EFFECT OF GLYCOGEN SYNTHASE KINASE-3 INHIBITION ON THE ACQUISITION AND EXPRESSION OF COCAINE-CUE CONDITIONED ACTIVITY AND SPONTANEOUS LOCOMOTION IN RODENTS

By

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A thesis submitted to the Department of Psychology

In conformity with the requirements for

The degree of Master of Science

Queen`s University

Kingston, Ontario, Canada

September, 2014

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Abstract

Pairing environmental stimuli with pro-dopaminergic drugs can produce conditioned effects on locomotor behaviour in rodents, an effect that is used to model incentive learning and drug craving in laboratory animals. One of the targets of dopamine signaling is glycogen synthase kinase-3 (GSK-3), a nearly constitutively active enzyme involved in a variety of cellular activities and signaling pathways. Recently, GSK-3 has been shown to be modulated by activity at the dopamine D2 and D3 receptors, both of which have been implicated in the acquisition and expression of conditioned activity. Here, it was hypothesized that the selective GSK-3 inhibitor, A-1070722 (Abbott Laboratories, Ludwigshafen, Germany), would block the acquisition and expression of cocaine-cue conditioned activity in Wistar rats.

Results indicated a block of the expression, but not acquisition, of conditioned activity at 10 and 20 mg/kg doses of A-1070722, but not 0, 1, or 5 mg/kg doses. The 10 mg/kg dose also appeared to decrease spontaneous locomotion, suggesting that the effect of A-1070722 on the expression of conditioned activity may be due an effect on locomotor behaviour more generally, rather than being due to a selective attenuation of the expression of incentive learning produced by cocaine-associated environmental stimuli.

Acknowledgements

I would like to thank our Animal Training Co-ordinator, Lisa Willberforce, for her excellent training and for her help throughout this project. I would also like to thank my thesis committee members, Dr. Cella Olmstead and Dr. Janet Menard for their invaluable input and support; Patrick Cote, for his love and for encouraging me throughout my University career. I would like to thank my family for always believing in me -- especially my sister, Mindy Rocca. A special thanks to Connie Reed, my best friend since high school, who encouraged me to take Brain and Behaviour in my second year of University, and who is quite possibly responsible for the career path I am on.

Most of all, I would like to thank Dr. Richard J. Beninger, my supervisor, for believing in me and for giving me the opportunity to work in his lab and to contribute to the science that I love. To him I will always be grateful. I look forward to working with him on future projects, and am happy to be continuing on in his lab for my PhD.

All original work presented here was made possible by a grant to Dr. Beninger from The Natural Sciences and Engineering Council of Canada.

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List of Abbreviations

AMPH – amphetamine

ACQ – acquisition

Akt – also known as protein kinase B

cAMP – cyclic adenosine monophosphate

CPP – conditioned place preference

D2 – dopamine D2 receptor

D3 – dopamine D3 receptor

EXP - expression

GSK-3 – Glycogen synthase kinase-3

PKA – protein kinase A

Chapter 1

Introduction

Overview: Conditioned activity is a context-specific enhancement of locomotion following multiple pairings of environmental stimuli with motor-activating drugs. This paradigm has been used to study drug reward and incentive learning (Beninger, 1983) as well as to model drug-craving in laboratory animals (Le Foll, Frances, Diaz, Schwartz, & Sokoloff, 2002). Dopamine signaling is crucial for the development and expression of conditioned activity. Dopamine D2-like receptor antagonists such as haloperidol more effectively block the acquisition than the expression of conditioned activity (Banasikowski et al., 2010; Beninger & Herz, 1986) while selective dopamine D3 antagonists and partial agonists can more effectively block expression than acquisition of conditioned activity (Banasikowski et al., 2010; Le Foll et al., 2002).

Recently, a target of D2-like receptor-signaling, glycogen synthase kinase-3 (GSK-3), has gained a great deal of attention for its role in dopamine-mediated behaviours. GSK-3 is inhibited by D2-receptor antagonists (reviewed in Beaulieu, 2012) and has altered expression in D3 knock-out mice (Beaulieu et al., 2007). GSK-3 has been found to modify the reconsolidation of cocaine reward memory (Wu et al., 2011; Shi, Miller, Harper, Pool, Gould, & Unterwald, 2014), and has been shown to attenuate the development of psychostimulant sensitization (Enman & Unterwald, 2012; Miller, Tallarida, & Unterwald, 2009; Xu et al., 2009), suggesting a role for GSK-3 in the neuroplastic changes that occur in response to psychostimulant use. In the current investigation, it was hypothesized that systemic delivery of the potent and selective GSK-3 inhibitor, A-1070722, would block the acquisition and expression of cocaine-cue conditioned activity. Furthermore, it was hypothesized that GSK-3 inhibition would not affect locomotion

per se, but would instead selectively influence the enhanced locomotion that occurs in response to cocaine or to conditioned stimuli.

1.1 Reward and Incentive Learning

Neutral stimuli preceding events that lead to obtaining primary reinforcers such as food or water can develop incentive motivational qualities, such that presentation of those conditioned stimuli alone can increase approach behaviours (Beninger, 1983). This also occurs with psychostimulant drugs that act as primary reinforcers. The ability of an organism to rapidly learn associations between stimuli that predict reward or punishment has adaptive value in that these associations can assist in obtaining biologically-important stimuli, as well as in avoiding harmful stimuli (Schultz, 2000).

The existence of a brain "reward system" was hinted at early on by Olds and Milner (1954) who showed that electrical stimulation can be self-administered by laboratory animals via a lever in a Skinner box if the electrodes are placed around the septum and lateral hypothalamus (Olds & Milner, 1954; Olds, 1956). Today, it is generally understood that reward and incentive learning involve activity within a neural network comprising the dopaminergic ventral tegmental area, the nucleus accumbens (NAc), the ventral pallidum, the amygdala, the prefrontal cortex, and others (reviewed in Di Chiara & Bassareo, 2007; Fields, Hjelmstad, Margolis, & Nicola, 2007; Koob, 1992).

Incentive learning may prove beneficial for organisms as they learn to navigate their environments and obtain useful reinforcers such as food or water, but certain situations arise in which the processes leading to this type of learning may prove harmful. For example, incentive learning occurs when formerly neutral stimuli are paired with drug states in human drug users,

and may be important for relapse to drug use in those who are abstinent (Fuchs, Lasseter, Ramirez, & Xie, 2008). Participants with a history of cocaine use, for example, experience physiological changes such as increased heart rate and decreases in skin temperature when shown cocaine-associated images (such as drug use paraphernalia) as opposed to opiate-associated or neutral non-drug related stimuli, and report increased subjective feelings of craving and withdrawal (Ehrman, Robbins, Childress, & O'Brien, 1992). Using animal models of incentive learning may be useful for identifying pharmacological compounds that could help individuals struggling with addiction (Le Foll et al., 2002).

1.2 Conditioned Activity and Incentive Learning

In conditioned activity, a rewarding drug such as cocaine is given in a distinct environment. Animals with a history of pairings of the drug with the environment show heightened locomotion compared to control animals following a drug-free injection (Pickens & Crowder, 1967). Since control animals are also given the stimulant – though in a different environment, such as their homecages after daily conditioning sessions – it can be concluded that the heightened locomotor response is not due to a history of stimulant administration *per se* but due to the pairing of the drug with the test environment. Therefore, experimental animals are referred to as cocaine-*paired*, while controls are referred to as cocaine-*unpaired*.

Rewarding stimuli elicit approach and other behaviour in animals (such as heightened exploratory behaviour), and formerly-neutral stimuli paired with reward can develop incentive motivational properties such that their presentation alone can elicit conditioned approach responses (Beninger, 1983; Di Chiara & Bassareo, 2007). Since pro-dopaminergic drugs can elicit approach behaviours and stimulant-paired animals show conditioned locomotion in the absence of drug, conditioned activity can be viewed as a demonstration of the incentive

motivational properties environmental stimuli have developed as a consequence of repeated pairings with drug (Beninger, 1983; Beninger & Banasikowski, 2008). Pharmacological manipulations during the acquisition (conditioning) phase that prevent the development of conditioned activity are considered different in nature than manipulations that block the demonstration or expression of conditioned activity, since compounds that may block one do not necessarily block the other (for example, see Banasikowski et al., 2010). This would suggest that the neural mechanisms involved in acquiring or learning the associations between a drug state and a paired environment may be distinct from the mechanisms that drive or maintain that learned behaviour (Beninger, 1983).

1.3 Role of Dopamine

Dopamine is a catecholamine-neurotransmitter that is important for reward-related learning (Beninger, 1983; Koob, 1992; Schultz, 2000) and is crucially involved in the development of stimulant-conditioned locomotion (Gold, Swerdlow, & Koob, 1988). Indirect agonists such as amphetamine (AMPH), as well as selective D1-like or D2 agonists (e.g., SKF 38393 and quinpirole) can produce conditioned activity (Mazurski & Beninger, 1991), suggesting activity at either subtype of dopamine receptor is capable of influencing the development of conditioned responses. (For a description of dopamine receptor subtypes, see section 1.4.) Antagonism of D2 receptors has a stronger attenuating effect on the acquisition of conditioned activity than its expression (Banasikowski et al., 2010), whereas D3 antagonists (Banasikowski et al., 2010), partial agonists (Le Foll et al., 2002), or other compounds such as cocaine- and amphetamine-regulated peptide protein (Yoon et al., 2010) have stronger attenuating effects on expression than acquisition. The intracellular signaling events that are set into motion by activity at different dopamine receptors that result in the development or expression of conditioned activity have not

been thoroughly assessed, though it has been shown that protein kinase A (PKA) inhibition in the nucleus accumbens is sufficient for prevention of the acquisition of AMPH conditioned activity (Sutton, McGibney, & Beninger, 2000). Roles for other signaling cascades such as the Akt-GSK-3 pathway (section 1.4) have not yet been evaluated.

1.4 Dopamine Receptor Subtypes

Originally, two subtypes of dopamine receptors were described: D1 and D2, with opposing effects on cAMP production (Stoof & Kebabian, 1981). Today at least 5 receptors have been described, categorized as either "D1-like" or "D2-like." The D1 like receptors, D1 and D5, couple with a Gs G-protein that activates the production of cyclic adenosine monophosphate (cAMP) from ATP by adenylate cyclase. Alternatively, the D2-like receptors (D2, D3, and D4) are coupled to Gi/o and inhibit the production of cAMP by adenylate cyclase (for review, see Beaulieu & Gainetdinov, 2011). Other signaling pathways that are cAMP-independent are also modulated by activity at dopamine receptors (section 1.4).

Both D1 and D2 receptors are found postsynaptically, though D2 receptors are also expressed presynaptically (reviewed in Beaulieu & Gainetdinov, 2011). D2 receptors exist in both long and short forms based on the length of the mRNA that encodes the receptor. The short form is mainly expressed presynaptically as autoreceptors, while the long version is mostly expressed postsynaptically (reviewed in Beaulieu & Gainetdinov, 2011). D3 receptors can also be expressed both pre- and postsynaptically, though with a spatial distribution somewhat different than D2; for example, while D2 receptors are abundant within the striatum, D3 receptors tend to be expressed in the ventral striatum (nucleus accumbens) more exclusively (Gurevich & Joyce, 1999).

1.5 Dopamine Receptors and Signaling Pathways

cAMP-Dependent Pathway

Activation of adenylate cyclase increases cAMP production, which is required for the activation of PKA (reviewed in Beaulieu, 2012). Targets of PKA such as cAMP response element-binding protein and cAMP-regulated phosphoprotein of 32 000 kDa (DARPP-32) are believed to be some of the downstream targets of dopamine receptor signaling that influence reward-related learning (Tropea, Kosofsky, & Rajadhyaksha, 2008; Chen, Chen, & Chiang, 2009).

β-Arrestin 2-Akt-GSK-3 Pathway

Dopamine receptors can also indirectly influence the phosphorylation state of GSK-3. When the D2 receptor is activated, G-protein coupled receptor kinases (GRKs) phosphorylate the receptor at sites that are recognized by arrestins such as β-Arrestin 2 (reviewed in Beaulieu & Gainetdinov, 2011). β-Arrestin 2 prevents further G-protein activation despite the receptor being stimulated by a ligand, and is involved in the occlusion of the receptor into the cell membrane through its interactions with clathrin (Beaulieu & Gainetdinov, 2011).

Once bound, β-Arrestin 2 can dephosphorylate Akt (also known as protein kinase B) – a kinase directly involved in the regulation of GSK-3 – through interactions with protein phosphatase 2 (PP2A) (see Fig. 1). Once dephosphorylated, Akt becomes inactive and can no longer phosphorylate its substrates (Kandel & Hay, 1999). Since phosphorylation *inhibits* rather than *activates* GSK-3, activity increases when Akt cannot phosphorylate it (Freyberg, Ferrando, & Javitch, 2010). Therefore, binding of dopamine to the D2 receptor can indirectly promote the sustained activity of GSK-3 by preventing Akt from phosphorylating it via its interactions with an activity-dependent β-Arrestin 2/PP2A protein complex.

Dopamine D3 receptors also may be involved in the regulation of GSK-3. Using western blotting and densitometric analyses of striatal tissue, Beaulieu et al. (2007) showed that drug-naïve D3 knock-out mice had significantly higher levels of phosphorylated (i.e., inhibited) GSK-3β than wild-types or D1 knock-out mice, as well as increased levels of phosphorylated Akt.

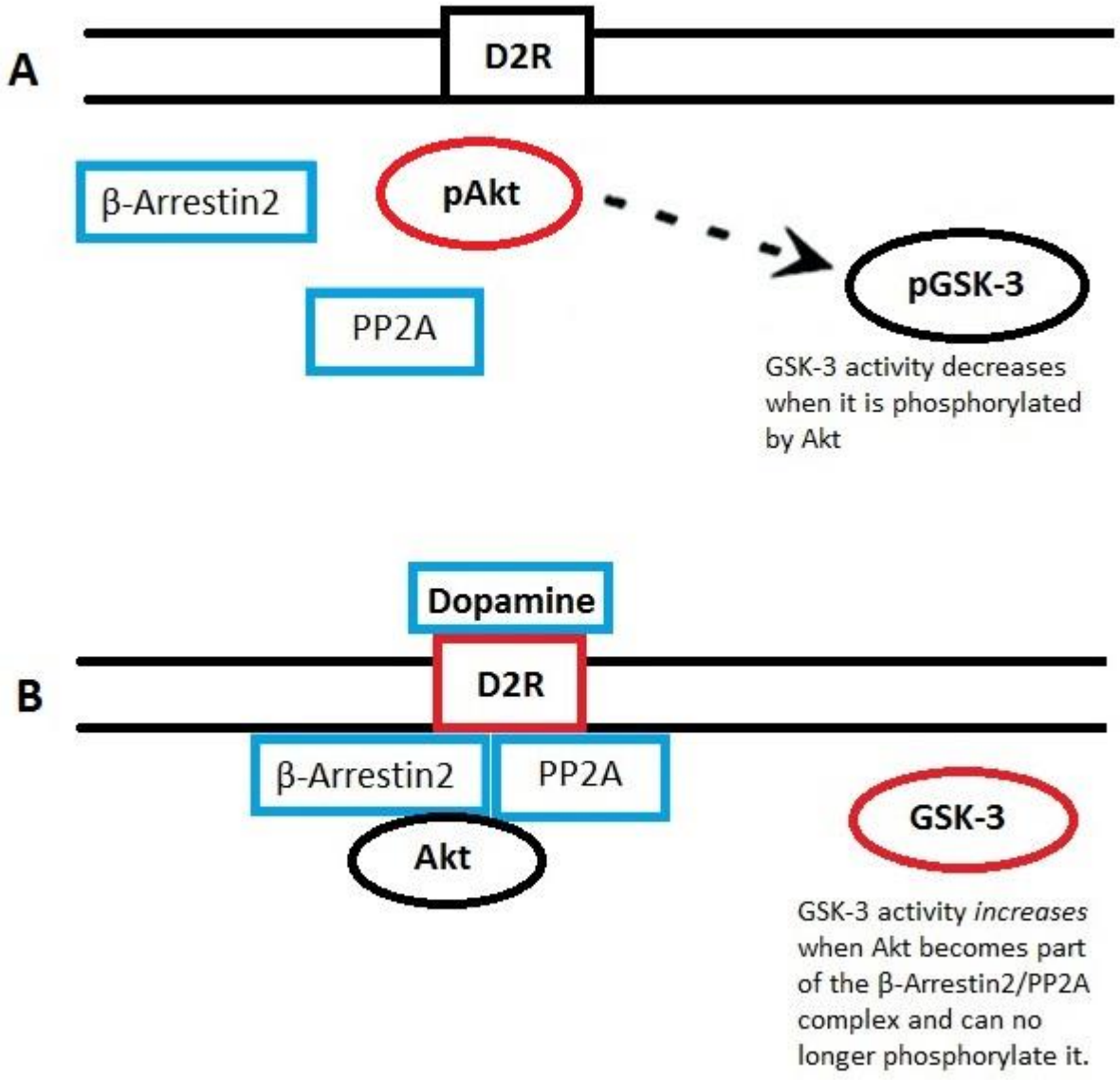

Fig. 1. Dopamine D2 Receptor and GSK-3 A) In the absence of dopamine activity at the D2R receptor, phosphorylated Akt can phosphorylate – and thus, inhibit – GSK-3. B) As a consequence of receptor stimulation by dopamine, the D2R receptor becomes occluded. A protein scaffolding complex consisting of β-Arrestin2, PP2A, and Akt forms. PP2A de-phosphorylates Akt, leaving it inactive and unable to inhibit GSK-3. GSK-3 activity increases. (Adapted from Freyberg et al., 2010.)

1.6 GSK-3

GSK-3 is a serine/threonine protein kinase that is nearly constitutively active. Both α and β isoforms have been identified in humans and other species (Ali, Hoeflich, & Woodgett, 2001). Phosphorylation of specific serine residues (serine 21 for GSK-3α and serine 9 for GSK-3β) can inhibit its activity, as well as dephosphorylation of tyrosine residues (reviewed in Eldar-Finkelman & Martinez, 2011 and Meijer, Flajolet, & Greengard, 2004). Phosphorylated GSK-3 recognizes its own N-terminus as a pseudo-substrate, leading it to autophosphorylate rather than to phosphorylate its target molecules (reviewed in Wu & Pan, 2009).

GSK-3 was first identified for its role in glucose metabolism (Embi, Rylatt, & Cohen, 1980) but has since been shown to be a target of various neuropsychiatric medications, such as lithium (Stambolic, Ruel, & Woodgett, 1996), valproate (Chen, Huang, Jiang, & Manji, 1999), haloperidol, clozapine, fluoxetine, and imipramine (Sutton & Rushlow, 2011), all of which have been shown to be inhibitors of GSK-3 (Beaulieu, 2012). Interestingly, many conditions that are treated by the above medications have been linked with aberrant GSK-3 activity, such as schizophrenia (Blasi et al., 2011; Emamian, 2012; Freyberg et al., 2010) and bipolar disorder (Valvezan & Klein, 2012) (Section 1.8). Other conditions, such as Alzheimer disease, may also involve GSK-3 (Hernandez & Avila, 2008). GSK-3 has been shown to be involved in the hyperphosphorylation of tau protein (Wagner, Utton, Gallo, & Miller, 1996) and could be involved in the formation of the neurofibrillary tangles found in neural tissue from affected individuals (Hernandez, Lucas, & Avila, 2013). Small but nonsignificant improvements in measures such as the Alzheimer's Disease Assessment Scale and others were noted in a pilot study with the GSK-3 inhibitor, tideglusib (Del Ser et al., 2013). A larger clinical trial with this drug is currently underway (Del Ser et al., 2013).

Role in Wnt Signaling

Aside from being regulated by dopamine receptor activity (section 1.4), GSK-3 is also influenced by insulin and Wnt signaling. Wnt is a family of glycoproteins that are important for developmental processes such as cell differentiation and pathological processes such as tumorigenesis (Clevers, 2006; Wu & Pan, 2009). An important downstream target of Wnt receptor signaling is the transcription factor, β-catenin. Under normal conditions, β-catenin is held in a "destruction complex" of proteins including axin, adenomatous polyposis coli (APC), and GSK-3, which facilitates GSK-3's ability to phosphorylate β-catenin (Freyberg et al., 2010; Wu & Pan, 2009). Phosphorylated β-catenin is targeted for proteolysis (breaking of protein bonds), while unphosphorylated β-catenin can accumulate and translocate to the cell's nucleus, where it can activate the transcription of various gene products (Wu & Pan, 2009).

Stimulation of Wnt receptors such as frizzled, along with stimulation of the co-receptor, low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) signal recruitment of a protein called dishevelled (Dvl) to the cell membrane, where it can interfere with the β-catenin destruction complex and prevent GSK-3 from phosphorylating it (Lovestone et al., as cited in Freyberg et al., 2010). Free GSK-3 can then be targeted by Akt for inhibitory phosphorylation (Fukumoto et al., 2001) or is recruited to the cell membrane where it phosphorylates the LRP5/6 co-receptor (Zeng et al., 2008, as cited in Wu & Pan, 2009).

Interestingly, the antipsychotics haloperidol and clozapine, believed to inhibit GSK-3 via interactions with the D2 receptor, appear also to increase the production of Dvl3 in the striatum and prefrontal cortices in rats (Sutton & Rushlow, 2011), suggesting that the inhibition of GSK-3 by these compounds may also involve alterations of Wnt signaling.

1.7 GSK-3: Involvement in Dopamine - Mediated Behaviour

Stimulant Sensitization

Animals with a history of psychostimulant administration show enhanced responses to psychostimulants over time, a process referred to as *sensitization*. Researchers Enman and Unterwald (2012) showed that mice given either the non-selective GSK-3 inhibitor, valproate, or the selective GSK-3 inhibitor, SB216763, had reduced locomotor responses to AMPH. Repeated administrations of AMPH over trials resulted in a typical sensitized response (i.e., enhanced locomotor and stereotypy responses to the drug), but only in animals that were not also treated with the GSK-3 inhibitors. Both these inhibitors were also shown to attenuate the development of cocaine sensitization, as well as the unconditioned locomotor response to it (Miller et al., 2009). However, research by Li, Han, Deng, Chen, and Liang (2004) failed to demonstrate a block of cocaine sensitization by valproate, in contrast to what was shown in Miller et al. (2009), but did show a block of sensitization to methamphetamine.

The ability of GSK-3 inhibitors to disrupt the development of sensitized responses appears to depend upon GSK-3 activity in the core of the nucleus accumbens (NAc). Administration of cocaine (10 mg/kg i.p.) results in heightened GSK-3β activity (i.e., reduced levels of phosphorylated GSK-3) within the core, but not shell, of the NAc (Xu et al., 2009). Pharmacological inhibition of GSK-3 using lithium (i.p) or infusions of SB216763 into the core (but not shell) prevented both the acquisition and the expression of cocaine-induced behavioural sensitization (Xu et al., 2009). Overall, this evidence strongly suggests a role for accumbal GSK-3 – specifically, the β isoform – in the development and expression of cocaine and AMPH behavioural sensitization.

Conditioned Place Preference (CPP)

Another way that stimulant-induced reward can be evaluated is with CPP, a paradigm that involves administration of drug or vehicle in distinct chambers of a test apparatus. More time spent on the drug-paired side is considered indicative of a rewarding response to the drug during conditioning (Carr, Fibiger, Phillips, & Liebman, 1989). Wu et al. (2011) found that inhibition of GSK-3 using infusions of SB216763 into the basolateral amygdala (BLA), but not the central amygdala (CeA), prevented the re-consolidation of cocaine reward memories in rats. Animals that had developed a cocaine-induced CPP spent relatively more time in the cocaine-paired chamber than in a saline-paired chamber. Treatment with SB216763 after the CPP test, however, resulted in no futher demonstrations of CPP after re-testing (Wu et al., 2011). Further, it was shown that exposure to the test environment itself resulted in increased GSK-3 activity in the BLA – but not the CeA. The fact that inhibition of this heightened GSK-3 activity in the BLA was associated with less evidence of CPP at re-test would suggest that GSK-3 within the BLA is important for reconsolidation of reward memories.

1.8 GSK-3: Involvement in Neuropsychiatric Conditions

Schizophrenia:

Recently, work from several labs has implicated GSK-3 and its targets in the pathophysiology of schizophrenia. A Scottish family with a high incidence of conduct disorder, schizophrenia, and other mental health conditions has been shown to have a high incidence of a chromosome 1 mutation resulting in a behavioural phenotype typical of individuals with schizophrenia and at-risk relatives, such as reductions in the P300 event-related potential (Blackwood et al., 2001). Disrupted in Schizophrenia 1 and 2 (DISC1 and DISC2) are proteins

believed to be involved in neurodevelopment and the development of schizophrenia-related phenotypes (reviewed in Blackwood et al., 2001). Work with this family has shown the chromosome 1 mutation to be linked to disruption of these proteins (Blackwood et al., 2001). DISC1 itself has been shown *in vitro* to be a direct modulator of GSK-3β activity and involved in neuronal progenitor cell proliferation (Mao et al., 2009), linking abnormal DISC1 activity and perhaps behavioural phenotypes of schizophrenia to GSK-3β.

A sample of 28 individuals with schizophrenia were found to have 68% lower levels of Akt1 (one of the isoforms of Akt) in lymphocytes compared to healthy controls, as well as a decrease in serine 9 phosphorylated (i.e., inhibited) GSK-3β (Emamian, Hall, Birnbau, Karayiorgou, & Gogos, 2004). Post-mortem tissue samples from patients with schizophrenia revealed reduced Akt1 levels as well as decreased levels of serine 9 phosphorylated GSK-3β, further suggesting this group may have alterations in GSK-3-related pathways (Emamian et al., 2004). Similarly, Blasi et al. (2010) found decreased Akt1 and phosphorylated GSK-3β protein levels in blood cells of patients with schizophrenia who had specific polymorphisms for genes encoding the dopamine D2 receptor and Akt1. Patients with a specific combination of D2 and AKT1 mutations had a tendency to be more responsive to the antipsychotic agent, olanzapine (Blasi et al., 2010), a known D2 receptor antagonist and GSK-3 inhibitor (Li et al., 2007, as cited in Beaulieu, 2012).

These findings draw a link between alterations in GSK-3β activity and other members of the GSK-3β pathway, such as Akt, in individuals with schizophrenia, but do not necessarily show these changes to be casually related to pathogenesis or symptomatology. Work with mice, however, has shown the selective GSK-3 inhibitors, SB216763 and 1-azakenpaullone, to decrease psychotomimetic effects of ketamine, an N-methyl-D-aspartate receptor antagonist

commonly used to model psychosis in animals and known to cause a worsening of psychotic symptoms in schizophrenia patients (Chan, Chiu, Lin, & Chen, 2012). This work suggests antipsychotic potential for inhibitors of GSK-3.

Bipolar Disorder:

With lithium being the first GSK-3 inhibitor identified, it is not surprising that many have asked if aberrant GSK-3 activity occurs in bipolar disorder and if lithium's efficacy in this condition is related to its effects on it (Stambolic et al., 1996; Valvezan & Klein, 2012). As reviewed in Valvezan & Klein (2012), an animal model demonstrating the cyclic nature of bipolar disorder has not been developed. The manic phase of bipolar disorder, however, may be modeled using AMPH-induced hyperactivity, and the depressive phase can be modelled using the forced swim task or others (reviewed in Valvezan & Klein, 2012). AMPH-induced hyperactivity, as discussed above, can often be attenuated by lithium and structurally unrelated mood stabilizers/GSK-3 inhibitors, such as SB216763 (Miller et al., 2009). Interestingly, depressive-like responses in the forced swim task can also be attenuated by lithium as well as antidepressant drugs such as SSRIs (reviewed in Valvezan & Klein, 2012), compounds also shown to be inhibitors of GSK-3 α and β isoforms (Sutton & Rushlow, 2011). Lithium and antidepressant drugs can promote neurogenesis in the hippocampus (Chen, Rajkowska, Du, Seraji-Bozorgzad, & Manji, 2000; Malberg, Eisch, Nestler, & Duman, 2000), and the behavioural effects of antidepressants can be abolished by prevention of neurogenesis due to hippocampal irradiation (Santarelli et al., 2003; reviewed in Valvezan & Klein, 2012). Given that GSK-3 inhibitors show the ability to reduce animal hyperactivity (e.g., Miller et al., 2009; Xu et al., 2009) and themselves display antidepressant-like effects in the forced swimming task (Gould, Einat, Bhat, & Manji, 2004), it stands to reason that GSK-3 and related signaling

molecules may be involved in the ability of various neurotropic compounds to treat the symptoms of bipolar disorder (Valvezan & Klein, 2012).

1.9 Hypotheses

A review of the literature suggests a role for GSK-3 in dopamine-mediated behaviours that are indicative of reward, such as cocaine and AMPH sensitization and CPP. In conditioned activity, block of specific dopamine receptors can attenuate both its acquisition and expression. In general, D2 antagonists more effectively block the acquisition than the expression of conditioned activity, while other compounds such as D3 antagonists more effectively block its expression than its acquisition (Banasikowski et al., 2010). It could be hypothesized that the block of acquisition of conditioned activity is related to GSK-3 activity since it is known that D2 receptor activity regulates GSK-3 phosphorylation (Beaulieu & Gainetdinov, 2011). However, evidence from Beaulieu et al. (2007) and Salles et al. (2013) also suggests the D3 receptor may play a role in regulation of GSK-3 activity. Since it has been shown that D2 and D3 blocking agents differentially affect the acquisition and expression of conditioned activity (Banasikowski et al., 2010), it could be postulated that the differences between selective compounds in their ability to block acquisition or expression of conditioned activity may be related to different influences on intracellular signaling pathways. Here, it was hypothesized that the acquisition and expression of cocaine-cued conditioned activity would be blocked by GSK-3 inhibition using a novel and selective compound, A-1070722. It was also hypothesized that A-1070722 would not affect locomotion *per se*, but would instead only produce between-group differences in cocaine-paired animals (i.e., in animals that should show conditioned activity under normal circumstances).

Chapter 2

METHODS

2.1 Subjects

Three month old male Wistar rats (N = 126) weighing approximately 250 g were ordered from Charles River Laboratories (Montreal, QC, Canada). Rats were housed three per cage in large Plexiglas cages (50 x 40 x 20 cm) and were given *ad libitum* access to water and food (LabDiet 5001, PMI Nutrition International, Brentwood, MO) throughout the experiment. Animals were kept on a reverse light cycle with lights off from 7:00 to 19:00 hr. The temperature in the colony room averaged 21°C with a humidity level between 40 and 80%. Rats were handled for 1-5 min per day for 5 consecutive days prior to the initiation of experiments. Animals were treated in accordance with the guidelines set forth by the Animals for Research Act and the Canadian Council on Animal Care. Experimental procedures were approved by the Queen's University Animal Care Committee.

2.2 Drugs

Cocaine hydrochloride [3b-hydroxy-1a*H*,5a*H*-tropane-2b-carboxylic acid methyl ester benzoate hydrochloride] (Sigma-Aldrich, Oakville, ON) was dissolved in a 0.9% saline solution. A-1070722 was obtained from Abbott Laboratories (Ludwigshafen, Germany) and was dissolved in either 100% dimethyl sulfoxide (Sigma-Aldrich) or suspended in a 20:80 mixture of Kolliphor EL (Sigma-Aldrich) and distilled water. Dimethyl sulfoxide was used in the cocaine paired + 10 mg A-1070722 in Acquisition and Expression groups (see below) but was replaced with the Kolliphor solution for subsequent groups. All injections were given intraperitoneally in a 1 ml/kg volume.

2.3 Apparatus

Six clear Plexiglas automated activity chambers (41.0 x 50.0 x 37.0 cm) were used to assess locomotion (see Fig. 2). The chambers are housed in wooden boxes insulated with Styrofoam. Each chamber is equipped with seven infrared light emitters and detectors, positioned 5 cm above the metal grid floor. Three pairs of emitters and detectors are placed at 10-cm intervals along the 41.0-cm long walls and four pairs are found at 10-cm intervals on the front and back of the 50.0 cm long walls. The chambers are ventilated with a fan and contain a small 2.5-W incandescent light bulb. Each horizontal beam break (disruption of the signal from the emitter to the detector) caused by the rat during locomotion was recorded by a 1 GHz IBM (White Plains, NY) computer running Windows® XP operating system software. The sampling rate was kept at 80 Hz. For a full description see Beninger et al. (1985).

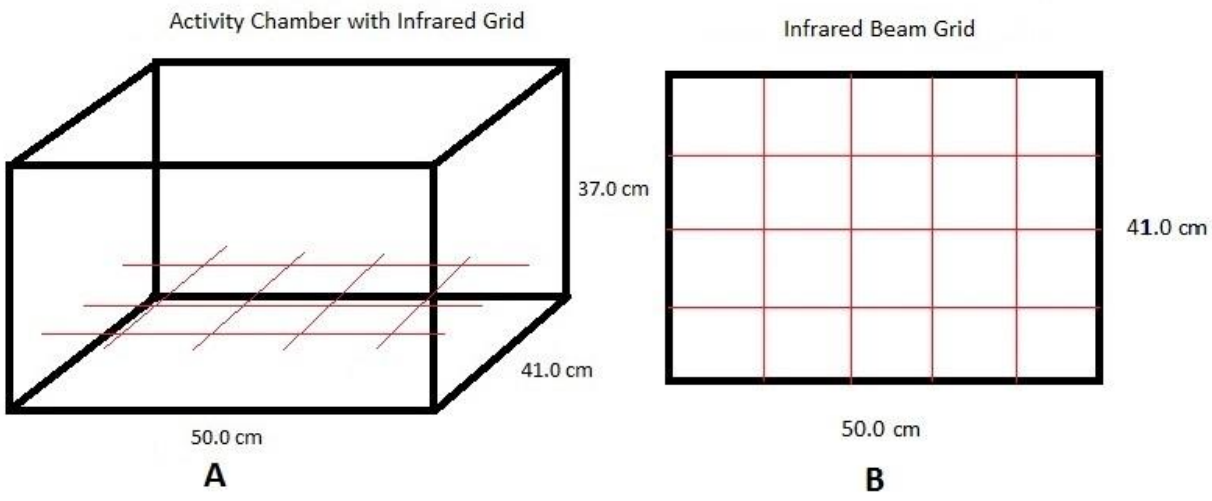

Fig. 2. A) Illustration of a locomotor activity chamber from eye level. Red lines indicate the infrared beams generated from the array of sensors. B) View of the floor of an activity chamber and the infrared grid generated from the array of sensors. As the animal moves through the grid, the computer records each beam break. A tally of beam breaks is then used to assess locomotor activity per unit of time.

2.4 Procedure

Experiment 1: Establishing Conditioned Activity

The conditioned activity paradigm was demonstrated using two groups (n = 9 each). All animals were pre-exposed to the activity chambers for 1 hr/day for 3 days consecutively (pre-exposure days 1, 2, and 3) (see Fig. 3). Beginning on the fourth day, animals in the paired group were given 10 mg/kg of cocaine immediately before being placed in the activity chamber for 1 hr. A saline injection (1 ml/kg) was given approximately 1 hr after they had been returned to their home cages. This procedure was repeated for three days (conditioning days 1, 2, and 3). In the unpaired group, the same procedure was used except that saline (1 ml/kg) was given immediately before placement in the activity chambers and cocaine (10 mg/kg) was given 1 hr after being returned to their home cages. Finally, all animals were tested for 1 hr following a saline injection the day after the last conditioning trial (day 7, test day). Conditioned activity would be demonstrated should the paired group show greater activity than the unpaired group.

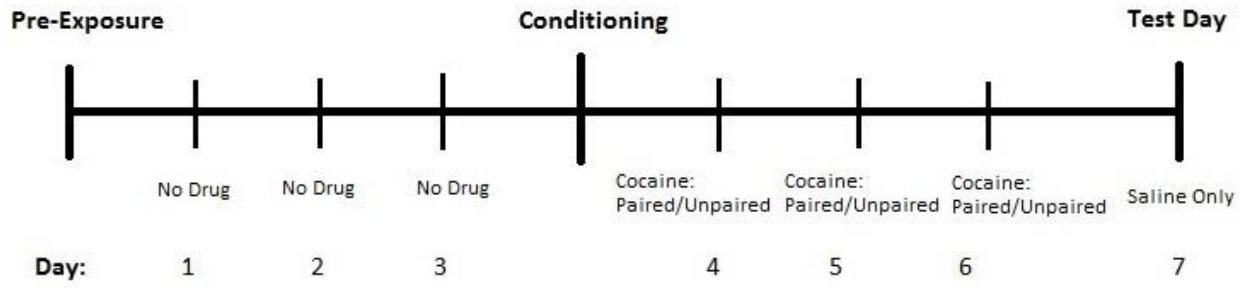

Fig. 3. Timeline for Experiment 1. Three 1 hr drug-free sessions are conducted over the first three days, followed by three days of 1 hr conditioning sessions. Paired animals receive cocaine directly before placement in the activity chambers and saline 1 hr after being returned to their homecage. Unpaired animals receive saline before placement in the chambers, and cocaine 1 hr after being returned to their homecage. All animals are tested with saline only on the test day.

Experiment 2: Effect of A-1070722 on the Acquisition and Expression of Conditioned Activity

The effect of the GSK-3 inhibitor, A-1070722, on conditioned activity when given in either the conditioning phase (acquisition [ACQ]) or on the test day (expression [EXP]) was assessed in cocaine-paired rats. All rats were pre-exposed and then given 10 mg/kg of cocaine immediately before being placed in the activity chambers on conditioning days 1, 2, and 3, and were given a 1 ml/kg saline injection approximately 1 hr after being returned to their home cages (See Fig. 4). On the test day, all animals were tested for conditioned activity following saline (1 ml/kg). During conditioning, rats in the ACQ groups were given either 0, 1, 5, 10, or 20 mg/kg A-1070722 30 min before cocaine (10 mg/kg) injections. The EXP groups were given the same doses of A-1070722 on the test day 30 min prior to injection with saline.

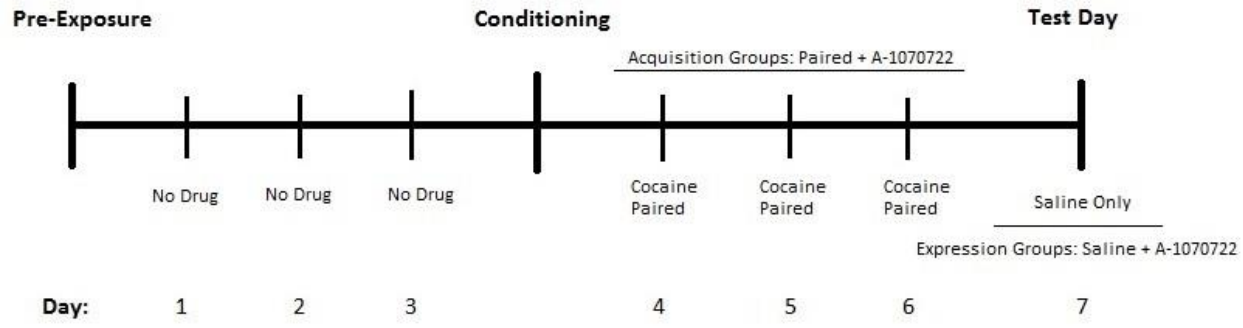

Fig. 4. Timeline for Experiment 2. Three 1 hr drug-free sessions were conducted over the first three days, followed by three days of 1 hr conditioning sessions. All animals were cocaine-paired. The acquisition groups received 0, 1, 5, 10, or 20 mg/kg A-1070722 0.5 hr before conditioning sessions. Expression groups received the same doses but on the test day.

Experiment 3: Assessing Effect of A-1070722 on Spontaneous Locomotion in Cocaine-Unpaired Animals

A final experiment was conducted to assess the effects of GSK-3 inhibition on locomotion *per se.* Two groups of cocaine-*unpaired* rats (n = 9 each) were pre-exposed to the activity chambers (see Fig. 5). During the conditioning phase, both groups were treated with saline before placement in the activity chambers, then treated with 10 mg/kg of cocaine approximately 1 hr

after being returned to their home cages. The ACQ group was also treated with 10 mg/kg of A-1070722 30 min before being placed in the chambers. On the test day, both groups were tested with saline. The EXP group was treated with 10 mg/kg of A-1070722 30 min prior to testing. It was hypothesized that beam breaks in animals given A-1070722 in either ACQ or EXP should not differ significantly from beam breaks in the cocaine-unpaired group from Experiment 1 if A-1070722 does not affect locomotion *per se*.

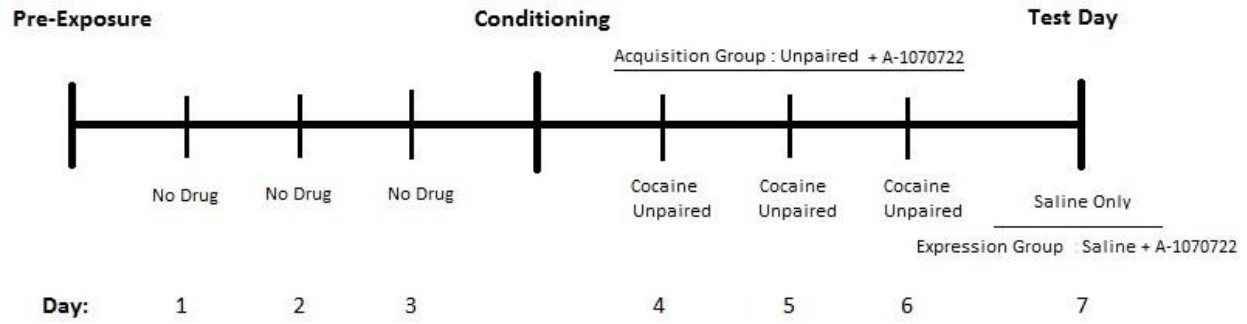

Fig. 5. Timeline for Experiment 3. Experimental protocol was the same as for Experiment 2 except that animals were cocaine-unpaired. The acquisition group received 10 mg/kg A-1070722 0.5 hr before conditioning sessions. The expression group received the same dose but on the test day.

2.5 Statistical Analysis

All statistical analyses were conducted using IBM SPSS Statistics 22 for Windows® (SPSS Inc., Chicago, IL). In each experiment, one-way ANOVA or ANCOVA were used to compare beam breaks among groups recorded during pre-exposure, conditioning, and test phases. Experiment 2 ANOVA and ANCOVA included the paired group from experiment 1, as well as all paired ACQ and EXP groups (total number of groups = 11). Analyses for experiment 3 included the unpaired group from experiment 1 as well as the 10 mg/kg unpaired ACQ and EXP groups (total groups = 3). Significant omnibus findings from ANOVA/ANCOVA from experiments 2 and 3 were followed up with Tukey's HSD post hoc analysis or simple contrast planned comparisons, with each group compared to the paired or unpaired reference group.

Chapter 3

Results

Experiment 1: Establishing Conditioned Activity

On the last day of the pre-exposure phase, cocaine-paired and cocaine-unpaired groups (Table 1) did not differ significantly on total beam breaks, $F(1,16) = 2.556$, $p = .129$. During conditioning, beam breaks (Table 1) were significantly higher for cocaine-paired rats than for cocaine-unpaired rats, $F(1,16) = 28.901$, $p < .001$.

Table 1. Mean beam breaks per 60 min with standard error in parentheses for paired and unpaired groups from Experiment 1.

	Last Day of Pre-Exposure	Conditioning
Paired	883.78 (34.0)	1673.4 (150.0) **
Unpaired	779.44 (55.7)	787.2 (68.4)

**Differs from unpaired ($p < .001$).

Evidence of conditioned activity was revealed by a one-way ANOVA on activity data from the test day. Paired rats had significantly higher beam breaks than unpaired rats, $F(1,16) = 6.675$, $p = .02$ (Fig. 6). Thus, conditioned activity was established in the cocaine-paired group.

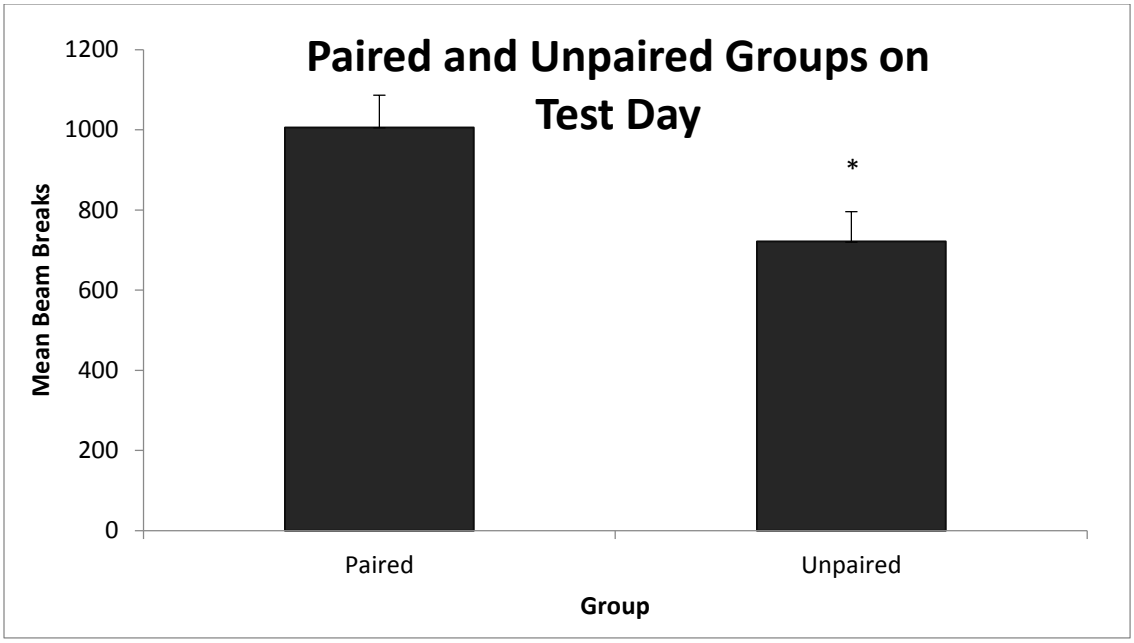

Fig. 6. Mean (± SEM) beam breaks per 60 min during the test day for paired and unpaired groups from Experiment 1.
*Significantly different than paired, $p < .05$.

Experiment 2: Effect of A-1070722 on the Acquisition and Expression of Conditioned Activity

Activity during the last day of pre-exposure differed significantly among groups, $F(10,88) = 3.030$, $p = .002$ (Table 2). Tukey's HSD post hoc analysis revealed significant differences among the 1, 5, and 20 mg EXP groups, the activity of the 5 and 20 mg groups being significantly lower than 1 mg group. This variable was entered in as a covariate for analyses of the average conditioning day activity and test day data in order to control for this baseline difference in activity.

ANCOVA on the three-day average of conditioning day activity (with the last day of pre-exposure entered in as a covariate) yielded no significant differences among groups, $F(10, 87) = .749$, $p = .677$ (Table 2).

Table 2. Mean beam breaks with standard error in parentheses for paired and paired acquisition (ACQ) and expression (EXP) groups from Experiment 2. Conditioning data are estimated marginal means which have been adjusted by using the last day of pre-exposure data as a covariate.

	Last Day of Pre-Exposure	Conditioning
Paired	883.78 (34.02)	1557.81 (158.75)
ACQ 0 mg	736.33 (44.72)	1551.31 (156.13)
ACQ 1 mg	813.67 (69.51)	1710.10 (156.12)
ACQ 5 mg	772.33 (68.21)	1493.58 (155.74)
ACQ 10 mg	890.44 (48.63)	1547.03 (159.13)
ACQ 20 mg	751.67 (57.81)	1452.51 (155.88)
EXP 0 mg	711.56 (54.89)	1219.24 (156.78)
EXP 1 mg	955.89 (96.71)	1659.20 (163.95)
EXP 5 mg	620.11 (47.99) *	1463.74 (161.83)
EXP 10 mg	726.89 (38.38)	1588.81 (156.34)
EXP 20 mg	665.00 (56.75) *	1689.17 (158.85)

*Significantly different ($p < 0.05$) from 1 mg/kg in expression by Tukey HSD multiple comparisons following significant effect of group by one-way analysis of variance.

ANCOVA on activity from the test day revealed a significant effect of group, $F(10,87) = 3.722$, $p < .001$ (Fig. 7). A simple contrast was used to determine which groups differed significantly from the paired reference group. No significant differences from paired were found for the 0, 1, 5, 10, and 20 mg ACQ groups, as well as the 0, 1, and 5 mg EXP groups. The 10 mg EXP group differed from paired, however, contrast estimate = -343.46, $p = .001$, as did the 20 mg EXP group, contrast estimate = -385.84, $p < .001$.

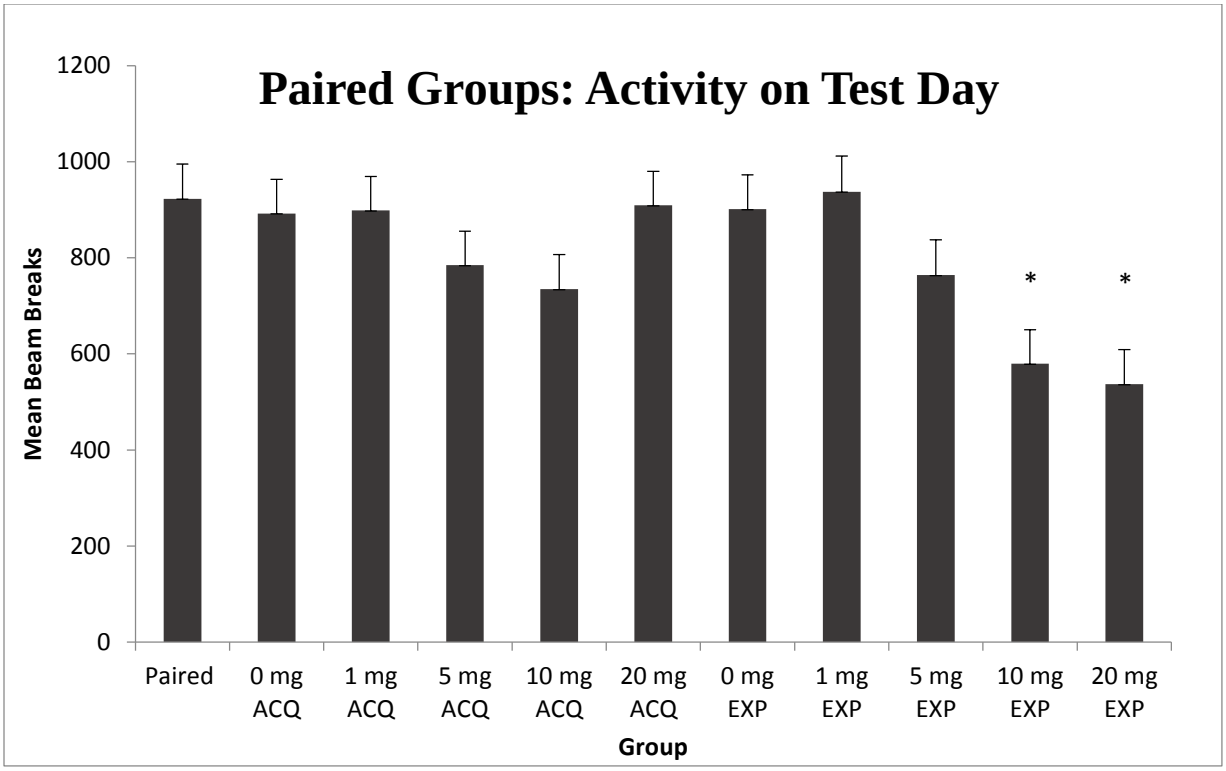

Fig. 7. Estimated marginal means (± SEM) of beam breaks per 60 min during the test day for all cocaine – paired groups from Experiment 2. Means were adjusted using the last day of pre-exposure scores as a covariate.
*Significantly different ($p < 0.05$) from paired group by planned contrasts following significant omnibus result of one-way analysis of covariance.

Experiment 3: Assessing Effect of A-1070722 on Spontaneous Locomotion

Beam breaks (Table 3) did not differ significantly among groups on the last day of pre-exposure, $F(2,24) = 1.079$, $p = .356$, but did they differ during the conditioning phase, $F(2,24) = 5.623$, $p = .01$. A planned contrast comparing the unpaired group given A-1070722 during ACQ and the unpaired control group accounted for this difference in means, contrast estimate = -257.9, $p = .005$.

Table 3. Mean beam breaks per 60 min with standard error in parentheses for the unpaired acquisition (ACQ) and expression (EXP) groups from Experiment 3 plus the corresponding data for the unpaired group from Experiment 1.

	Last Day of Pre-Exposure	Conditioning
Unpaired	879.56 (57.89)	787.2 (68.4)
Unpaired ACQ 10 mg	779.44 (55.69)	529.3(55.6)*
Unpaired EXP 10 mg	757.67 (72.78)	752.2 (51.7)

*Significantly different than Unpaired, ($p < .05$).

Visual inspection of the test day data (Fig. 8) indicates a trend towards lowered locomotion in both the ACQ and EXP groups compared to the unpaired group from Experiment 1, though the results of the one-way ANOVA were nonsignificant, $F(2,24) = 2.627$, $p = .093$.

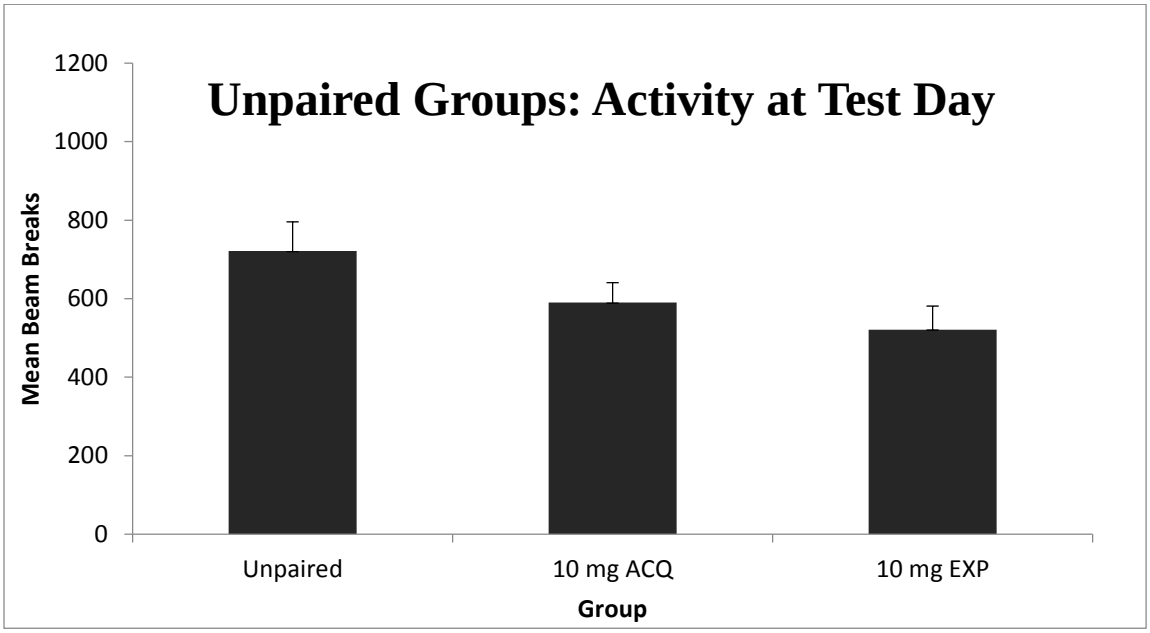

Fig. 8. Mean (± SEM) beam breaks per 60 min during the test day for cocaine – unpaired groups from Experiment 3.

Chapter 4

Discussion

As expected, results from Experiment 1 indicated a conditioned effect on activity in animals that had daily cocaine injections paired with the test environment, the paired group showing higher activity than the unpaired group. Mean activity did not differ on the final day of pre-exposure, indicating no baseline differences in activity existed between groups before conditioning began. Activity during conditioning was much higher in the paired group, given that they had received cocaine. This group difference (though smaller in magnitude) was maintained on test day despite all animals being treated with saline only, indicating a conditioned response to the environment had occurred. Overall, this effect is comparable to results found in other cocaine- and AMPH-conditioned activity experiments (Aujla, Sokoloff, & Beninger, 2002; Banasikowski et al., 2010; Le Foll et al., 2002; Mazurski & Beninger, 1990; Sutton et al., 2000; Beninger & Hahn, 1983; Beninger & Herz, 1986; Pickens & Crowder, 1967; Fontana, Post, Weiss, & Pert, 1993; Yoon et al., 2010).

The fact that the cocaine-unpaired group displayed lower activity than the cocaine-paired group indicates that the increased response seen in animals with a history of receiving cocaine is environment-specific, and is not a more general locomotor sensitization effect. Yoon et al. (2010) used a different conditioned activity design, having only one day of conditioning followed by a cocaine-challenge test, and compared activity among paired, unpaired, and saline-only treated animals. Activity in the paired group was higher than both the unpaired and saline-only animals, indicating a conditioned effect on locomotion. The unpaired and saline-only groups had similar levels of activity. This was also noted in Sutton et al. (2000), in which saline-only animals showed similar activity levels to animals with a history of environment-unpaired central infusions of AMPH. These data support the conclusion that the locomotor-enhancing

effect seen in cocaine- or AMPH-paired animals is due to pairing of drug with the environment, and not due to a more general enhancement of locomotion in animals with a history of receiving that drug.

In Experiment 2, block of the conditioned activity response was indicated by significant differences from the cocaine-paired reference group, since failure of A-1070722 to block conditioned activity should result in activity levels comparable to it. No dose of A-1070722 given during the acquisition phase (i.e., conditioning days 4, 5, and 6) blocked the conditioned activity response, though the 10 and 20 mg/kg doses given on the saline-only test day resulted in a block of the expression of conditioned activity (Fig. 7).

Significant differences existing on the last day of pre-exposure indicated a baseline difference among groups, the 5 and 20 mg/kg expression groups having showed lower activity than the 1 mg/kg expression group. This baseline difference was controlled for by entering the data from this day as a covariate for all subsequent analyses. Similar results were found when a one-way ANOVA was used on difference scores, in which the data from the last day of pre-exposure were subtracted from the test-day data rather than being entered as a covariate (data not shown). Using this method, the 10 and 20 mg/kg expression groups were significantly lower than paired, similarly with what was found here with the ANCOVA from Experiment 2.

No differences were detected on the average activity during the conditioning phase (Table 2), indicating no dose of A-1070722 reduced the locomotor response to cocaine. This was different than what was found with studies of other GSK-3 inhibitors. Previously, it has been shown that locomotor responses to cocaine are attenuated by clozapine in male ICR mice (Park, Cui, Hwang, & Kang, 2010), and that lithium, (Xu et al., 2009), valproate, and SB216763 attenuate responses in CD-1 mice and Sprague Dawley rats (Miller et al., 2009; Xu et al., 2009).

Not all studies have found this effect, however. Li et al. (2005) found valproate failed to inhibit the acute response to cocaine in Kunming mice, but dose-dependently reduced the development of cocaine sensitization. Similarly, Kalinichev & Dawson (2011) found valproate as well as SB216763 failed to inhibit the acute response to AMPH on horizontal locomotion in male Black Swiss mice. The GSK-3 inhibitors AZ1080 and Compound A were found to have no effect on hyperactivity induced by AMPH in male C57B1/6 J mice (Caberlotto et al., 2013), in contrast to effects of other GSK-3 inhibitors, such as lithium (Caberlotto et al.,2013) and SB216763 in CB-1 mice (Enman & Unterwald, 2012) (but not in Black Swiss mice [Kalinichev & Dawson, 2011]). It would appear that the effects of GSK-3 inhibition on hyperlocomotion induced by psychostimulants varies by drug used and across studies, and perhaps across species and species strain. In agreement with this line of thinking, Gould, O'Donnell, Picchini, & Manji (2007) found the attenuation of AMPH-induced hyperactivity was attenuated by lithium in some, but not all, strains of mice tested.

Previous work from our lab has shown that the cAMP-dependent pathway is involved in the acquisition of conditioned activity. AMPH-induced conditioned activity is attenuated by intraaccumbal infusions of the PKA inhibitor, Rp-cAMPS (Sutton et al., 2000; Gerdjikov, Giles, Swain, & Beninger, 2007). Systemic administration of the dopamine D2-receptor antagonist, haloperidol, also blocks the acquisition of conditioned activity (e.g., Banasikowski et al., 2010). Under normal conditions, dopamine activity at the D2 receptor leads to an inhibition of cAMP production, which should reduce levels of active PKA (for review, see Beaulieu & Gainetdinov, 2011). Blockade of this receptor, therefore, should lead to an *increase* in PKA production in D2-containing neurons. That a D2-receptor antagonist and a PKA inhibitor produce similar effects on the acquisition of conditioned activity appears to be at odds, and left room for alternative

signaling pathways, such as the Akt/GSK-3 pathway, as a potential mediator of the effects of haloperidol on conditioned activity. The failure of A-1070722 to block acquisition of cocaine-cue conditioned activity (Experiment 2) would suggest that this pathway is perhaps not as important for conditioned activity to develop as other signaling pathways.

The ability of A-1070722 to block the *expression* of conditioned activity is not surprising given recent work published by Shi et al. (2014), which highlighted the importance of GSK-3 in reward memory. Mice that were exposed to the side of a CPP apparatus that had been previously paired with cocaine showed an elevation of the activity of GSK-3α and β and alterations in levels of other enzymes in GSK-3 pathways in both the hippocampus and the nucleus accumbens. The importance of GSK-3 activity in the reconsolidation of reward memories was tested by treating mice that had showed a cocaine CPP with the GSK-3 inhibitor, SB216763, directly after the CPP test. Animals given SB216763 following the test did not show a place preference when tested a second time, suggesting that the increase in GSK-3 activity that occurs upon exposure to reward-associated environments may be important for reconsolidation of reward-related memory (i.e., incentive learning), and that inhibition with SB216763 could disrupt these memories while they are in a labile state (Shi et al., 2014). It is possible, then, that the block of the expression of conditioned activity found in Experiment 2 could suggest, similarly to what Shi et al. (2014) found, that GSK-3 activity is important for incentive learning. Obvious differences exist between these experiments and paradigms, however. CPP uses not just a reward-paired environment, but also another contextually different environment in which only vehicle is given. CPP also uses different dependent variables (i.e., time spent on each side) than conditioned activity. Still, some similarities between these paradigms could be expected since both look at the results of environmental pairings of reward and can be used to model incentive learning (Beninger &

Banasikowski, 2008). Another difference that could be important is that Shi et al. (2014) treated animals with a GSK-3 inhibitor *after* the demonstration of incentive learning had already taken place, whereas here animals were treated directly before test. To see if these phenomena are related, future studies could investigate if a similar pattern of GSK-3 activation occurs in animals upon exposure to the cocaine-paired test environment used in conditioned activity, and if delivery of a GSK-3 inhibitor *after* the demonstration of conditioned activity prevents it from being displayed again upon retest.

The conclusion that A-1070722 *specifically* blocked the expression of conditioned activity unfortunately cannot be made here because of a significant effect of the drug on spontaneous locomotion observed in Experiment 3 (Table 3). Here, the first dose found effective in blocking conditioned activity from Experiment 2 (10 mg/kg) was tested in cocaine-*unpaired* animals. Activity among the three groups tested (unpaired, 10 mg ACQ, 10 mg EXP) did not differ on the last day of pre-exposure. When the three days of conditioning were averaged, in which the 10 mg ACQ group received A-1070722 half an hour before activity sessions, significant reductions in activity were found when planned contrasts compared activity against the average activity displayed by the unpaired reference group. This indicated a locomotor suppressing effect of A-1070722. When activity was analysed on the test day, a trend towards lowered activity was observed in the 10 mg ACQ and EXP groups, $p = .093$. It should be noted that although activity in the 10 mg EXP group failed to reach significance when compared to the unpaired reference group, both the ACQ and EXP groups appeared to be displaying a nonsignificant reduction of activity levels (Fig. 8). It is possible that a conditioned *inactivity* response had developed in the ACQ group due to three days of pairing the effects of A-1070722 with the locomotor chambers. It is possible that this reduction in activity drowned out the effect

of A-1070722 given to the 10 mg EXP group on test day, resulting in a nonsignificant overall effect by one-way ANOVA. The effects of the dopamine D2 receptor antagonist and GSK-3 inhibitor, haloperidol, on conditioned inactivity has been investigated by our lab (Beninger & Xu, unpublished work). Future studies could investigate whether A-1070722 or other GSK-3 inhibitors can produce conditioned suppressions of locomotion.

The effect of A-1070722 on spontaneous locomotion described here stands in contrast to the effects of several other GSK-3 inhibitors, such as valproate, olanzapine, and SB216763 (Kalinichev & Dawson, 2011). Lithium was found not to have an effect on spontaneous locomotion in Black Swiss mice in Kalinichev & Dawson (2011) and Gould et al. (2007), but did show an effect in Caberlotto et al. (2013) in male C57B1/6 J mice. Again, this would suggest effects of drug may differ across strain, at least in mice.

Reductions in spontaneous locomotion were found with the GSK-3 inhibitor Compound A, which also failed to reduce AMPH-induced locomotion (Caberlotto et al., 2013). AR-A014418 reduced spontaneous horizontal locomotion at 10, but not 5 mg/kg (Gould et al., 2004; Kalinichev & Dawson, 2011) as well as spontaneous rearing behaviour at 20 mg/kg (Kalinichev & Dawson, 2011). Similar to this last finding, the GSK-3 inhibitor TDZD-8 reduced spontaneous rears in mice, but unlike AR-A014418, failed to reduce horizontal locomotion (Kalinichev & Dawson, 2011).

In light of these findings, it is not unprecedented that a GSK-3 inhibitor should reduce spontaneous locomotion as A-1070722 has here in Experiment 3, though this finding tends to be the exception rather than the rule, with only AR-A014418, TDZD-8, Compound A, and sometimes lithium showing this effect. Because of this finding, the results of A-1070722 found in Experiment 2 on the block of expression of cocaine-cue conditioned activity should be

interpreted with caution, since the effect on locomotion may not be specific to *conditioned* locomotion so much as *locomotion per se*. The procedure used here should be replicated using other GSK-3 inhibitors which have not been found to suppress spontaneous locomotion, such as SB216763, in order to determine if the block of the expression of conditioned activity seen here was due to a block of the demonstration of the incentive learning that takes place following drug plus environment pairings, or was due to a more general effect on locomotion.

If the effect of A-1070722 was in fact due to block of cocaine-cue conditioned activity, results reported here would be similar to findings from our lab (e.g., Aujla et al., 2002; Banasikowski et al., 2010) and others (e.g., Le Foll et al., 2002) on the effects of dopamine D3 receptor antagonists and partial agonists on conditioned activity (for review, see Beninger & Banasikowski, 2008). Dopamine D3 antagonists and partial agonists given both centrally as well as systemically block the expression of conditioned activity while having less of an effect on its acquisition (Aujla et al., 2002; Banasikowski et al., 2010; Le Foll et al., 2002). The similarity of these findings to what was seen here in Experiment 2 with a GSK-3 inhibitor leave open the possibility that the effects of D3 antagonists/partial agonists on conditioned activity may be mediated by an influence on GSK-3 activity. Beaulieu et al. (2007) showed that the D3 receptor is important for control of GSK-3β activity in mice. Using western blotting and densitometry, it was found that D3-knock out mice had more pAkt and pGSK-3β (i.e., inhibited GSK-3β) levels under basal conditions than wild-type mice, suggesting that under normal conditions the D3 receptor plays a role in the activation of GSK-3β by dopamine. Since GSK-3β activation has been seen in animals exposed to the cocaine-paired compartment of a CPP apparatus (Shi et al., 2014), and that other indicators of the incentive learning produced by cocaine such as conditioned activity are blocked by D3 antagonists/partial agonists (Banasikowski et al., 2010;

Le Foll et al., 2002) and the GSK-3 inhibitor, A-1070722 (Experiment 2), it could be postulated that GSK-3 activity required for expression of conditioned activity may be modulated by D3 preferring compounds. Future studies should investigate whether GSK-3β is activated by exposure to the activity chambers used in the conditioned activity paradigm in a fashion similar to what was found with exposure to the cocaine-paired chamber in CPP (Shi et al., 2014) and whether D3 antagonism/partial agonism modulates GSK-3 activity in animals that demonstrate conditioned activity.

It should be noted, though, the role of the dopamine D3 receptor in inhibition of GSK-3β activity is far from clear. Work by Salles et al. (2013) has shown that the dopamine D3 preferring *agonist*, quinelorane, also increases pGSK-3β levels. That knock out of this receptor or stimulation of it by an agonist drug both produce inhibitions of GSK-3β would indicate that the role of the D3 receptor in regulation of GSK-3β is complex. The exact role of D3 receptors in the regulation of GSK-3β activity needs further clarification. It seems that effects of dopamine agonists on GSK-3β activity mediated through D2 and D3 receptor subtypes depends on a variety of factors, such as time. Salles et al. (2013) found inhibition of GSK-3β activity at 10 min after quinelorane injection, but not 20 min after. MacLeod, Moores, & Beninger (2012) found that AMPH, an indirect dopamine agonist, *inhibited* GSK-3β 15 min after administration, whereas Beaulieu et al. (2007) noted an *activation* of GSK-3β approximately 80 after AMPH. From this, it can be concluded that the regulation of GSK-3β by dopamine and dopamine agonists varies based on the time that the drug has been active, and regulation involves a complex mix of activation and inhibition. Therefore, the fact that work by Beaulieu et al. (2007) and Salles et al. (2013) on the role of the dopamine D3 receptor in GSK-3β regulation conflict

with one another is not completely unexpected, given the complex nature of GSK-3β's regulation.

Summary

Work presented here has found a block of the conditioned activity produced by cocaine in animals treated with 10 and 20 mg/kg of A-1070722, a selective GSK-3 inhibitor. The effect of A-1070722 on locomotion was found to be non-specific to conditioned activity, as it also reduced locomotion more generally. It is possible that the effect of this drug was due entirely to a locomotor-suppressing effect. Alternatively, it is possible that these results highlight an important role for GSK-3 in conditioned responses to cocaine-paired environments. Work by other labs (e.g., Shi et al., 2014) indicates an activation of GSK-3β in animals exposed to cocaine-paired environments, and that inhibition of GSK-3β following this activation blocked further demonstrations of CPP. If the findings reported here were in fact due to effects on conditioned activity and not simply due to an effect on locomotion *per se*, these results together with work by Shi et al. (2014) would demonstrate an important role for active GSK-3β in cocaine-reward memory and incentive learning.

The fact that GSK-3 inhibition with A-1070722 produced effects on conditioned activity that are similar to results obtained from studies with dopamine D3 antagonists/partial agonists could hint at a role of the D3 receptor in regulation of GSK-3 that may be required for the expression of conditioned activity. The role of the D3 receptor in GSK-3 regulation is a complex one, with some studies finding its absence (Beaulieu et al., 2007) or its activation (Salles et al., 2013) can lead to an inhibition of GSK-3β activity. Future studies should further elucidate the importance of this receptor subtype in GSK-3 regulation, and attempt to investigate GSK-3

activity as a potential mechanism by which D3 antagonists/partial agonists exert their blocking effect on the expression of cocaine-cue conditioned activity.

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