic forebrain (Chen et al., 2007). Repeated administration of methamphetamine however results in an increase in GSK3 activity with no change Akt (Chen et al., 2007). Thus, activation or deactivation of GSK3 is contingent upon pharmacological and genetic manipulations of dopaminergic receptors in specific brain regions.

Previous data from our laboratory demonstrate that selective inhibition of GSK3 with SB 216763 reduced acute cocaine-induced ambulatory and stereotypic activity in mice with EC50 values of 1.21 mg/kg ± 0.18 mg/kg for ambulation and 1.28 mg/kg ± 0.21 mg/kg for stereotypy (Miller et al., 2009a). Further, SB 216763 (7.5 mg/kg) reduced cocaine-stimulated activity to baseline levels (Miller et al., 2009a). In the present study, 7.5 mg/kg SB 216763 attenuated SKF-82958-induced ambulation by about 53% and stereotypy by about 60%. This indicates that SB 216763 is more potent yet less effective at reducing dopamine D1 receptor-induced hyperactivity as compared to cocaine-induced hyperactivity. Taken together, these studies suggest that activation of GSK3 is critical to the hyper-motor response associated with increased extracellular dopamine and dopaminergic receptor stimulation as pharmacological (Miller et al., 2009a; Beaulieu et al., 2004; Kozikowski et al., 2007)) and genetic (Beaulieu et al., 2004) manipulations of GSK3 attenuate dopamine mediated hyperactivity.

The data presented herein demonstrate that administration of the selective GSK3 inhibitor SB 216763 attenuated the heightened ambulatory and stereotypy responses produced by selective dopamine D1 receptor stimulation. This suggests that a signaling pathway comprising GSK3 is activated by dopamine D1 receptor stimulation and is critical for the behavioral response to dopamine D1 receptor agonists. Given the behavioral and neurochemical implications of GSK3, further elucidation of the specific mechanisms by which this kinase functions to modulate dopaminergic behaviors is warranted.

CHAPTER 4

THE ROLE OF GSK3 IN COCAINE-CONDITIONED REWARD

Introduction

Cocaine is a psychostimulant that produces a number of behavioral and neurochemical modifications. Further, continued use of cocaine can lead to drug addiction, which is characterized by drug craving and relapse into compulsive drug-seeking behavior (Jaffe et al., 1989). Cocaine causes alterations in dopaminergic transmission via blockade of the dopamine transporter, therefore resulting in an increase in extracellular dopamine in the synapse (Heikkila et al., 1975). Specifically, cocaine-induced increases in synaptic dopamine levels within the mesolimbic dopamine pathway are associated with the rewarding properties of the drug (Kuhar et al., 1991). Cocaine also increases extracellular glutamate levels in the ventral tegmental area, nucleus accumbens, striatum and prefrontal cortex (Kalivas and Duffy, 1995; Smith et al., 1995; Reid and Berger, 1996; Reid et al., 1997).

The rewarding properties of cocaine have been tested extensively using animal models such as the place conditioning paradigm. Place conditioning measures the appetitive value of a drug (Tzschentke, 1998) and the conditioned response to a drug is proposed to model drug-seeking behavior and relapse (Robinson and Berridge, 2001). Relapse to drug seeking can be precipitated by exposure to the drug or by environmental cues previously associated with the drug (O'Brien et al., 1990; Childress et al., 1999). Following the acquisition of drug-associated memories, exposure to the previous conditioned stimulus in the absence of the unconditioned stimulus, reactivates previously learned memories resulting in reconsolidation or strengthening of the memory (Mactutus et al., 1979; Przybyslawski and Sara, 1997). During this process, however, memory traces are labile and can be manipulated pharmacologically (Nader et al., 2000; Valjent et al., 2006). Previous evidence indicates that the retrieval of cocaine-associated contextual memory cues can be altered by perturbations of specific intracellular signaling proteins and transcription factors (Miller and Marshall, 2005). As these cues can trigger relapse to drug-seeking behaviors, a therapeutic goal in the treatment of cocaine addiction is to interfere with the retrieval of cocaine-associated memories. As such, the goal of the present study was to establish a novel molecular target that can alter the development and retrieval of cocaine conditioned reward.

Glycogen synthase kinase-3 (GSK3) has gained attention as being an important molecular substrate involved in psychostimulant-induced behaviors. GSK3 is expressed in all tissues with abundant levels in the brain (Woodgett, 1990) and its activity is regulated via phosphorylation of the serine-21 (α-isoform) or serine-9 (β-isoform) residues (Grimes and Jope, 2001). Recent work has established the role of GSK3 in behaviors associated with dopaminergic (Alimohamad et al., 2005; Beaulieu et al., 2007) and glutamatergic transmission (Chen et al., 2007) and drugs of abuse (Beaulieu et al., 2004; Russo et al., 2007; Perrine et al., 2008; Miller et al., 2009a). Although there are a number of studies indicating the importance of GSK3 in the behavioral underpinnings associated with drugs of abuse, research focusing on the role of this kinase as it pertains to cocaine-conditioned reward is lacking. As such, we investigated whether inhibition of GSK3 would modulate the development, retrieval and reinstatement of cocaine-conditioned reward.

Methods

Animals

Male CD-1 mice (8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed five per Plexiglass cage (28 x 18 x 14 cm) without additional enrichment objects in a temperature and relative humidity-controlled room with a 12-hr light/dark cycle (lights on at 7:00 a.m. All animals had access to standard laboratory chow and tap water *ad libitum*. Animals were housed for five days prior to behavioral testing and were handled and weighed daily. All animal testing was conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and with an approved protocol from Temple University School of Medicine Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrochloride, (3 ml/kg) generously supplied by the National Institute on Drug Abuse, was dissolved in sterile saline (0.9% NaCl). SB 216763 (10 ml/kg) (Tocris; Ellisville, MO) was dissolved in propylene glycol and brought up to volume in distilled water (70:30). Sterile saline (0.9% NaCl) or 70% propylene glycol were used for control injections.

Development of Cocaine Conditioned Place Preference

A randomized unbiased conditioned place preference procedure was used. Conditioned place preference chambers were rectangular in shape (45 x 20 x 20 cm) and consisted of two compartments, separated by a removable door. One compartment had a smooth floor with white walls and vertical black stripes, while the other had a sandpaper floor and black walls. On days 1-4, mice were pretreated with vehicle or SB 21673 (2.5

mg/kg, i.p.) in their home cages. After 5 minutes, mice were injected with saline (3 ml/kg, i.p.) or cocaine (2.5-30 mg/kg, i.p.) and placed into alternate sides of the conditioning chamber for 30 minutes. This was repeated for 4 days with mice receiving 2 pairings with saline and 2 pairings with cocaine on alternate sides of the conditioning chamber. On test day (day 5), mice were given access to both sides of the conditioning chamber for 30 minutes in a drug-free state and time in each side was recorded. Preference scores were determined by subtracting the amount of time spent in the saline-paired compartment from the cocaine-paired compartment.

Retrieval of Cocaine Conditioned Place Preference

An unbiased conditioned place preference procedure was used. On days 1-8, mice were injected on alternating days with saline or cocaine (10 mg/kg, i.p.) and placed in alternate sides of the conditioning chamber. Place preference was assessed on day 9 (test day 1) with mice given access to both sides of the conditioning chamber in a drug-free state and time spent on each side recorded for 30 minutes. Four hours following testing for initial preference mice were injected with vehicle or SB 216763 (2.5 mg/kg, i.p.) and once again on day 10 in their home cages. On day 11 (test day 2), mice were once again tested for place preference being given free access to both sides of the conditioning chamber for 30 minutes in a drug-free state. The retrieval of place preference was assessed by subtracting the amount of time spent on the saline-paired compartment from the cocaine-paired compartment.

Reinstatement of Cocaine Conditioned Place Preference

An unbiased conditioned place preference procedure was used. On days 1-8, mice were injected with saline (3 ml/kg, i.p.) or cocaine (10 mg/kg, i.p.) and placed in alternate sides of the conditioning chamber for 30 minutes. Place preference was assessed on day 9 as described above. Following testing for initial preference, mice underwent extinction training on days 10-17 during which mice were injected with saline and placed into alternate sides of the conditioning chamber. On either days 10-11 or days 10-17, mice were injected with vehicle or SB 216763 (2.5 mg/kg, i.p.) in their home cages 4 hours after extinction training. On day 18, place preference was measured to assess extinction of the initial cocaine place preference. On day 19, reinstatement was tested by administration of cocaine (10 mg/kg, i.p.) and measurement of place preference.

Data Analysis

Data were analyzed using two-way ANOVA with pretreatment and treatment factors followed by Bonferroni test for multiple comparisons or by an unpaired two-tailed Student t-test as indicated (GraphPad Prism 4, La Jolla, CA).

Results

Inhibition of GSK3 prior to cocaine conditioning prevented the development of cocaine-induced place preference

Prior to testing the role of GSK3 in the development of cocaine conditioned place preference, we performed a dose-response study with cocaine to assess which dose of cocaine would produce the most robust preference. Figure 4.1 shows that 10 mg/kg of cocaine produced the strongest preference (<350 seconds) for the cocaine-paired side therefore 10 mg/kg of cocaine was used to test the effect of SB 216763 on the development of cocaine place preference. The role of GSK3 in cocaine-conditioned reward was evaluated by pretreatment with the selective GSK3 inhibitor SB 216763 prior to conditioning with cocaine in a conditioned place preference paradigm. Two-way ANOVA of the place preference data (Figure 4.2A) revealed a significant interaction and treatment effect (Interaction: $F(1,42)=6.829$, $p=0.0124$; Pretreatment: $F(1,42)=1.987$, $p=0.1661$; Treatment: $F(1,42)=4.977$, $p=0.0311$). Bonferroni post-hoc analysis indicated that animals conditioned with cocaine demonstrated a significant preference toward their cocaine-paired side as compared to saline controls (**$p<0.01$; veh/sal vs. veh/coc). Animals pretreated with SB 216763 five minutes prior to cocaine administration showed no preference toward their cocaine-paired side as compared to animals pretreated with vehicle prior to cocaine (**$p<0.01$; SB/coc vs. veh/coc). SB 216763 alone had no effect on place preference. The effects of SB 216763 on two other doses of cocaine were also tested. Figure 4.2B shows that there was no significant place preference in mice pretreated with SB 216763 (2.5 mg/kg, i.p.) followed by any dose of cocaine (2.5-30 mg/kg). Thus, selective inhibition of GSK3 prior to cocaine conditioning prevented the development of cocaine-induced place preference.

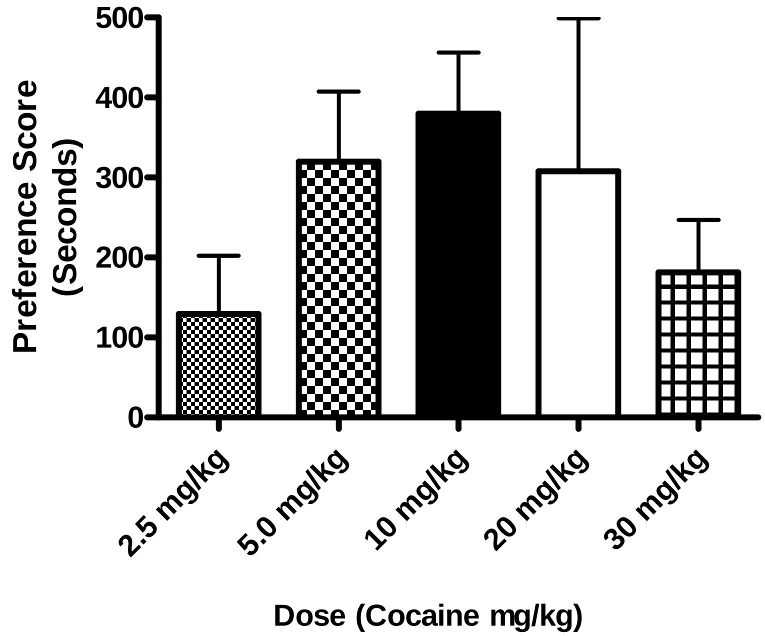

Figure 4.1: Dose-response of cocaine-induced conditioned place preference. Mice were conditioned with varying doses of cocaine (2.5-30 mg/kg, i.p.) using a 4-day conditioning paradigm. On test day (day 5), preference score (time spent on cocaine-conditioned side minus time spent on saline-conditioned side was determined. Cocaine induced a biphasic place preference score with mice conditioned with 10 mg/kg of cocaine yielding the most robust place preference.

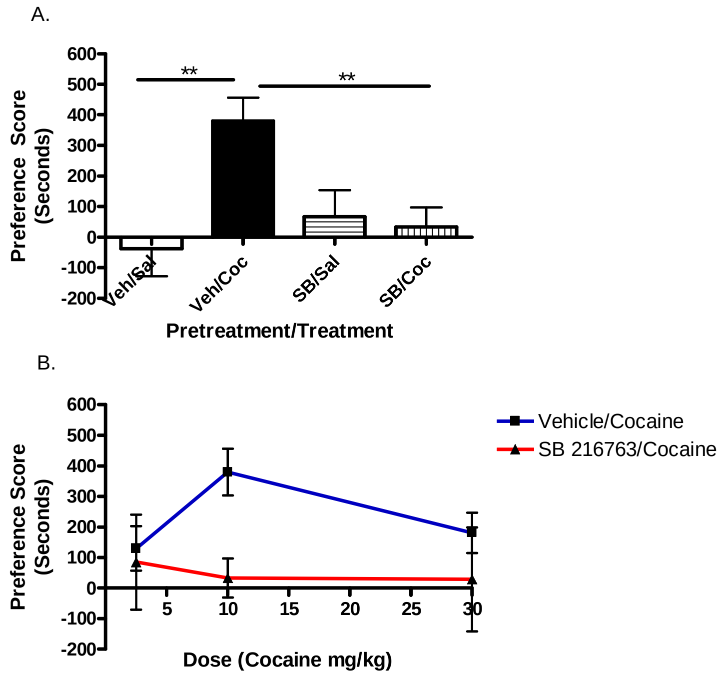

Figure 4.2: Inhibition of GSK3 prevented the development of cocaine-induced conditioned place preference. Mice were pretreated with vehicle or SB 216763 (2.5 mg/kg, i.p.) followed by cocaine (10 mg/kg, i.p.) using a 4-day conditioning paradigm. On test day (day 5), preference score (time spent on cocaine-conditioned side minus time spent on saline-conditioned side) was determined. (4.2A) Mice conditioned with cocaine (10 mg/kg, i.p.) showed a significant place preference toward their cocaine-paired side as compared to saline controls (veh/sal vs veh/coc, $^{**}p<0.01$). Pretreatment of mice with SB 216763 significantly prevented the development of cocaine-induced conditioned place preference as compared to mice pretreated with vehicle (SB/coc vs veh/coc, $^{**}p<0.01$). SB 216763 alone had no effect on preference. (4.2B) There was no significant place preference in mice pretreated with SB 216763 (2.5 mg/kg, i.p.) followed by any dose of cocaine (2.5-30 mg/kg). Data were analyzed by two-way ANOVA and Bonferroni post-hoc analysis. All data points are represented as means ± SEM (n=9-18/group).

Inhibition of GSK3 prevented the retrieval of cocaine associated contextual memories

The role of GSK3 on the retrieval of cocaine-associated memories was investigated using the selective GSK3 inhibitor SB 216763. Following an 8-day conditioning paradigm, mice showed a preference toward their cocaine-paired side (Figure 4.3A). Following administration of vehicle for 2 days in their home cages, mice conditioned with cocaine were able to retrieve cocaine-associated memories and successfully maintained their preference toward the cocaine-paired side. In contrast, treatment with SB 216763 (2.5 mg/kg, i.p.) for 2 days in the home cage abrogated the retrieval of cocaine-associated memories and significantly attenuated preference toward the cocaine-paired side as compared to vehicle injected controls (t_{21}=3.394, p=0.0027; Figure 4.3B).

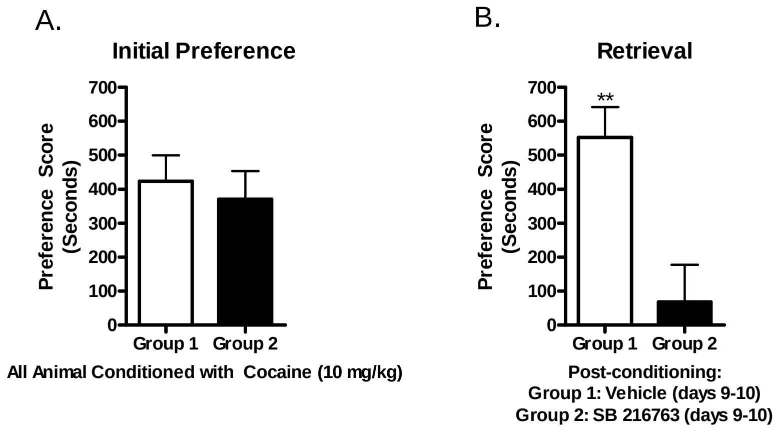

Figure 4.3: Inhibition of GSK3 prevents the retrieval of cocaine-associated memories. Two groups of adult male CD-1 mice were conditioned with saline and cocaine (10 mg/kg, i.p.) on alternate days using an 8-day conditioning paradigm. (4.3A) On test day (day 9) preference scores (time spent on cocaine-conditioned side minus time spent on saline-conditioned side) were determined. Conditioning with cocaine yielded a significant preference for the cocaine-paired side in both groups. Following testing, one group of mice were administered an injection of vehicle in their home cages on days 9-10 and the other group was administered SB 216763 (2.5 mg/kg, i.p.) in their homes cages. Twenty-four hours later (day 11), preference scores (time spent on cocaine-conditioned side minus time spent on saline-conditioned side) were determined. SB 216763 significantly attenuated cocaine-induced place preference as compared to vehicle ($p<0.01$). Data were analyzed by an unpaired two-tailed Student t-test. Data are represented as means ± SEM (8-11/group).

Inhibition of GSK3 during extinction training does not prevent reinstatement to cocaine place preference

The effect of SB 216763 (2.5 mg/kg, i.p.) administration after the first 2 of 8 days of extinction training on reinstatement was tested. Figures 4.4A shows that mice conditioned with cocaine (days 1-8) showed an initial preference toward their cocaine-paired side (day 9). Following 8 days (days 10-17) of extinction training consisting of saline administration paired with alternate sides of the conditioning chamber, followed by injections of vehicle or SB 216763 (2.5 mg/kg, i.p.) in the home cages 4 hours later on days 10 and 11 of training, mice in both groups showed an extinction of cocaine-induced place preference (**$p<0.01$, initial preference vs. extinction) (day 18; Figure 4.4B). On day 19, a priming injection of cocaine (10 mg/kg, i.p.) produced a reinstatement of cocaine conditioned place preference (#$p<0.05$, extinction vs. reinstatement) (Figure 4.4C). This was not altered by administration of SB 216763 on days 10-11 (Figure 4C, $p>0.05$, initial preference vs. reinstatement).

The effect of SB 216763 (2.5 mg/kg, i.p.) administration after all 8 days of extinction training on reinstatement was also tested. Figures 4.5A shows that mice conditioned with cocaine (days 1-8) showed an initial preference toward their cocaine-paired side (day 9). Following 8 days (days 10-17) of extinction training consisting of saline administration paired with alternate sides of the conditioning chamber, followed by injections of vehicle or SB 216763 (2.5 mg/kg, i.p.) in the home cages 4 hours later on days 10-17 of training, mice in both groups showed an extinction of cocaine-induced place preference (**$p<0.01$, initial preference vs. extinction) (day 18; Figure 4.5B). On day 19, a priming injection of cocaine (10 mg/kg, i.p.) produced a reinstatement of cocaine conditioned place preference (#$p<0.05$, extinction vs. reinstatement) (Figure 4.5C). This was not altered by administration of SB 216763 on days 10-17 (Figure 4.5C, $p>0.05$, initial preference vs. reinstatement).

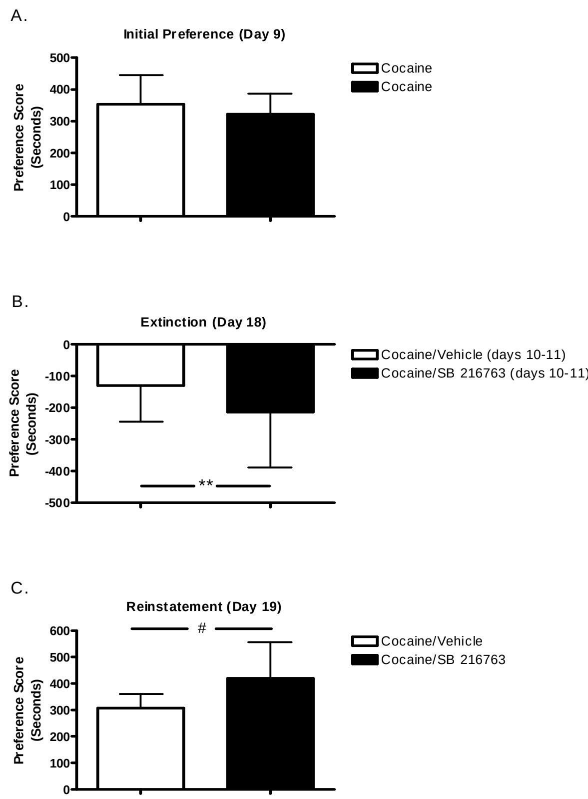

Figure 4.4: Inhibition of GSK3 for 2 days coupled with extinction training does not prevent cocaine-primed reinstatement in mice. (4.4A) Mice conditioned with cocaine (days 1-8; 4 cocaine pairings) showed an initial preference toward their cocaine-paired side (day 9). (4.4B) Following 8 days (days 10-17) of extinction training consisting of saline injections paired to alternate sides of the conditioning chamber and injections of vehicle or SB 216763 (2.5 mg/kg, i.p.), in the home cage on days 10 and 11 of training, mice in both groups showed an extinction of cocaine-induced place preference (**$p<0.01$) (day 18). (4.4C) A priming injection of cocaine, 10 mg/kg reinstated cocaine-induced place preference (#$p<0.05$), thus injections of SB 216763 on day 10 and 11 did not prevent reinstatement to a priming injection of cocaine on day 19 as compared to vehicle-injected controls ($p>0.05$). Data were analyzed using a paired two-tailed Student t-test. Data represent mean ± SEM (n=8/group).

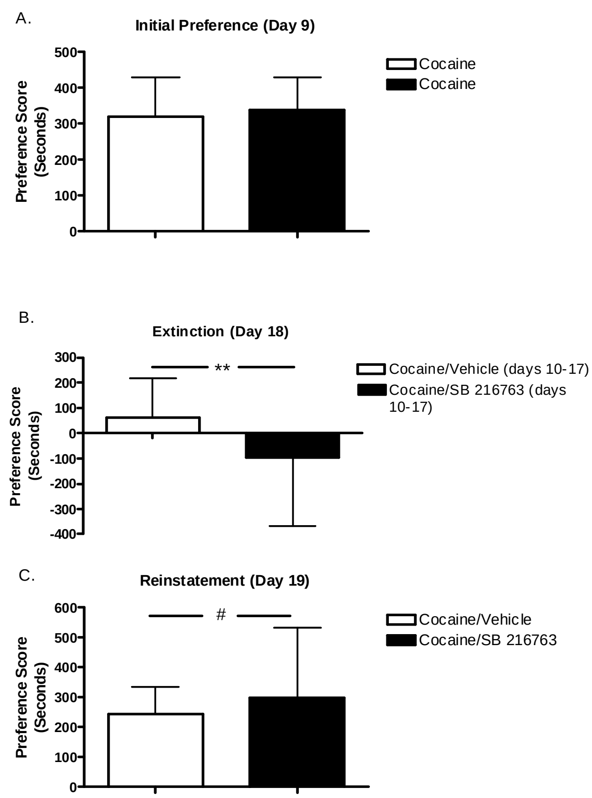

Figure 4.5: Inhibition of GSK3 for 8 days coupled with extinction training does not prevent cocaine-primed reinstatement in mice. (4.5A) Mice conditioned with cocaine (days 1-8; 4 cocaine pairings) showed an initial preference toward their cocaine-paired side (day 9). (4.5B) Following 8 days (days 10-17) of extinction training consisting of saline injections paired to alternate sides of the conditioning chamber and injections of vehicle or SB 216763 (2.5 mg/kg, i.p.), in the home cage throughout extinction training, mice in both groups showed an extinction of cocaine-induced place preference (**$p<0.01$) (day 18). (4.5C) A priming injection of cocaine, 10 mg/kg reinstated cocaine-induced place preference (#$p<0.05$) thus injections of SB 216763 on days 10-17 did not prevent reinstatement to a priming injection of cocaine on day 19 as compared to vehicle-injected controls ($p>0.05$). Data were analyzed using a paired two-tailed Student t-test. Data represent mean ± SEM (n=8/group).

Discussion

Previous studies indicate that the behavioral abnormalities associated with addiction are contingent upon perturbations of specific intracellular signaling proteins and transcriptions factors. Alterations in protein and gene expression mediate the function of individual neurons and larger neural circuits (Nestler, 2004) thus drug addiction can be classified as a form of drug-induced plasticity (Nestler et al., 1993). Recent evidence suggests that a number of intracellular signaling proteins and transcription factors mediate the development (Valjent et al., 2000), retrieval (Miller and Marshall, 2005) and reinstatement (Valjent et al., 2006) of cocaine-conditioned reward. Given the modulation of GSK3 activity by psychostimulants (Perrine et al., 2008) and its role in psychostimulant-induced hyper-locomotion (Beaulieu et al., 2004; Miller et al., 2009a) the overall aim of the present study was to investigate the role of GSK3 in various facets of cocaine-conditioned reward.

The data presented herein are the first to show that selective inhibition of GSK3 prevented the development of cocaine conditioned place preference. Previous investigations from our lab highlight the importance of this kinase in cocaine-induced hyperactivity. For example, non-selective (valproate) and selective (SB 216763) inhibition of GSK3 dose-dependently attenuates acute cocaine-induced hyper-locomotion in mice (Miller et al., 2009a). Selective inhibition of GSK3 with SB 216763 also prevents the development of cocaine-induced sensitization (Miller et al., 2009a). It is well established that both dopaminergic and glutamatergic transmission contribute to the development of cocaine-conditioned reward. Given the role of GSK3 in dopamine and glutamate mediated signal transduction, it may be that both dopamine and glutamate contribute to the effect of GSK3 on cocaine-conditioned reward.

The induction of cocaine conditioned place preference is contingent upon dopamine D1 receptor stimulation as antagonism of the D1 receptor during cocaine conditioning prevents the acquisition of cocaine place preference (Cervo and Samanin, 1995). The dopamine D1 receptor also functions as a primary reward in cocaine-naïve animals as dopamine D1 receptor agonists induce place preference (Graham et al., 2007) and are self-administered (Self and Stein, 1992). Interestingly, pharmacological inhibition of the dopamine D2 receptor does not affect the induction of cocaine place preference (Cervo and Samanin, 1995; Baker et al., 1996) and administration of the dopamine D2 receptor agonist quinpirole fails to produce place preference in cocaine-naïve animals (Graham et al., 2007). Glutamatergic NMDA receptors are also critical to the induction of cocaine-induced place preference as pharmacological (Kim et al., 1996; Harris and Aston-Jones, 2003) and genetic (Heusner and Palmiter, 2005) inhibition of NMDA receptors prevents the development of cocaine-induced place preference.

Previous investigations indicate that GSK3 mediates dopaminergic and glutamatergic function. Pharmacological inhibition (Beaulieu et al., 2004; Alimohamad et al., 2005) and genetic deletion (Beaulieu et al., 2007) of dopamine D2 receptors decrease the activity of GSK3 as evidenced by an increase in its phosphorylation. As previous studies suggest that the dopamine D2 receptor does not participate in the induction of cocaine place preference it seems unlikely that the mechanism by which SB 216763 alters cocaine place preference is related to dopamine D2 receptor activation of GSK3.

Investigations as to whether dopamine D1 receptors signal through GSK3 are conflicting. Administration of the dopamine D1 receptor antagonist SCH-23390 to mice lacking the dopamine transporter has no effect on pGSK3α/β in the striatum (Beaulieu et al., 2004) suggesting that dopamine D1 receptors are not linked to GSK3 activity. In contrast, data from our laboratory suggest that selective inhibition of GSK3 attenuates dopamine D1 receptor agonist-induced hyper-locomotion (Miller et al., 2009b). Further, acute cocaine administration to mice lacking the dopamine D1 receptor reduces the expression of β-catenin in the caudate putamen (Zhang et al., 2002) suggesting that the dopamine D1 receptor is critical to cocaine-induced activation of GSK3 and subsequent expression of β-catenin, as GSK3 negatively regulates β-catenin (Ikeda et al., 1998). Thus indirect activation of the dopamine D1 receptor and subsequent activation of GSK3 by cocaine may influence the development of cocaine place preference.

NMDA receptors and GSK3 act in a feedback mechanism to regulate one another. For example, inhibition of GSK3 activity causes NMDA receptor internalization (Chen et al., 2007), whereas repeated administration of the NMDA receptor antagonist MK-801 decreases the activity of GSK3 in the frontal cortex of rats (Seo et al., 2007). Given the role of GSK3 in maintaining the function of the NMDA receptor, it may be that inhibition of GSK3 during the conditioning phase of cocaine place preference reduces the function of NMDA receptors, therefore preventing the development of cocaine place preference.

Following the expression of cocaine preference, mice injected with SB 216763 for 2 days in their home cages no longer expressed a cocaine place preference 24 hours following the last injection of SB 216763. This suggests that inhibition of GSK3 reduced cocaine-seeking behavior by preventing the reconsolidation of cocaine-associated contextual memories. Our results are consistent with previous investigations highlighting the importance of glutamatergic transmission in the retrieval and reconsolidation of cocaine contextual memories. Acute systemic administration of the NMDA receptor antagonist MK-801 immediately following the expression of cocaine place preference (during memory reconsolidation) blocks the subsequent expression of context elicited preference (Itzhak, 2008). The neural circuitry associated with drug seeking involves structures such as the nucleus accumbens, cortex and basolateral amygdala (Weiss et al., 2000; Carelli and Ijames, 2001). The basolateral amygdala contains a high concentration of NMDA receptors and directly impacts the reconsolidation of contextual memories (Monaghan and Cotman, 1985). Interestingly, binge pattern cocaine administration increases the activity of GSK3 in the amygdala (Perrine et al., 2008). Given the role of GSK3 in maintaining the function of NMDA receptors (Chen et al., 2007), it may be that inhibition of GSK3 acting on NMDA receptors in the amygdala prevents the reconsolidation and retrieval of cocaine-associated contextual memories.

Relapse to drug-seeking behavior can be precipitated following exposure to stress, contextual cues or the drug itself. In the present study, inhibition of GSK3 in a neutral environment during extinction training did not prevent cocaine-induced reinstatement when tested 24 hours or 5 days later. This suggests a differential role for GSK3 in the reconsolidation and subsequent retrieval of cocaine contextual memories following exposure to the conditioned stimulus (environment) as compared to memory reactivation following exposure to the previously unconditioned stimulus (cocaine). Previous investigations demonstrate that following extinction training memory reactivation to the conditioned and unconditioned stimulus is required for blockade of cocaine-primed reinstatement by the NMDA receptor antagonist MK-801 (Brown et al., 2008). Re-exposure of animals to the conditioned stimulus (place preference chamber) and unconditioned stimulus (cocaine) in the presence of MK-801 prevents cocaine-primed reinstatement of place preference when tested a day later (Brown et al., 2008). Further, introducing cocaine in a neutral environment (home cage) in the presence of MK-801 does not prevent cocaine-primed reinstatement the following day (Brown et al., 2008). This is interesting in that pharmacological blockade of NMDA receptors inhibits the activity of GSK3. Thus, it may be that in order for the inhibition GSK3 to prevent cocaine-primed reinstatement, animals must be re-exposed to the conditioned and unconditioned stimulus in the presence of SB 216763 prior to testing.

In summary, the results presented here demonstrate that inhibition of GSK3 prevented the development of cocaine-induced place preference. Further, inhibition of GSK3 prevented the retrieval of cocaine-associated contextual memories. These results provide support for further exploration of this kinase in the various behavioral and molecular adaptations associated with cocaine-conditioned reward. In addition, further work elucidating the role of GSK3 in cocaine-primed and stress-induced reinstatement could provide new insights into the mechanism of relapse to drug-seeking behaviors.

CHAPTER 5

REGULATION OF AKT/GSK3 BY COCAINE IN MICE: ROLE OF DOPAMINERGIC AND GLUTAMATERGIC RECEPTORS

Introduction

Akt (protein kinase B) is a serine/threonine protein kinase involved in a number of cellular processes including metabolism (Cross et al., 1995) and apoptosis (Marte and Downward, 1997; Alessi and Cohen, 1998). Activation of Akt requires recruitment to the plasma membrane by PI (3,4,5)P3 and PI (3,4) which have a high affinity for the PH domain of Akt (Burgering and Coffer, 1995; Franke et al., 1995). Recruitment of Akt to the plasma membrane facilitates the activation of Akt via phosphorylation of two regulatory (Thr. 308) and (Ser. 473) sites (Alessi et al., 1996). Akt (Thr. 308) lies within the T loop of the kinase and its phosphorylation is catalyzed by PDK-1 (Alessi et al., 1997; Stephens et al., 1998). Akt (Ser. 473) is located within a hydrophobic region near the carboxyl terminus of the kinase (Alessi et al., 1996), and the mechanism by which this residue is phosphorylated is currently unknown. One mechanism, by which Akt facilitates cellular processes such as cell survival and metabolism, is by negatively regulating the activity of glycogen synthase kinase-3 (GSK3) (Cross et al., 1995).

Originally identified for its regulation of glycogen metabolism (Embi et al., 1980), GSK3 has been since shown to be critical for a number of cellular processes including apoptosis (Cross et al., 2001), and synaptic plasticity (Peineau et al., 2008) and in disease states such as schizophrenia (Emamian et al., 2004). GSK3 is regulated via phosphorylation of the N-terminal serine-21 (GSK3α) or serine-9 (GSK3β) and is highly expressed in regions of the brain including the frontal cortex, nucleus accumbens, caudate putamen, hippocampus and amygdala (Leroy and Brion, 1999). Regulation of GSK3 activity is critical in that it phosphorylates more than 40 substrates (Jope and Johnson, 2004) including the transcription factor CREB (Grimes and Jope, 2001), which serves an important role in the addictive properties of drugs of abuse (Carlezon et al., 1998).

Previous investigations have demonstrated the importance of the Akt/GSK3 signaling pathway in dopaminergic transmission. For example, pharmacological inhibition and genetic deletion of the dopamine D2 receptor increases phosphorylated Akt (Thr. 308) and GSK3β in the striatum (Beaulieu et al., 2004; Beaulieu et al., 2007). Psychostimulants effecting dopaminergic transmission also modulate the activity of Akt and/or GSK3. Amphetamine, cocaine and methylphenidate induce a distinct temporal and spatial regulation of Akt and/or GSK3 activity in the brain, an effect that is contingent upon acute or repeated drug exposure (Beaulieu et al., 2004; Beaulieu et al., 2006; Shi and McGinty, 2007; Perrine et al., 2008). Given the role of the Akt/GSK3 signaling cascade in dopaminergic transmission and psychostimulant-induced behaviors, we measured the effects of acute cocaine on levels of phosphorylated Akt and GSK3 in the caudate putamen, nucleus accumbens and frontal cortex of mice. Further, we characterized the role of the dopamine D1 and D2 receptors and the glutamatergic N-methyl-D-aspartate (NMDA) receptor in cocaine-induced phosphorylation of Akt and GSK3.

Methods

Animals

Male CD-1 mice (8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed five per plastic cage (28 x 18 x 14 cm) without additional enrichment objects in a temperature- and relative humidity-controlled room with a 12-hr light/dark cycle (lights on at 7:00 a.m.). Animals were housed for seven days prior to testing and were handled and weighed daily. All animals had access to standard laboratory chow and tap water *ad libitum*. All animal testing was conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and with an approved protocol from Temple University Institutional Animal Care and Use Committee.

Drugs

Cocaine hydrochloride was generously supplied by the National Institute on Drug Abuse, SCH-23390, eticlopride hydrochloride and MK-801 were purchased from Sigma-Aldrich (St. Louis, MO) and all compounds were dissolved in sterile saline (0.9% NaCl) and injected at a volume of 3 ml/kg.

Drug Administration

To investigate the effect of acute cocaine on Akt and GSK3 activity in the brain, mice were administered saline or cocaine (20 mg/kg, i.p.) and euthanized 30 minutes post-injection. The involvement of the dopamine D1 and D2 receptor and the glutamatergic NMDA receptor in cocaine-induced modulation of Akt and GSK3 in the brain were assessed using selective receptor antagonists. Mice were injected with the dopamine D1 receptor anatgonist SCH-23390 (0.1 mg/kg), the dopamine D2 receptor antagonist eticlopride hydrochloride (1.0 mg/kg, i.p.) or the NMDA receptor antagonist MK-801 (1.0 mg/kg, i.p.) 30 minutes prior to saline or cocaine (20 mg/kg, i.p.). For receptor antagonist studies, all mice were euthanized 30 minutes following saline or cocaine injection.

Immunoblotting

Following drug administration, the frontal cortex, nucleus accumbens and caudate putamen were rapidly dissected on ice. Tissues were immediately sonicated in boiling 1% SDS, boiled for 5 minutes, aliquotted and stored at –80˚C until assayed. Protein concentrations were determined using the Lowry assay (Lowry et. al, 1951). Protein extracts (20-50 μg) were subjected to SDS-polyacrylamide gel electrophoresis (7.5% Tris-HCl BioRad Ready-gels, Hercules, CA) and transferred for 95 minutes to nitrocellulose membranes. Membranes were subsequently blocked for 1 hour in blocking solution consisting of 5% nonfat dry milk and Tween-TBS and then incubated overnight at 4˚C in the following antibodies, phospho-GSK3α-β (1:2000-1:5000, Cell Signaling, Beverly, MA), phospho-Akt (Ser. 473) (1:1000, Cell Signaling, Beverly, MA), phospho-Akt (Thr. 308) (1:1000, Cell Signaling, Beverly, MA), total Akt (1:2000; Cell Signaling, Beverly, MA) total GSK3α-β (1:10000; Santa Cruz, Santa Cruz, CA) or anti-tubulin antibody (1:20000; Sigma, St. Louis, MO). Following overnight incubation in primary antibodies, membranes were washed in Tween-TBS and incubated in either anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Immunoreactivity was visualized by chemiluminescence following incubation in Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) with bands being quantified using the FujiFilm Intelligent Dark Box II, IR LAS-100 Pro V3.1, and Image Gauge V4.22 equipment and software packages.

Data Analysis

Immunoblotting data were expressed as a ratio of phosphorylated protein/total protein. Data were analyzed using one-way ANOVA followed by a Bonferroni test for multiple comparisons or an unpaired two-tailed Student t-test (GraphPad Prism 4, La Jolla, CA).

Results

Acute cocaine administration decreased phosphorylated Akt (Thr. 308) in the caudate putamen but not the nucleus accumbens or frontal cortex

Levels of phosphorylated and total Akt were determined by Western blot analysis. Representative images from the caudate putamen of pAkt (Thr. 308), pAkt (Ser. 473) and total Akt are shown in figure 5.1. Levels of pAkt (Thr. 308) were significantly decreased in the caudate putamen 30 minutes following acute cocaine administration as compared to saline controls (t_{23}=3.943, p=0.0006) (Figure 5.2A). In contrast, acute cocaine administration had no effect on pAkt (Thr. 308) in the nucleus accumbens (Figure 5.2C) or frontal cortex (Figure 5.2E) ($p>0.05$). Acute cocaine had no effect on phosphorylated Akt (Ser. 473) in any brain region (Figures 5.2B, 5.2D, 5.2F) ($p>0.05$). Levels of total Akt were also unchanged in all brain regions following cocaine (data not shown). These data show that acute cocaine administration regulates the activity of Akt by decreasing the phosphorylation of Akt (Thr. 308) and not Akt (Ser. 473), selectively in the caudate putamen.

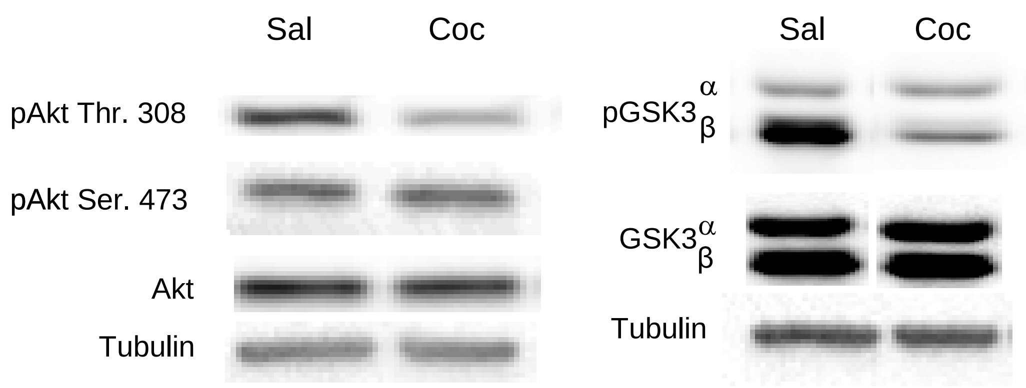

Figure 5.1: Representative immunoblots of caudate putamen tissue from saline (sal) and cocaine (coc) treated mice. Bands represent pAkt (Thr. 308), pAkt (Ser. 473), total Akt, pGSK3α-β, total GSK3α-β and tubulin (from top to bottom).

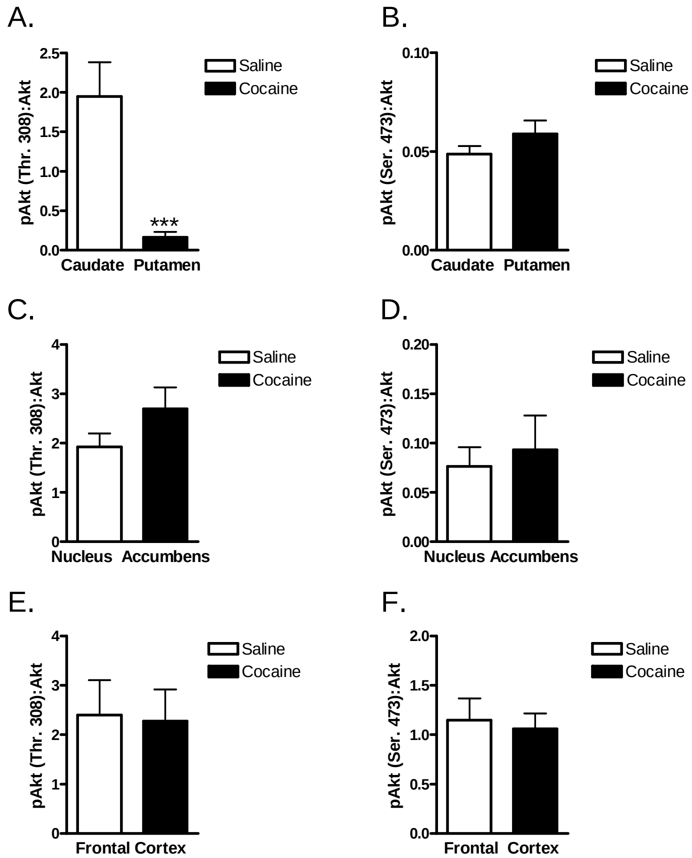

Figure 5.2: Acute cocaine administration selectively decreased pAkt (Thr. 308) in the caudate putamen of mice. Levels of pAkt (Thr. 308) and pAkt (Ser. 473 were measured by Western blot analysis in the caudate putamen, nucleus accumbens and frontal cortex of mice 30 minutes following acute cocaine administration. (5.2A) Acute cocaine significantly decreased pAkt (Thr. 308) (***$p<0.001$) and (5.2B) had no effect on pAkt (Ser. 473) in the caudate putamen of mice. (5.2C&D) Levels of pAkt (Thr. 308) and pAkt (Ser. 473) were not changed in the nucleus accumbens or (5.2E&F) frontal cortex. Bars represent the mean ± SEM (N=7-13/group) and are expressed as a ratio of pAkt:total Akt. Data were analyzed by an unpaired two-tailed Student t-test.

Phosphorylated GSK3β is reduced in the caudate putamen but not the nucleus accumbens or frontal cortex following acute administration of cocaine

Protein extracts from the caudate putamen were analyzed for levels of phospho-GSK3α/β and total GSK3α/β by immunoblot. Figure 5.1 shows representative immunoblots of tissue extracts from the caudate putamen probed with antibodies recognizing phospho-GSK3α (51 kDa) and -GSK3β (48 kDa) and total GSK3α/β. As shown in Figure 5.3A, animals administered cocaine had significantly less phosphorylated GSK3β in the caudate putamen as compared to saline controls (t_{10}=3.586, p=0.0050) indicating that acute cocaine enhanced the activity of GSK3β. In contrast, levels of pGSK3α in the caudate putamen were not changed ($p>0.05$; Fig. 5.3B). Phosphorylated GSK3α/β in the nucleus accumbens and frontal cortex following cocaine administration were not significantly different from saline controls ($p>0.05$; Fig. 5.3C-F). Levels of total GSK3α/β:tubulin were unchanged in all brain regions following acute administration of cocaine (data not shown).

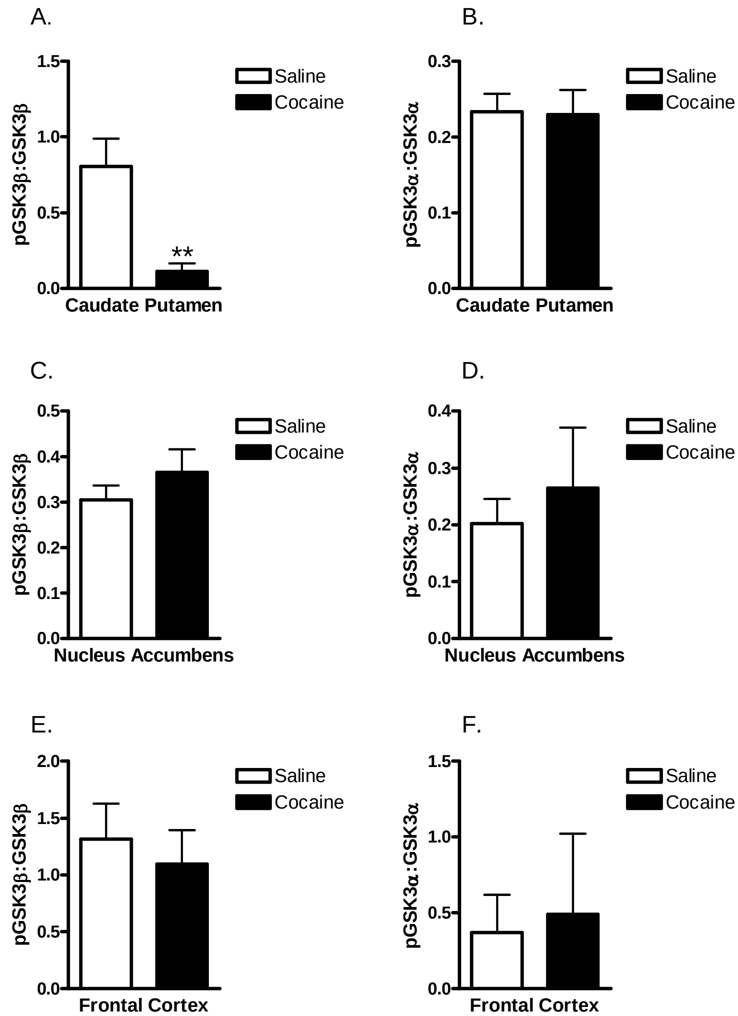

Figure 5.3: Phosphorylation of GSK3β but not GSK3α is reduced in the caudate putamen following acute administration of cocaine. Acute administration of cocaine reduced the phosphorylation of GSK3β but not GSK3α as compared to saline administration in the caudate putamen of mice (**p<0.01) (5.3A&B). Levels of phosphorylated GSK3α/β were unchanged in the nucleus accumbens (5.3C&D) and frontal cortex (5.3E&F) following cocaine. Bars represent the mean ± SEM, (n=6-9/group) and are expressed as a ratio of pGSK3α/β:total GSK3α/β. Data were analyzed by an unpaired two-tailed Student t-test.

Cocaine-induced attenuation of phospho-Akt (Thr. 308) and phospho-GSK3β (Ser. 9) is prevented by eticlopride

The role of the dopamine D2 receptor in cocaine-induced modulation of phosphorylated Akt was assessed using the dopamine D2 receptor antagonist eticlopride. Mice were pretreated with eticlopride (1.0 mg/kg, i.p.) 30 minutes prior to cocaine (20 mg/kg, i.p.) and animals were euthanized 30 minutes following cocaine administration. One-way ANOVA revealed a significant difference between groups [$F(3,27)=5.102$, $p=0.0071$; Fig. 5.4A]. Bonferroni post-hoc analysis showed that cocaine significantly decreased pAkt (Thr. 308) levels in the caudate putamen as compared to saline controls (sal/sal vs. sal/coc, $p<0.05$). Interestingly, administration of eticlopride prior to cocaine prevented the cocaine-induced reduction of phosphorylated Akt (Thr. 308) indicating that the dopamine D2 receptor is critical to cocaine-induced inactivation of Akt (sal/coc vs. etic/coc, $p<0.001$). Eticlopride alone had no effect on the levels of pAkt (Thr. 308) in the caudate putamen (sal/sal vs. etic/sal, $p>0.05$). There were no significant differences between groups on pAkt (Ser. 473) levels in the caudate putamen [$F(3,32)=0.1048$, $p=0.9566$; Fig. 5.4B] as determined by one-way ANOVA.

In the nucleus accumbens, one-way ANOVA revealed that there were no significant changes in the levels of pAkt (Thr. 308) [$F(3,29)=2.389$, $p=0.0918$; Fig. 5.4C] or pAkt (Ser. 473) [$F(3,32)=1.001$, $p=0.4063$; Fig. 5.4D] between groups. In the frontal cortex, the levels of phosphorylated Akt (Thr. 308) [$F(3,28)=0.2258$, $p=0.8776$; Fig. 5.4E] and (Ser. 473) [$F(3,29)=0.8162$, $p=0.4966$; Fig. 5.4F] were also not significantly changed between groups as determined by one-way ANOVA. No significant differences were observed in the levels of total Akt in mice pretreated with eticlopride as compared to mice pretreated with saline in any brain region tested (data not shown).

In addition to investigating the role of the dopamine D2 receptor in cocaine-induced inactivation of Akt, we assessed the importance of this receptor in the activation of GSK3 by cocaine. One-way ANOVA revealed a significant difference between groups in the levels of pGSK3β [$F(3,25)=4.562$, $p=0.0125$; Fig. 5.5A], but not pGSK3α [$F(3,31)=0.8790$, $p=0.4638$; Fig. 5.5B]. Bonferroni post-hoc analysis indicated that cocaine decreased the phosphorylation of GSK3β in the caudate putamen (sal/sal vs. sal/coc, $p<0.05$), therefore activating the kinase. However, administration of eticlopride prior to cocaine prevented cocaine-induced activation of GSK3β as evidenced by an increase in the phosphorylation of GSK3β (sal/coc vs. etic./coc, $p<0.01$). Basal levels of pGSK3α/β in the caudate putamen were not changed following pretreatment with eticlopride (sal/sal vs. etic/sal, $p>0.05$).

In the nucleus accumbens, there were no significant differences between groups as indicated by one-way ANOVA in pGSK3β [$F(3,29)=2.218$, $p=0.1099$; Fig. 5.5C] or pGSK3α [$F(3,30)=2.873$, $p=0.0547$; Fig. 5.5D] levels. In the frontal cortex, one-way ANOVA revealed that there were also no significant differences in the levels of pGSK3β [$F(3,26)=0.4129$, $p=0.7453$; Fig. 5.5E] or pGSK3α [$F(3,28)=0.3693$, $p=0.7758$; Fig. 5.5F] between groups. Mice pretreated with eticlopride showed no significant differences in the levels of total GSK3α/β as compared to mice pretreated with saline in any brain region tested (data not shown).

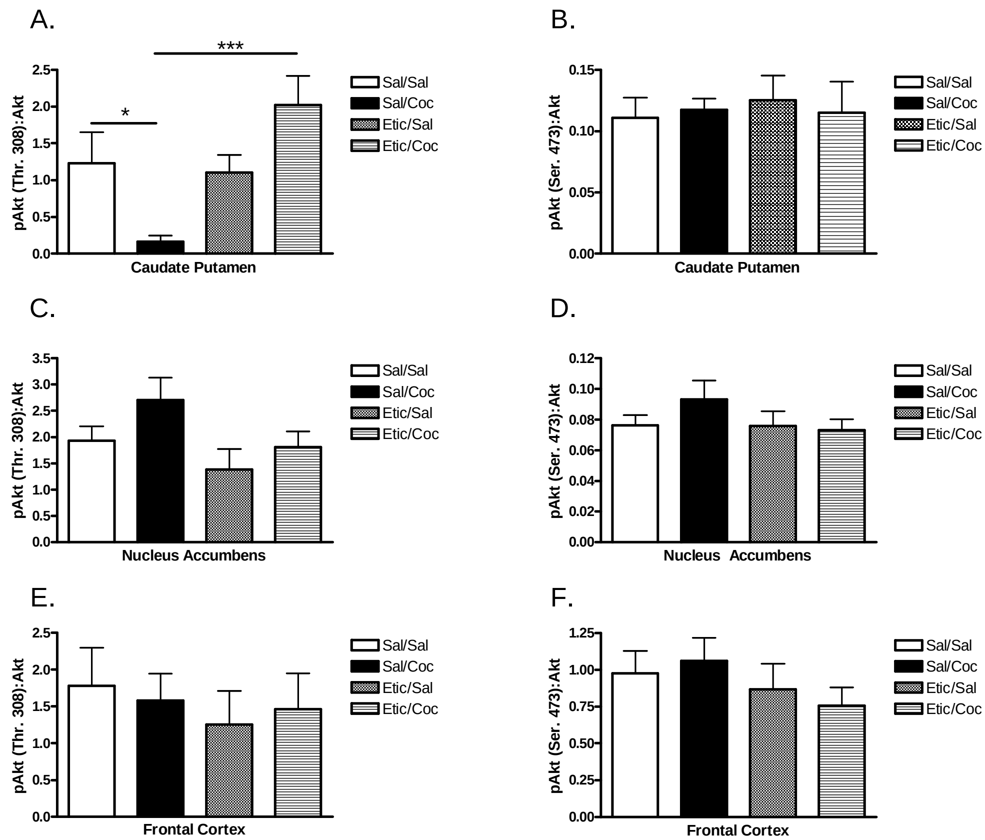

Figure 5.4: The cocaine-induced reduction of Akt (Thr. 308) phosphorylation in the caudate putamen is prevented by eticlopride. (5.4A) Acute cocaine administration decreased the phosphorylation of Akt (Thr. 308) in the caudate putamen (*$p<0.05$). Administration of eticlopride prior to cocaine prevented the cocaine-induced reduction of Akt (Thr. 308) phosphorylation (***$p<0.001$). Levels of pAkt (Ser. 473) were not changed in any experimental group in the caudate putamen (5.4B). Eticlopride had no effect on pAkt (Thr. 308) or pAkt (Ser. 473) in the nucleus accumbens or frontal cortex (5.4C-5.4F). Bars represent the mean ± SEM, (n=5-8/group) and are expressed as a ratio of pAkt:total Akt. Data were analyzed by a one-way ANOVA followed by a Bonferroni test for multiple comparisons.

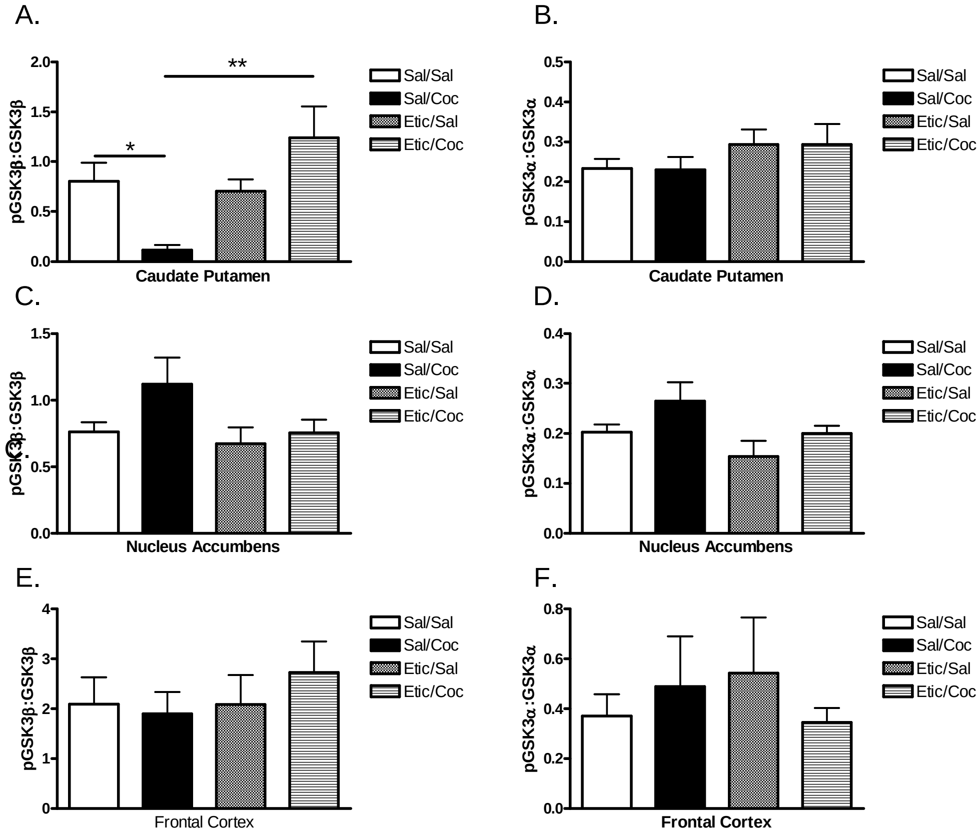

Figure 5.5: Eticlopride prevented the cocaine-induced attenuation of pGSK3β in the caudate putamen. (5.5A) Acute cocaine administration significantly reduced phosphorylated GSK3β levels in the caudate putamen (*p<0.05). Pretreatment with eticlpride prevented the cocaine-induced attenuation of pGSK3β in the caudate putamen (**p<0.01). (5.5B) Levels of pGSK3α were not changed in any experimental group in the caudate putamen. Eticlopride had no effect on pGSK3α/β in the nucleus accumbens or frontal cortex (5.5C-5.5F). Bars represent the mean ± SEM, (n=6-9/group) and are expressed as a ratio of pGSK3α/β:total GSK3α/β. Data were analyzed by a one-way ANOVA followed by a Bonferroni test for multiple comparisons.

The dopamine D1 receptor antagonist SCH-23390 prevented the cocaine-induced attenuation of GSK3β but not Akt (Thr. 308) phosphorylation in the caudate putamen

To determine the role of the dopamine D1 receptor in cocaine-induced activation of GSK3β and inactivation of Akt, mice were pretreated with the dopamine D1 receptor antagonist SCH-23390 (0.1 mg/kg, i.p.) 30 minutes prior to cocaine. One-way ANOVA revealed a significant difference between groups in the levels of pGSK3β [$F(3,25)=3.756$, $p=0.0256$; Fig. 5.7A] but not pGSK3α [$F(3,32)=0.6795$, $p=0.5717$; Fig. 5.7B]. Bonferroni post-hoc analysis indicated that acute cocaine administration significantly attenuated the levels of pGSK3β in the caudate putamen (sal/sal vs. sal/coc, $p<0.05$). Further, blockade of the dopamine D1 receptor prior to cocaine prevented the cocaine-induced attenuation of phosphorylated GSK3β levels in the caudate putamen (sal/coc vs. sch/coc, $p<0.05$). Administration of SCH-23390 alone had no effect on the levels of pGSKα/β (sal/sal vs. sch/sal, $p>0.05$).

In the nucleus accumbens, one-way ANOVA also revealed that there were no significant differences between groups in pGSK3β [$F(3,33)=2.785$, $p=0.0578$; Fig. 5.7C] or pGSK3α [$F(3,34)=1.354$, $p=0.2749$; Fig. 5.7D] levels. There was also no effect in the frontal cortex on the phosphorylation of GSK3β [$F(3,29)=0.05999$, $p=0.9803$; Fig. 5.7E] or GSK3α [$F(3,32)=0.1169$, $p=0.9495$; Fig. 5.7F] as determined by one-way ANOVA.

To assess whether dopamine D1 receptor modulation of pGSK3β levels in the caudate putamen were contingent upon modulation of Akt phosphorylation, we investigated the effect of D1 receptor blockade in the cocaine-induced attenuation of Akt phosphorylation. One-way ANOVA revealed significant differences between groups [$F(3,29)=3.935$, $p=0.0193$; Fig. 5.6A]. Bonferroni post-hoc analysis showed that cocaine administration decreased the phosphorylation of Akt (Thr. 308) in the caudate putamen as compared to saline controls (sal/sal vs. sal/coc, $p<0.05$). Blockade of the dopamine D1 receptor prior to cocaine, however, did not significantly effect Akt (Thr. 308) phosphorylation (sal/coc vs. sch/coc, $p>0.05$). Administration of SCH-23390 alone also had no effect on the phosphorylation of Akt (Thr. 308) (sal/sal vs. sch/sal, $p>0.05$). Further, there were no significant differences between groups on the phosphorylation of Akt (Ser. 473) in the caudate putamen [$F(3,33)=0.07535$, $p=0.9728$; Fig. 5.6B]. Thus, the mechanism by which the dopamine D1 receptor blocks cocaine-induced activation of GSK3β is not contingent upon activation of Akt.

In the nucleus accumbens, one-way ANOVA indicated that there were no significant differences between groups in the phosphorylation of Akt (Thr. 308) [$F(3,30)=2.492$, $p=0.0814$; Fig. 5.6C] or Akt (Ser. 473) [$F(3,34)=0.8519$, $p=0.4763$; Fig. 5.6D]. Similarly, there was no change between groups in phosphorylated Akt (Thr. 308) [$F(3,25)=1.365$, $p=0.2796$; Fig. 5.6E] or phosphorylated Akt (Ser. 473) [$F(3,33)=0.4467$, $p=0.7214$; Fig. 5.6F] in the frontal cortex. Blockade of the dopamine D1 receptor in animals had no significant effects on the levels of total GSK3α/β or Akt as compared to control animals in any brain region tested (data not shown).

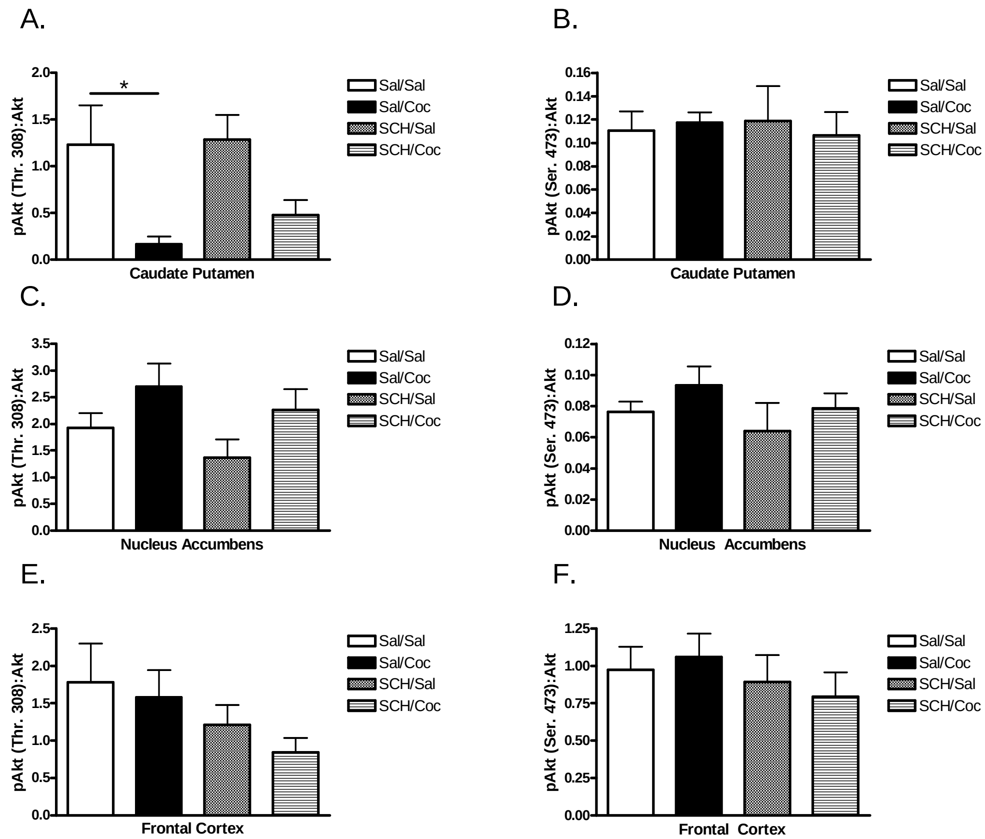

Figure 5.6: SCH-23390 did not prevent cocaine-induced attenuation of pAkt (Thr. 308). Administration of the dopamine D1 receptor antagonist SCH-23390 (0.1 mg/kg) prior to cocaine did not effect cocaine-induced attenuation of pAkt (Thr. 308) in the caudate putamen (5.6A). SCH-23390 also had no effect pAkt (Thr. 308) in the nucleus accumbens (5.6C) or frontal cortex (5.6E). Levels of pAkt (Ser. 473) were not changed in the caudate putamen (5.6B), nucleus accumbens (5.6D) or frontal cortex (5.6F). Bars represent the mean ± SEM, (n=6-10/group) and are expressed as a ratio of pAkt:total Akt. Data were analyzed by a one-way ANOVA followed by a Bonferroni test for multiple comparisons.

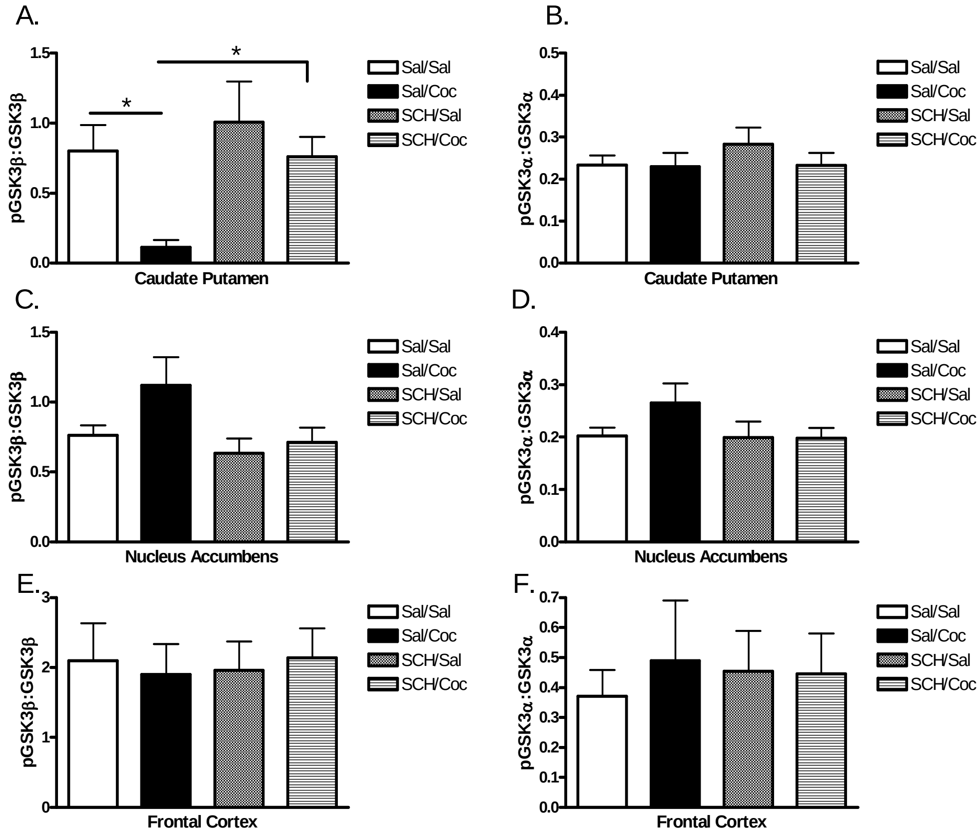

Figure 5.7: Blockade of the dopamine D1 receptor prior to cocaine increased GSK3β phosphorylation in the caudate putamen. (5.7A) Administration of SCH-23390 prior to cocaine blocked the reduction of pGSK3β (*$p<0.05$) in the caudate putamen with no effect on pGSK3α (5.7B). SCH-23390 had no effect on pGSK3α/β in the nucleus accumbens (5.7C&D) or frontal cortex (5.7E&F). Bars represent the mean ± SEM, (n=6-10/group) and are expressed as a ratio of pGSK3α/β:total GSK3α/β. Data were analyzed by a one-way ANOVA followed by a Bonferroni test for multiple comparisons.

Cocaine-induced attenuation of GSK3β phosphorylation in the caudate putamen is prevented by the glutamatergic NMDA receptor antagonist MK-801

In addition to the role of the dopamine D1 and D2 receptors, we investigated whether glutamatergic NMDA receptors were involved in the cocaine-induced activation of GSK3β. Mice were pretreated with the NMDA receptor antagonist MK-801 (1.0 mg/kg, i.p.) 30 minutes prior to cocaine and the phosphorylation of GSK3α/β was assessed by Western blot analysis. In the caudate putamen, one-way ANOVA indicated significant differences between groups with respect to the phosphorylation of GSK3β [$F(3,22)=4.978$, $p=0.0103$; Fig. 5.9A] but not GSK3α [$F(3,29)=0.7448$, $p=0.5352$; Fig. 5.9B]. Bonferroni post-hoc analysis showed that acute cocaine attenuated GSK3β phosphorylation in the caudate putamen (sal/sal vs. sal/coc, $p<0.01$). Further, mice pretreated with MK-801 prior to cocaine showed a significant increase in GSK3β phosphorylation as compared to saline pretreated controls (sal/coc vs. MK-801/coc, $p<0.05$). Pretreatment with MK-801 alone had no effect on GSK3β phosphorylation in the caudate putamen (sal/sal vs. MK-801/sal, $p>0.05$). The results of this study indicate that the NMDA receptor is critical to cocaine-induced activation of GSK3β in the caudate putamen.

In the nucleus accumbens, one-way ANOVA revealed no significant differences between groups on the levels of GSK3β [$F(3,29)=2.473$, $p=0.0840$; Fig. 5.9C] or GSK3α [$F(3,29)=1.124$, $p=0.3574$; Fig. 5.9D] phosphorylation. There were also no significant differences in the phosphorylation of GSK3β [$F(3,26)=0.1376$, $p=0.9365$; Fig. 5.9E] or GSK3α [$F(3,29)=0.1421$, $p=0.9338$] between groups in the frontal cortex as determined by a one-way ANOVA.

The involvement of the NMDA receptor in cocaine-induced inactivation of Akt in the caudate putamen was also investigated using the NMDA receptor antagonist MK-801. In the caudate putamen, significant differences in the phosphorylation of pAkt (Thr. 308) were found between groups [$F(3,26)=3.319$, $p=0.0377$; Fig. 5.8A] as determined by one-way ANOVA. Bonferroni post-hoc analysis showed that administration of cocaine significantly decreased Akt (Thr. 308) phosphorylation, therefore inactivating the kinase (sal/sal vs. sal/coc, $p<0.05$). However, blockade of the NMDA receptor prior to cocaine did not prevent cocaine-induced attenuation of Akt (Thr. 308) phosphorylation (sal/coc vs. MK-801/coc, $p>0.05$). Administration of MK-801 alone also had no effect on the levels of Akt (Thr. 308) phosphorylation in the caudate putamen (sal/sal vs. MK-801/sal, $p>0.05$). In addition, there were no significant effects on Akt (Ser. 473) phosphorylation as determined by one-way ANOVA [$F(3,31)=0.1804$, $p=0.9088$]. Overall, the data presented herein demonstrate that the inhibition of cocaine-induced GSK3 activation by blocking the NMDA receptor is not dependent on the activity of Akt.

Similar to GSK3, one-way ANOVA revealed no significant differences between groups in the levels of phosphorylated Akt (Thr. 308) [$F(3,27)=1.527$, $p=0.2329$; Fig. 5.8C] or Akt (Ser. 473) [$F(3,31)=0.6627$, $p=0.5819$; Fig. 5.8D] in the nucleus accumbens. In the frontal cortex, there were also no significant differences between groups in the levels of phosphorylated Akt (Thr. 308), [$F(3,28)=0.5293$, $p=0.6663$] or Akt (Ser. 473) [$F(3,31)=0.7652$, $p=0.5232$] as determined by one-way ANOVA. Blockade of the NMDA receptor with MK-801 did not affect the total levels of GSK3α/β or Akt in any brain region tested as compared to saline controls (data not shown).

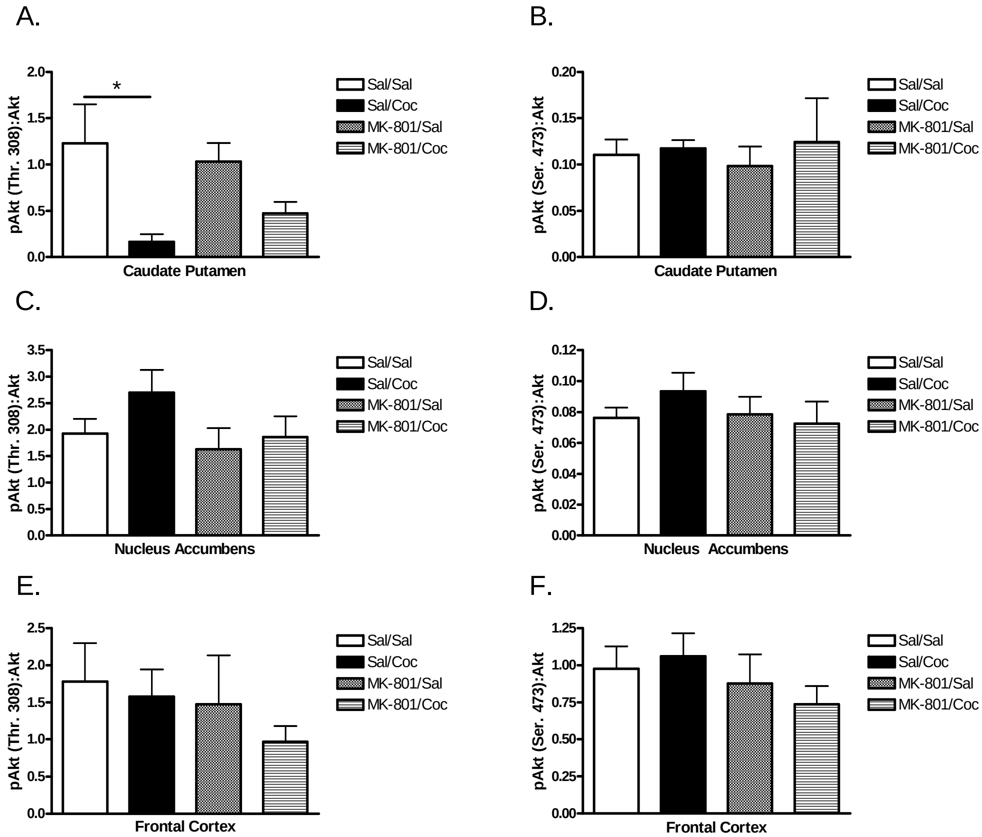

Figure 5.8: Pretreatment with MK-801 does not effect pAkt levels following cocaine. (5.8A) MK-801 administration prior to cocaine had no effect on cocaine-induced reductions in pAkt (Thr. 308) in the caudate putamen. Administration of MK-801 had no effect on pAkt (Ser. 473) in the caudate putamen (5.8B). Levels of pAkt were also unchanged in the nucleus accumbens (5.8C&D) and frontal cortex (5.8E&F). Bars represent the mean ± SEM, (n=6-9/group) and are expressed as a ratio of pAkt:total Akt. Data were analyzed by a one-way ANOVA followed by a Bonferroni test for multiple comparisons.

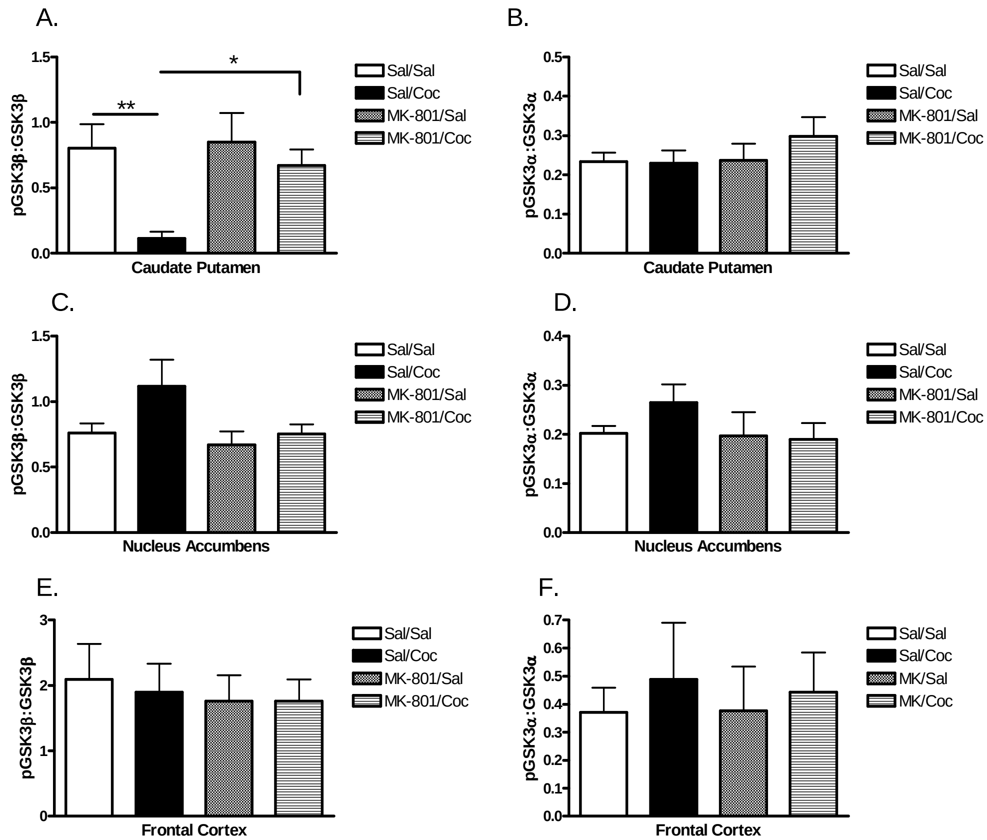

Figure 5.9: MK-801 prevented the cocaine-induced attenuation of pGSK3β levels in the caudate putamen. (5.9A) Administration of MK-801 prior to cocaine prevented the cocaine-induced attenuation of pGSK3β in the caudate putamen (*$p<0.05$). Levels of pGSK3α were not changed in the caudate putamen (5.9B). MK-801 had no effect on pGSK3α/β in the nucleus accumbens (5.9C&D) or frontal cortex (5.9E&F). Bars represent the mean ± SEM, (n=5-9/group) and are expressed as a ratio of pGSK3α/β:total GSK3α/β. Data were analyzed by a one-way ANOVA followed by a Bonferroni test for multiple comparisons.

Discussion

Dopaminergic and glutamatergic receptors are critical to the behavioral and neuromodulatory effects of cocaine. Cocaine indirectly activates dopamine and glutamate receptors by increasing extracellular dopamine (Heikkila et al., 1975) and glutamate (Vanderschuren and Kalivas, 2000) in various brain regions. Cocaine increases extracellular dopamine by binding to and blocking the dopamine transporter, whereas the increase in extracellular glutamate is thought to be mediated by dopamine D1 receptors (Kalivas and Duffy, 1995). Activation of dopaminergic and glutamatergic receptors following cocaine alters a number of intracellular signaling proteins and subsequently gene transcription. The data presented herein demonstrate that acute cocaine administration regulates Akt and GSK3 activity in the caudate putamen. Further our data show that this effect is contingent upon activation of dopamine D1, D2 and glutamatergic NMDA receptors.

The Akt/GSK3 signaling cascade plays an important role in the behavioral effects of psychostimulants, including cocaine. Heterozygote GSK3β mice display an attenuated hyper-locomotor response to acute amphetamine (Beaulieu et al., 2004) and selective inhibition of GSK3 prevents the hyper-locomotor response in mice lacking the dopamine transporter (Beaulieu et al., 2004). Recent evidence from our laboratory indicates that GSK3 is critical to the acute and sensitized hyper-locomotor response of cocaine (Miller et al., 2009a). The data presented herein shows that acute cocaine administration reduced pAkt (Thr. 308) and pGSK3β levels in the caudate putamen of mice, without effecting pAkt (Ser. 473) or pGSK3α. These results are consistent with those indicating that acute amphetamine attenuates pAkt (Thr. 308) and pGSK3β (Beaulieu et al., 2004, 2005) in the striatum of mice. In addition, we show no changes in Akt or GSK3 phosphorylation in the nucleus accumbens or frontal cortex. Previous studies suggest that the Akt/GSK3 pathway may be regulated within the nucleus accumbens or frontal cortex following repeated exposure to psychostimulants. For example, repeated cocaine administration reduces Akt activity in the nucleus accumbens (Pulipparacharuvil et al., 2008) and increases PI3K activity in the frontal cortex (Zhang et al., 2006). Evidence suggests that extracellular dopamine regulates pAkt and pGSK3β as mice lacking the dopamine transporter that display an increased level of extracellular dopamine (Giros et al., 1996) show a decrease in pAkt (Thr. 308) and pGSK3β in the striatum (Beaulieu et al., 2004). Therefore, cocaine-induced effects on Akt and GSK3 are likely due to increased extracellular dopamine.

The effects of dopamine are mediated by dopamine D1-like (D1 and D5) and D2-like (D2, D3 and D4) dopamine receptors. Dopamine D1 and D2 receptors are highly expressed in brain regions receiving dopaminergic innervation (Meador-Woodruff, 1994) and exert changes in intracellular signaling by coupling to specific G-proteins (Sibley et al., 1993). Evidence suggests that dysregulation of Akt and GSK3 activity by dopamine and dopamine D2 receptors may be involved in diseases such as schizophrenia. Postmortem samples of frontal cortex show that subjects with schizophrenia exhibit approximately 40% lower GSK3β mRNA levels (Kozlovsky et al., 2004) and GSK3β protein levels (Kozlovsky et al., 2000) as compared to controls. Akt protein levels in postmortem frontal cortex samples of schizophrenic patients are also lower than controls (Emamian et al., 2004). Therapeutics used in the treatment of schizophrenia such as the antipsychotic and dopamine D2 receptor antagonist haloperidol increase the phosphorylation of pAkt (Thr. 308) without changing pAkt (Ser. 473) (Emamian et al., 2004). Genetic manipulation of dopamine D2 receptors also modulates the activity of Akt and GSK3. Mice lacking the dopamine D2 receptor display an increase in pAkt (Thr. 308) and pGSK3β and a decrease in pAkt (Ser. 473) in the striatum (Beaulieu et al., 2007). Further, administration of the dopamine D2 receptor antagonist raclopride increases phosphorylated Akt (Thr. 308) and GSK3β in the striatum of mice lacking the dopamine transporter (Beaulieu et al., 2004). The data presented here are consistent with previous studies suggesting that dopamine D2 receptors regulate the Akt/GSK3 signaling pathway. Administration of the dopamine D2 receptor antagonist eticlopride prevented the cocaine-induced attenuation of pAkt (Thr. 308) and pGSK3β in the caudate putamen without effecting basal phosphorylation levels of either kinase. Thus, activation of dopamine D2 receptors is essential to cocaine-induced regulation of Akt and GSK3 activity.

The present study provides the first evidence indicating that the dopamine D1 receptor is involved in cocaine-induced regulation of pGSK3β. Recent evidence from our laboratory indicates that GSK3 plays a role in dopamine D1 receptor mediated behaviors. Selective inhibition of GSK3 attenuates hyperactivity produced by administration of the D1 receptor agonist SKF-82958 (Miller et al., 2009b). Previous investigations, however, focusing on the relationship between the dopamine D1 receptor and the Akt/GSK3 signaling cascade are contradictory. Administration of SCH-23390 to mice lacking the dopamine transporter has no effect on the pAkt (Thr. 308) or pGSK3α/β in the striatum (Beaulieu et al., 2004). Mice lacking the dopamine D1 receptor however show a decrease in pAkt (Ser. 473) in the striatum (Beaulieu et al., 2007). In contrast, dopamine D1 receptor agonists increase pAkt (Thr. 308) in primary striatal neuronal cultures (Brami-Cherrier et al., 2002).

In the present study, we show that pretreatment with the dopamine D1 receptor antagonist SCH-23390 prior to cocaine prevented cocaine-induced attenuation of pGSK3β but not pAkt (Thr. 308) in the caudate putamen. This suggests that the dopamine D1 receptor differentially regulates the activity of Akt and GSK3. Given the lack of pAkt (Thr. 308) regulation, it is tempting to speculate that another kinase may be regulating pGSK3β in response to dopamine D1 receptor activation. It may be that GSK3 regulation following cocaine is contingent upon cAMP or calcium-dependent signal transduction and not solely on Akt. Dopamine D1 receptors influence calcium-dependent signal transduction by coupling to the Gq protein (Wang et al., 1995) and releasing calcium from intracellular stores (Bergson et al., 2003). Activation of Gq increases the activity of GSK3β and is not dependent on Akt (Fan et al., 2003). Thus, blockade of the dopamine D1 receptor and inactivation of the Gq protein may be regulating the activity of GSK3 in the caudate putamen following cocaine.

Interestingly, other receptor types modulate the activity of Akt and GSK3 suggesting that the acute effect of cocaine on Akt and GSK3 may involve other, non-dopaminergic receptors. Since cocaine increases extracellular glutamate in the caudate putamen (McKee and Meshul, 2005), we investigated whether the NMDA receptor antagonist MK-801 would prevent the modulation of Akt and GSK3 activity following acute cocaine. Here, we show that blockade of the glutamatergic NMDA receptor prevented the cocaine-induced attenuation of pGSK3β in the caudate putamen. Our results agree with previous investigations highlighting the relationship between the

NMDA receptor and GSK3. Stimulation of the NMDA receptor activates GSK3 via protein phosphatase-1 in the adult mouse brain (Szatmari et al., 2005). In addition, inhibition of GSK3 can cause NMDA receptor internalization (Chen et al., 2007). Blockade of the NMDA receptor prior to cocaine does not affect the cocaine-induced inactivation of Akt in the caudate putamen, suggesting that regulation of Akt 30 minutes following acute cocaine is not contingent upon activation of the NMDA receptor. Although the NMDA receptor does not effect acute cocaine-induced regulation of Akt, the NMDA receptor may be involved in Akt plasticity following repeated cocaine exposure. Blockade of the NMDA receptor prevents the development of cocaine-induced behavioral sensitization (Karler et al., 1989) and repeated MK-801 administration increases Akt activity (Seo et al., 2007). Thus, as repeated cocaine exposure produces sensitization and decreases Akt activity (Pulipparacharuvil et al., 2008), blockade of the NMDA receptor during the development of cocaine sensitization may increase Akt activity, therefore preventing cocaine sensitization.

In summary, the data presented herein demonstrate that Akt and GSK3 are selectively regulated by cocaine in the caudate putamen but not the nucleus accumbens or frontal cortex of mice. Activation of GSK3 in the caudate putamen is contingent upon dopamine D1 and D2 receptors and the glutamatergic NMDA receptor, whereas activation of Akt activity is dependent upon dopamine D2 receptors. Our data provide further evidence indicating that psychostimulants such as cocaine modulate the activity of Akt and GSK3. Future studies investigating the spatial and temporal pattern of psychostimulant-induced regulation of Akt/GSK3 are needed in order to elucidate the mechanisms in which manipulation of Akt/GSK3 may affect psychostimulant-induced behaviors.

CHAPTER 6

GENERAL DISCUSSION

Overview

Cocaine exposure causes a number of behavioral and neuromodulatory effects with repeated use potentially culminating in drug addiction. The behaviors characterizing addiction such as drug-seeking, craving and relapse are influenced by neuroadaptations of molecular substrates involved in reward-related and learning memory processes (Nestler, 2004; Miller and Marshall, 2005; Thomas et al., 2008). Thus, elucidating new molecular mechanism underlying acute/occasional drug use and repeated drug taking is essential for understanding addiction. As such, the aim of the experiments presented in this thesis was to investigate the potential neuromodulatory effect of the intracellular signaling protein, glycogen synthase kinase 3 (GSK3) on cocaine-induced behaviors.

Previous investigations suggest that GSK3 is critical to the hyper-locomotor responses associated with acute amphetamine (Beaulieu et al., 2004; Gould et al., 2004) and in mice lacking the dopamine transporter (Beaulieu et al., 2004). It is well established that acute cocaine causes a dose-dependent increase in locomotor activity and rearing in animals (Ushijima et al., 1994). As such, we hypothesized that GSK3 would regulate the hyper-locomotor response following acute cocaine administration. The data presented herein demonstrate a role for GSK3 in acute cocaine-induced locomotion. Specifically, we show that both valproate, which inhibits GSK3, and the selective inhibitor of GSK3 SB 216763 dose-dependently attenuated acute cocaine-induced hyperactivity (Table 6.1). Repeated cocaine administration elicits a sensitized or increased response to the locomotor-stimulating properties of the drug (Post and Rose, 1976; Robinson and

Berrridge, 1993). In addition to the role of GSK3 in the acute hyper-locomotor response of cocaine, we show that GSK3 activity is necessary for the development of the sensitized locomotor response associated with repeated cocaine administration as selective inhibition of GSK3 prevented the development of cocaine-induced behavioral sensitization (Table 6.1).

Table 6.1: Inhibition of GSK3 modulates cocaine-induced behavioral responses.

Behavioral Paradigm	Effect
Acute Cocaine-Induced Locomotion	↓
Cocaine-Induced Locomotor Sensitization	↓
Development of Cocaine Place Preference	↓
Retrieval of Cocaine Context Memories	↓
Reinstatement of Cocaine Place Preference	NC

↓=Decrease, NC=No Change

The dopamine D1 receptor is critical to cocaine-induced hyper-locomotion as systemic and intra-accumbens infusions of the dopamine D1 receptor antagonist SCH-23390 attenuate acute cocaine-induced hyper-locomotion (Cabib et al., 1991; Baker et al., 1998). Data presented herein demonstrate that administration of the selective GSK3 inhibitor SB 216763 attenuated the heightened ambulatory and stereotypy responses produced by selective dopamine D1 receptor stimulation. This provided the first evidence suggesting that a signaling pathway comprising GSK3 is activated by dopamine D1 receptor stimulation and is critical for the behavioral response to dopamine D1 receptor agonists. Taken together, the results of this study suggest that either the direct activation of dopamine D1 receptors or the indirect activation of D1 receptors by cocaine activates GSK3 to elicit a hyper-locomotor response.

Previous investigations highlight the importance of the Akt/GSK3 signaling pathway in regulating amphetamine-induced behaviors (Beaulieu et al., 2004; Gould et al., 2004). Additional studies presented in this dissertation have shown that acute cocaine activates GSK3β selectively in the caudate putamen as evidenced by a reduction in phosphorylated GSK3β 30 minutes following cocaine administration. Alternatively, acute cocaine inactivates Akt by reducing the levels of phosphorylated Akt (Thr. 308) in the caudate putamen (Table 6.2). Interestingly, Akt (Ser. 473) phosphorylation was not altered in the caudate putamen following acute cocaine administration. These results are consistent with previous studies indicating that increased extracellular dopamine selectively modulates Akt (Thr. 308) but not Akt (Ser. 473) phosphorylation in the striatum (Beaulieu et al., 2004, 2005). As Akt (Thr. 308) phosphorylation is contigent upon PDK-1 (Alessi et al., 1997; Stephens et al., 1998), cocaine acting upon PDK-1 may cause the reduction of Akt (Thr. 308) phosphorylated levels. To date, the mechanism by which Akt (Ser. 473) is phosphorylated has yet to be determined although investigations suggest that regulation may occur via the protein kinase mTOR (Sarbassov et al., 2005). Thus, future investigations focusing on mTOR induced regulation of pAkt (Ser. 473) levels may provide insight into the mechanism by which cocaine regulates Akt activity.

Table 6.2: The effects of acute cocaine on phosphorylated levels of GSK3 and Akt in the brain.

Brain Region	pGSK3 (Ser. 9)	pGSK3 (Ser. 21)	pAkt (Ser. 473)	pAkt (Thr. 308)
Caudate Putamen	↓	NC	NC	↓
Nucleus Accumbens	NC	NC	NC	NC
Frontal Cortex	NC	NC	NC	NC

↓= Decrease, NC= No Change

The ability of cocaine to activate GSK3 is contingent upon dopamine D1 receptor stimulation, as pretreatment with the D1 receptor antagonist SCH-23390 prevented cocaine-induced activation of GSK3 in the caudate putamen. Interestingly, cocaine-induced activation is also mediated by the glutamatergic NMDA receptor and the dopamine D2 receptor (through the upstream kinase Akt) (Table 6.3). Thus, the neuromodulatory effects of cocaine on GSK3 signaling through dopaminergic and glutamatergic receptors are essential to the behavioral manifestations of the drug.

Table 6.3: The effects of pretreatment with dopaminergic or glutamatergic receptor antagonists on cocaine-induced reductions of phosphorylated GSK3 (Ser. 9) and Akt (Thr. 308) levels in the caudate putamen.

Protein	Antagonist	Effect
pGSK3 (Ser. 9)	Eticlopride	Prevents cocaine-induced reduction
	SCH23390	Prevents cocaine-induced reduction
	MK-801	Prevents cocaine-induced reduction
pAkt (Thr. 308)	Eticlopride	Prevents cocaine-induced reduction
	SCH23390	No Effect
	MK-801	No Effect

The behavioral abnormalities associated with addiction are contingent upon perturbations of specific intracellular signaling proteins and transcriptions factors. Recent evidence suggests that a number of intracellular signaling proteins and transcription factors mediate the development (Valjent et al., 2000), retrieval (Miller and Marshall, 2005) and reinstatement (Valjent et al., 2006) of cocaine-conditioned reward. Given the neuromodulatory effects of GSK3 in cocaine-induced locomotor responses, we assessed the role of GSK3 in various facets of cocaine-conditioned reward.

Our results provide the first evidence demonstrating that the development of cocaine-conditioned reward is prevented by selective inhibition of GSK3. These data suggest that activation of GSK3 by cocaine is essential to the development of cocaine place preference (Table 6.1). Interestingly, following the expression of cocaine preference, inhibition of GSK3 in a neutral environment for 2 days prevented the expression of cocaine place preference 24 hours later (Table 6.1). This suggests that inhibition of GSK3 reduced cocaine-seeking behavior by preventing the reconsolidation of cocaine-associated contextual memories. Our results agree with investigations highlighting the importance of glutamatergic transmission in the retrieval and reconsolidation of cocaine contextual memories. For example, administration of the NMDA receptor antagonist MK-801 immediately following the expression of cocaine place preference (during memory reconsolidation) blocks the subsequent expression of context-elicited preference (Itzhak, 2008). The effect of GSK3 on reconsolidation and retrieval of cocaine-associated contextual memories may be contingent on regulation of GSK3 and NMDA receptors in the basolateral amygdala. The basolateral amygdala contains a high concentration of NMDA receptors and directly impacts the reconsolidation of contextual memories (Monaghan and Cotman, 1985). Interestingly, binge pattern cocaine administration increases the activity of GSK3 in the amygdala (Perrine et al., 2008). Given the role of GSK3 in maintaining the function of NMDA receptors (Chen et al., 2007), GSK3 acting on NMDA receptors in the amygdala may be an underlying mechanism preventing the reconsolidation and subsequent retrieval of cocaine-associated contextual memories.

Our results also indicated a differential role for GSK3 in the reconsolidation and subsequent retrieval of cocaine contextual memories following exposure to the conditioned stimulus (environment) as compared to memory reactivation following exposure to the previously unconditioned stimulus (cocaine). Inhibition of GSK3 in a neutral environment during extinction training did not prevent cocaine-primed reinstatement of place preference (Table 6.1). As relapse to drug-seeking behavior can be precipitated following exposure to stress, contextual cues, or the drug itself, the role of GSK3 in reinstatement to drug-seeking behavior may be similar to the substrates it regulates such as CREB, therefore being effective at preventing reinstatement following exposure to stress (Kreibich and Blendy, 2004). Overall, the data presented herein demonstrate that GSK3 is a neuromodulator of cocaine-induced behavior responses and future investigations focusing on GSK3 may provide new insights into the mechanisms underlying cocaine addiction.

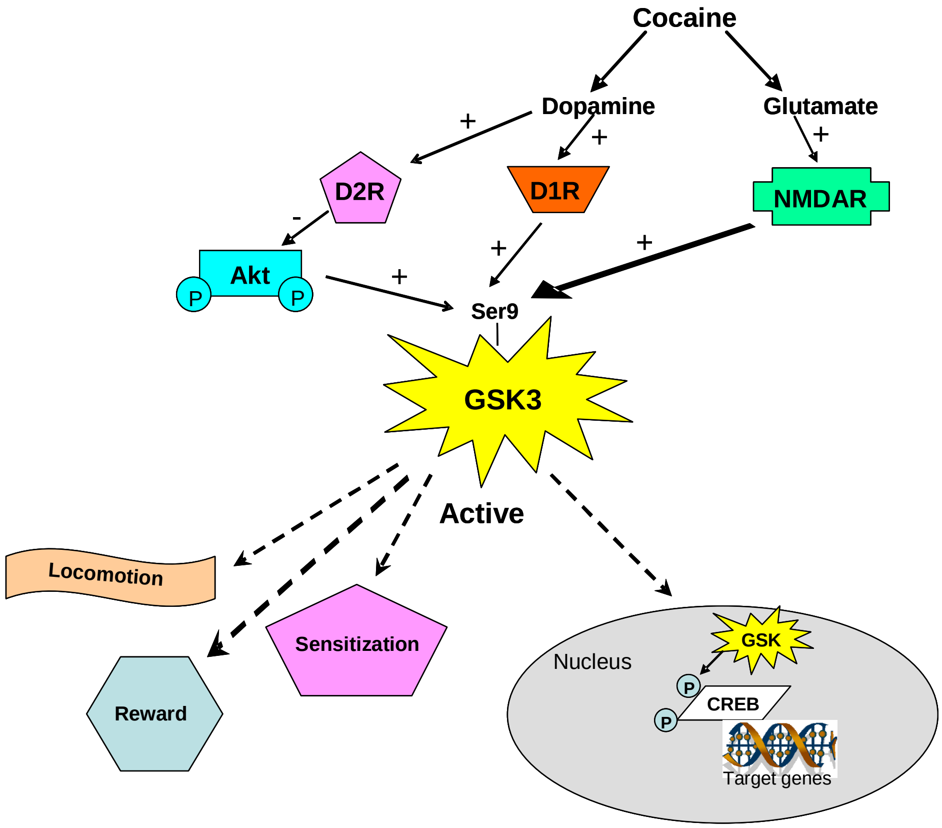

Figure 6.1: Schematic representation of the role of GSK3 in the behavioral and neuromodulatory effects of cocaine. Abbreviations: D2R=dopamine D2 receptor, D1R=dopamine D1 receptor, NMDAR=N-methyl-D-aspartate receptor, Akt=protein kinase B, GSK3=glycogen synthase kinase-3, CREB=cyclic AMP response element binding protein.

Future Directions

The results presented in this dissertation have shown the importance of GSK3 in cocaine-induced acute and sensitized locomotor responses, conditioned reward and reconsolidation. Further, we show that cocaine regulates the Akt/GSK3 signaling cascade, an effect that is contingent upon dopaminergic and glutamatergic receptors. Given the role of the mu opioid receptor in the behavioral (Schroeder et al., 2007; Soderman and Unterwald, 2008) and neuromodulatory effects of cocaine, it would be interesting to investigate the role of the mu opioid receptor on the regulation of Akt and GSK3. Previous evidence indicates that repeated administration of morphine increases the activity of GSK3 and decreases the activity of Akt in the ventral tegmental area (Russo et al., 2007). Further, selective inhibition of GSK3 using SB 216763 blocks the development of tolerance to the analgesic effects of morphine (Parkitna et al., 2006).

Preliminary data from our laboratory suggests that mice lacking the mu opioid receptor (MOR-KO) have an attenuated response to the acute locomotor stimulating effects of cocaine (Figure 6.2). Further, MOR-KO mice display a decreased expression of GSK3α/β protein levels in the caudate putamen (Figure 6.2). Interestingly, decreased expression of GSK3 attenuates amphetamine-induced hyper-locomotion, whereas transgenic mice expressing a constitutively active form of GSK3 show an increase in locomotion as compared to controls (Prickaerts et al., 2006). Given the importance of GSK3 in cocaine-induced hyper-locomotion and reward, future investigations focusing on how mu opioid receptors signal through GSK3 may provide new insights with respect to the neuromodulatory effects of the kinase in cocaine behaviors. In addition, the behavioral paradigms outlined in this dissertation would be useful in investigating the importance of GSK3 in morphine-induced locomotion and reward.

To further assess the role of GSK3 in drug-seeking behavior, it would be interesting to assess which brain regions may specifically be involved in modulation of drug-seeking behavior by GSK3. Previous investigations have identified the importance of the basolateral amygdala in drug-seeking behavior and repeated cocaine exposure activates GSK3 in this brain region (Perrine et al., 2008). Thus, direct injections of GSK3 inhibitors or siRNA into the basolateral amygdala following the initial expression of cocaine-induced place preference would identify whether inhibition of GSK3 in the basolateral amygdala is one mechanism by which GSK3 modulates reconsolidation and the renewal of drug-seeking behavior during exposure to the conditioned stimulus. In addition, it would be interesting to assess the activity of GSK3 following the expression of drug-seeking behavior. Here, we hypothesize that the activity of GSK3 would be increased during the expression of place preference as inhibition of GSK3 during cocaine conditioning prevented the development of cocaine-induced place preference whereas inhibition of GSK3 in a neutral environment following the initial expression of place preference prevented the renewal of drug-seeking behavior.

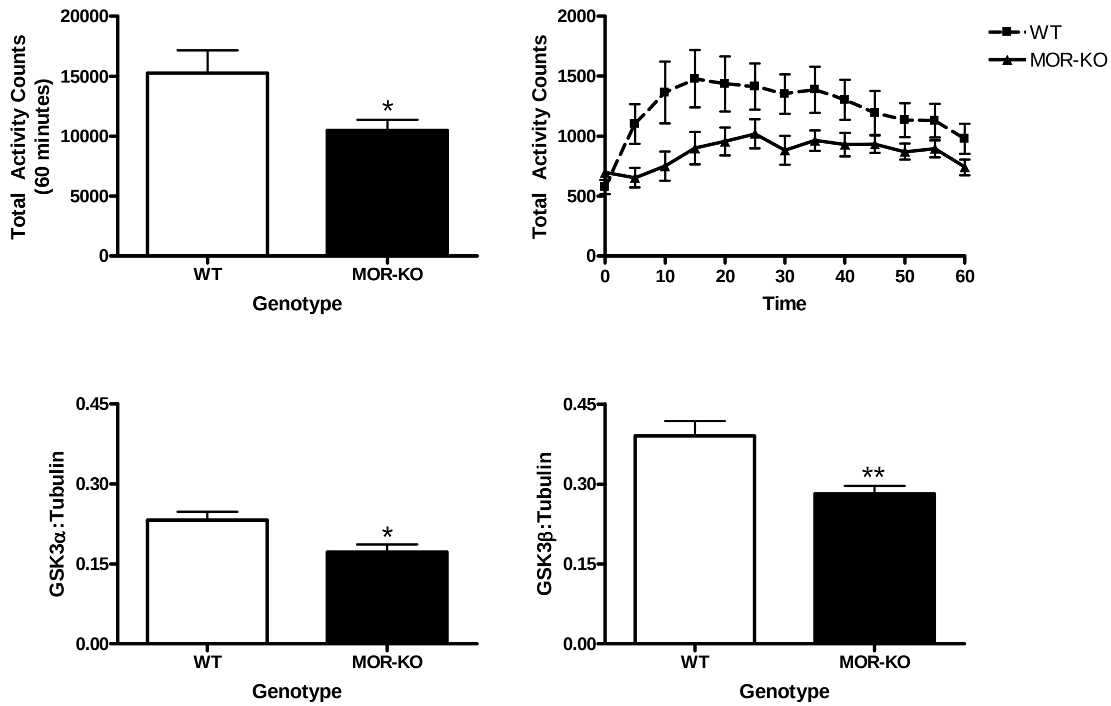

Figure 6.2: MORs regulate cocaine-induced behavior and the expression of GSK3α/β. MOR-KO mice display a significantly attenuated locomotor response to acute cocaine (t_{19}=2.362, p=0.0290) and have a decreased expression of GSK3α/β protein levels in the caudate putamen (GSK3α; t_{19}=2.703, p=0.0141); (GSK3β; t_{19}=3.135, p=0.0054). Abbreviations: WT= wild-type, MOR-KO=Mu opioid receptor knockout.

As inhibition of GSK3 prevents the reconsolidation and retrieval of cocaine-associated contextual memories, it would be interesting to assess whether GSK3 can attenuate the extinction of cocaine-induced reward. Extinction refers to a form of new and active learning that aims to reduce the resumption of drug-seeking following exposure to stress, contextual cues or the drug itself. Animal models of extinction training consist of repeated daily testing and repeated exposure to the previously drug-paired environment (conditioned stimulus) in the absence of drug (unconditioned stimulus), therefore resulting in a decline in place preference (Mueller and Stewart, 2000). Given the role of GSK3 in cocaine-conditioned reward, we hypothesize that inhibition of GSK3 in the drug-paired environment (conditioned stimulus) may decrease learning and memory, thereby slowing the extinction of cocaine place preference.

Further, our data show that acute administration of cocaine activates GSK3 in the caudate putamen as evidenced by a decrease in GSK3 phosphorylation without effecting GSK3 activity in the nucleus accumbens. Given the importance of the nucleus accumbens in cocaine-induced reward (Roberts et al., 1977, 1980) as well as the role of GSK3 in cocaine-induced place preference, it would be interesting to assess the activity of GSK3 in the nucleus accumbens during the development of cocaine-induced place preference. Local injections of GSK3 inhibitors or siRNA into the nucleus accumbens during the development of cocaine-induced place preference will provide evidence as to the importance of GSK3 activity in the nucleus accumbens during the acquisition of cocaine reward. Further, experiments focusing on the importance of GSK3 activity in the accumbens following the expression of cocaine place preference will further establish the role of GSK3 in cocaine-seeking behaviors during exposure to a conditioned stimulus.

Regulation of GSK3 activity is critical in that GSK3 phosphorylates more than 40 substrates (Jope and Johnson, 2004). Given the ability of GSK3 to regulate various proteins and transcription factors including CREB, it would be interesting to assess whether GSK3 can modulate proteins critical to the expression of cocaine-induced behaviors such as ΔFosB. Evidence using genetically modified mice suggests that ΔFosB regulates cocaine-induced behavioral responses (Haile et al., 2001; Hiroi et al., 1997, Kelz et al., 1999). For example, the over-expression of ΔFosB in the striatum of mice enhances behavioral responses to chronic cocaine (Kelz et al., 1999). In addition, FosB-KO mice exhibit increased locomotor activity following acute cocaine administration, yet fail to exhibit behavioral sensitization following repeated cocaine administration (Hiroi et al., 1997). ΔFosB is a splice variant of fosB with a truncated C-terminus (Nakabeppu and Nathans, 1991). Fos family members heterodimerize with Jun family transcription factors (c-Jun, JunB, JunD) to form the activator protein-1 (AP-1) complex (Gentz et al., 1989; Nakabeppu and Nathans, 1991). The AP-1 complex binds to specific DNA sequences in the promoters of various target genes and can act as either inducers or repressors of transcription (Chao and Nestler, 2004). Alternatively, GSK3 negatively regulates AP-1 activation by phosphorylating c-Jun at the Thr-239, Ser-243, and Ser-249 sites therefore decreasing DNA binding activity (Boyle et. al, 1991). In addition, there have been several studies in cultured cells and in the rat brain indicating an increase in AP-1 activity following treatment with the GSK3 inhibitor lithium (Hedgepeth et al., 1997; Jope and Song, 1997; Ozaki and Chuang, 1997; Asghari et al., 1998; Yuan et al., 1998). Thus, inhibition of GSK3 may enhance the upregulation of ΔFosB following cocaine administration, indicating a role for GSK3 in cocaine-induced plasticity.

Overall, the data presented herein demonstrate that GSK3 is essential to the behavioral effects of cocaine. Further, we show that cocaine selectively regulates GSK3 activity in the brain, an effect contingent upon dopaminergic and glutamatergic receptors. Thus, future investigations focusing on the behavioral and neuromodulatory implications of GSK3 on drugs of abuse may provide new insights into the mechanism of addiction.

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