The *in vivo* use of Dopamine Binding Aptamers in a Mouse Model of Dopamine Dysregulation

by
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Abstract

In the development of central nervous system therapeutics, delivery of agents across the blood-brain barrier remains one of the biggest challenges. In the present study, a liposome, surface-modified with an aptamer for the transferrin receptor, was used to facilitate delivery of a dopamine and norepinephrine binding aptamer from the periphery into the brain. Repeated, systemic administration of the modified aptamer produced no behavioral or neurodegenerative effects. In a behavioral experiment using cocaine administration to induce elevated concentrations of dopamine, systemic pre-treatment with the aptamer-loaded liposomes reduced cocaine-induced hyperlocomotion. Systemic pretreatment with the transferrin-negative liposome control nor transferrin-positive liposomes loaded with either a non-binding aptamer or a random oligonucleotide did not alter cocaine-induced hyperlocomotion. RT-PCR was used to detect the aptamer in brain tissue, confirming the delivery of the aptamer payload across the blood-brain barrier. Differential distribution within the brain of rhodamine fluorescence based on the presence or absence of the transferrin receptor aptamer on the surface of the rhodamine-tagged liposomes was observed. Results suggest that systemic administration of the modified liposomes led to delivery of the aptamer into the brain. The potential of this multi-aptamer payload/targeting system is not restricted to dopamine or norepinephrine and could be easily modified with any aptamer for a variety of neural targets.

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List of Abbreviations

ADHD – Attention deficit hyperactivity disorder

DA - dopamine

NAc - Nucleus Accumbens

CRF – corticotropin-releasing factor

VTA – ventral tegmental area

PFC – prefrontal cortex

5HT – serotonin

NPY – Neuropeptide Y

LTP – long-term potentiation

NSDUH – National Survey on Drug Use and Health

SUD – substance use disorder

DAT – dopamine transporter

BE – benzoylecgonine

EME – ecgonine methyl ester

CYP450 – cytochrome P450

NCOC – norcocaine

NCOC-NO – norcocaine nitroxide

CBT – cognitive behavioural therapy

ADH – alcohol dehydrogenase

ALDH – aldehyde dehydrogenase

NE – norepinephrine

IgG – immunoglobulin G

SNC – succinyl norcocaine

BBB – blood brain barrier

MPH – methylphenidate

SELEX – systematic evolution of ligands by exponential enrichment

PCR – polymerase chain reaction

RT-PCR – real-time polymerase chain reaction

ssDNA – single stranded DNA

VEGF – vascular endothelial growth factor

AuNP – gold nanoparticle

AMNV – aptamer-mediated nanovehicles

PEG – polyethylene glycol

siRNA – small interfering RNA

ICV – intracerebroventricular

CPP – cell penetrating peptides

qPCR – quantitative polymerase chain reaction

I.P. – intraperitoneal

K_d – dissociation constant

CCAC – Canadian Council on Animal Care

TfR – transferrin receptor

PFA – paraformaldehyde

Sub – substituted sequence

TRA- Transferrin receptor aptamer

DAL- Dopamine aptamer loaded

TRAM – Transferrin receptor aptamer modified liposome

DAL-TRAM – dopamine aptamer loaded-transferrin receptor aptamer modified liposome

Sub-TRAM – substituted aptamer loaded-transferrin receptor aptamer modified liposome

ROL-TRAM – random oligonucleotide loaded-transferrin receptor aptamer modified liposome

NTC – non-template control

Cq – quantitation cycle

HPLC – high performance liquid chromatography

GAGs – Glycisaminoglycans

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Introduction

Overview

This thesis examined the novel *in vivo* use of a dopamine and norepinephrine binding aptamer in a mouse model of dopamine dysregulation, as a result of cocaine exposure. It was hypothesized that the aptamer would bind to dopamine and reduce the locomotor effects induced by cocaine. The overall objective was to elucidate whether the aptamer could offer any therapeutic potential for cocaine dependence and as a proof of concept that the delivery system could deliver the aptamer to the central nervous system.

Drug Addiction

Drug addiction has proven to be a major health problem affecting an estimated 243 million people worldwide (Wingo et al., 2015). Addiction has an overwhelming cost on society via direct health care costs, loss in productivity, and increased crime rates (UNODC, 2013). A recent survey in the United States showed that in 2012 there were 2.8 million new drug users (UNODC, 2013). It is estimated that drug addiction treatment costs $250 billion per year worldwide (UNODC, 2013). Unfortunately, there have been major limitations in the efficacy of current pharmacotherapies leaving 5% of the population burdened with drug addiction (UNODC, 2013). The major setback to addiction treatments is a lack of understanding into the precise mechanisms that make certain individuals more

susceptible to addiction and more vulnerable to relapse compared to others (Buchta and Riegel, 2015).

Addiction is the end result of a number of different factors including the desire, abuse of and physical dependence on a particular drug manifested through continued or frequent use (Jupp and Lawrence, 2010). Addiction is considered a chronic disorder characterized by three major stages: 1- compulsion to seek and consume the drug, 2- loss of control and inability to regulate intake, and 3- onset of a negative emotional state in the absence of the drug (withdrawal) (Koob and Volkow, 2010). Craving, which contributes to the compulsion to seek and consume the drug, is a major contributor to relapse. In the specific case of cocaine addicts, many users admit that their reason for relapse is a direct result of their craving for cocaine and the high they experience (Belin et al., 2009).

Illicit Drugs, Learning, Memory and Craving

Drugs of abuse are thought to commandeer learning and memory processes which in turn lead to long-term addictive behaviours (Tipps and Buck, 2015). Repeated drug use leads to the development of strong associations between the physiological response produced by the drug, environmental cues present during the time of drug consumption when drug effects are experienced by a user and drug specific withdrawal symptoms (Cleva et al., 2010). With continued drug use, these associations become overlearned leading to drug

craving in the absence of the drug (Cleva et al., 2010). These drug-related associations are continually triggering craving, one of the leading causes of relapse. Extinguishing the motivation to seek substances of abuse, and the craving response, as a result of drug abuse is one ultimate goal of treatment. Unfortunately, to date there has been limited success in breaking strong drug-paired associations with environmental cues.

Cyclic phases and the Neurobiology of Addiction

Addiction can be viewed as a combination of impulsive and compulsive actions defined by the following cyclic phases: binge/intoxication, withdrawal/ negative affect, preoccupation/ anticipation (Koob and Volkow, 2010). The underlying neurocircuitry for each cyclic phase of the addiction cycle has been extensively studied and is presented below.

It has been suggested that addiction occurs as a result of a change in behavior from impulsive to compulsive (Belin et al., 2008). There has been deep interest in understanding the relationship between novelty-seeking behaviour, impulsivity, and drug addiction (Piazza et al., 1989; dela Peña et al., 2015). Novelty-seeking is defined as the tendency to explore and experience excitement in response to a novel stimulus. Impulsive behaviour reflects poor control, carelessness, and rapid responses to situations with little precaution. High impulsivity and novelty seeking behaviours may predict whether an individual is likely to switch from controlled to compulsive drug use (Belin et al., 2008).

Both novelty-seeking and impulsivity are behavioural symptoms associated with the most common neurodevelopmental disorder, attention deficit hyperactivity disorder (ADHD) (dela Peña et al., 2015). Given the similarities between individuals with ADHD and addiction, studies suggest that individuals diagnosed with ADHD may have an increased risk of compulsively consuming drugs of abuse because of their personality traits (dela Peña et al., 2015). Substance use is common in the parents of children with ADHD, and children with parents who consume illicit substances are at an elevated risk for ADHD (Dürsteler et al., 2015). Comorbid ADHD is common among a number of different substance-using patients (Dürsteler et al., 2015). It has been found that cocaine dependence is common among adult ADHD patients with higher impulsivity levels (dela Peña et al., 2015).

The binge/ intoxication stage is mediated in large part by neural changes occurring in the basal ganglia. The basal ganglia are known to be involved in voluntary motor control and learning of routine behaviours or habits (Koob and Mason, 2016) and play an important role in associating incentive to environmental stimuli (Koob and Mason, 2016). The release of dopamine (DA) in the ventral striatum (nucleus accumbens, NAc) has been linked to the reinforcing actions associated with drugs of abuse. Human studies have revealed that intoxicating doses of drugs of abuse release DA and opioid peptides in the NAc leading to habit formation and strengthening of associations between the

environment and drug responses via activity in the basal ganglia-globus pallidus-thalamic cortical loop (Koob and Mason, 2016).

During the transition from intoxication to withdrawal, neurochemical stress signals including corticotropin-releasing factor (CRF), norepinephrine, dynorphin, hypocretin, and substance P are recruited resulting in the physiological expression of stress-like states. While these neurochemical stress signals increase, anti-stress compounds are down regulated (Corominas et al., 2010). This neurochemical imbalance based on decreases in reward neurotransmitter function and recruitment of stress neurochemical signals increases the motivation to re-engage in drug taking and drug seeking behaviours to "normalize" this chemical imbalance.

There appear to be two underlying neurobiological processes mediating the withdrawal/ negative affect stage of the addiction cycle. A major stress effector, corticotrophin releasing factor (CRF) contributes to addiction by increasing locomotion, mediating the response to conditioned stimuli (Corominas et al., 2010). CRF induces an increase in DA activity in the ventral tegmental area (VTA) leading to an increase in DA release in the projection areas including the prefrontal cortex (PFC) and the nucleus accumbens (NAc) causing drug seeking behaviours and craving responses (Wanat et al., 2008; Corominas et al., 2010). It has been found that withdrawal periods following chronic administration of psychostimulants (opioids and alcohol) in rats results in a significant decrease in NAc DA and serotonin (5HT) levels, which have been

associated with dysphoria and the negative symptoms experienced during withdrawal (Jupp and Lawrence, 2010). Similarly, dysphoria, anxiety, depression, and stress have all been associated with changes in receptor expression and decreases in GABA, neuropeptide Y (NPY), DA, and 5HT levels (Koob and Le Moal, 2008; Jupp and Lawrence, 2010).

The preoccupation/anticipation stage of addiction occurs due to dysregulation of prefrontal cortical circuits and subsequent loss of executive function (Koob and Mason, 2016). There are two hypothesized brain circuits that become engaged during this stage of addiction. One circuit involves connections between the anterior cingulate cortex and the dorsolateral prefrontal cortex. Both of these regions are involved in the development of habits and are mediated by the basal ganglia (Koob and Mason, 2016). Habit-learning is one of the components of compulsive behavior and persists even in the face of significant adverse consequences (Koob and Volkow, 2010). Connections between the ventral prefrontal cortex and the orbitofrontal cortex make up the opposing systems responsible for inhibiting the basal ganglia and the extended amygdala stress system (Koob and Mason, 2016). These two opposing circuits become dysregulated in individuals with substance use disorders altering drug consumption into a habit as a result of impairments in executive functioning. Addicts are known to experience disturbances in their decision making abilities and impairments in behavioural inhibition, both of which contribute to drug craving (Koob and Mason, 2016).

Dopamine (DA) is one of the key neurotransmitters involved in addiction. Multiple drugs of abuse are known to enhance mesocorticolimbic DA activity (Feltenstein and See, 2008). DA plays a major role in the reward system, pleasure centers, and goal directed behaviours. DA is known to facilitate the learning of cues that can predict rewards as well as in reinforcing actions that lead to rewards (Bobzean et al., 2014; Buchta and Riegel, 2015). It is well established that the mesocorticolimbic pathway is involved in the rewarding properties of both natural stimuli (food, drink, sex) and addictive drugs (Feltenstein and See, 2008). This pathway consists of neural projections from cell bodies in the VTA to limbic structures including the amygdala, ventral pallidum, hippocampus, and NAc (Feltenstein and See, 2008). The mesocortical pathway consists of dopaminergic projections from the ventral midbrain, including the VTA and substantia nigra, to the PFC and is primarily involved in the regulation of emotional responses, cognitive control, and executive function (Volkow et al., 1993). Overall the mesocortical pathway plays an active role in the conscious drug experience, the drug craving, and a loss of behavioural inhibition related to compulsive drug seeking and drug taking behaviours (Feltenstein and See, 2008).

The mesolimbic DA projection is a major focus of addiction research because drugs of abuse, such as amphetamines and cocaine, increase DA concentrations in the NAc in a prolonged and unregulated manner by blocking DA reuptake transporters (Koob and Volkow, 2010). This pathway serves as a

gating pathway that modulates the limbic-motor interface. It has been suggested that the hippocampal-accumbens pathway plays an important role in adaptive behaviours like locomotor activity and exploratory goal-directed behaviours (Glangetas et al., 2015). It has been well documented that increases in DA concentration lead to changes in synaptic plasticity within the DA system and DA receptive neurons (Koob and Volkow, 2010). Drugs of abuse have been shown to induce short and long-term modifications in DA firing in the VTA and DA release in the NAc (Koob and Volkow, 2010; Tortorelli et al., 2015). In animal models, progressive and continual increase in locomotor activity have been observed following repeated drug administration even after periods of abstinence (Tortorelli et al., 2015). These behavioural responses to drug administration have been linked to burst firing of DA neurons in the VTA (Tortorelli et al., 2015). It has been shown that a single exposure to cocaine or amphetamine induces long-term potentiation (LTP) of AMPA mediated excitatory neurotransmission of DA neurons in the VTA (Ungless et al., 2001a) and dopaminergic neurons burst fire more frequently in the presence of a DA antagonist, haloperidol (Freeman et al., 1985). Ungless *et al.,* observed exposure to cocaine can significantly increase AMPAR to NMDAR ratios in the VTA which is seen up to 5 days following cocaine exposure and can be blocked upon administration of an NMDA receptor antagonist (Ungless et al., 2001b). Based on these results, it has been suggested that the involvement of NMDA receptor is responsible for increasing the frequency of burst firing in the VTA (Koob and Volkow, 2010). These changes in

plasticity are suggested to lead to a shift in neurocircuitry from normal learning mechanisms to habit-learning (Koob and Volkow, 2010).

Cocaine Addiction

The United Nations Office on Drugs and Crime reported that there were 17 million cocaine users worldwide in 2012 (Dürsteler et al., 2015). Illicit drug use is not only associated with its own detrimental outcomes but it has been associated with higher incidence of mental illness. Cocaine use has been linked with multiple psychiatric conditions, both physical and psychosocial including neurological disorders, psychotic symptoms, unintentional injuries, violent behaviours, and even premature death (Dürsteler et al., 2015). Recent epidemiological data suggest that cocaine users show a 4 to 8 times higher mortality rate than their age and gender matched peers within the same population (Wagner and Anthony, 2002; Dürsteler et al., 2015). The National Survey on Drug Use and Health (NSDUH) reported that in 2013, of adults who admitted to having a substance use disorder (SUD) within the past year, 37.8 % also had co-occurring mental illness whereas 16.7% of adults without substance use disorders reported mental illness (NSDUH, 2014). Substance use disorders were also found to be common among 23.1% of adults who reported having a serious mental illness within the past year (NSDUH, 2014). Furthermore, 13.5% of past cocaine users were reported as having serious suicidal tendencies (NSDUH, 2014). These reported findings demonstrate the national mental health

burden of substance use disorders. Cocaine addiction has evolved into being a major health problem in our society because, unfortunately to date, there are still no pharmaceutical treatments for those trying to abstain.

Cocaine is known to produce pleasure, euphoria, loss of control, and compulsive responses. These consequences have been traced to the effect of cocaine on frontal brain regions that comprise the limbic system (Nestler, 2005). It is known that cocaine reinforces drug seeking habits by increasing DA levels and this is believed to be an underlying explanation as to its addictive nature (Kramar et al., 2014). One specific region of the limbic system, the NAc, is known to produce feelings of pleasure and satisfaction when stimulated by DA (Nestler, 2005). This increase in DA as a result of cocaine consumption leads to intense feelings of pleasure. In animal studies, animals will choose to self-administer cocaine instead of eating to the point of starvation (Nestler, 2005). Acute inhibition of DA uptake due to cocaine results in increased DA activity whereas chronic cocaine consumption leads to dysregulation of striatal DA signaling (Dürsteler et al., 2015).

Cocaine Pharmacokinetics & Pharmacodynamics

Cocaine is commonly known for its ability to block the re-uptake of DA and thereby acts as an indirect agonist at DA synapses (Barrett et al., 2004). The regions with the highest DA content are the NAc and the striatum, which receive innervation from the VTA and substantia nigra, respectively (Barrett et al., 2004).

These regions show the highest accumulation of DA during cocaine exposure. The dopamine transporter (DAT) is a monoamine transporter that mediates the reuptake of DA into presynaptic terminals by sequential binding of 2 Na^+ and 1 Cl^- ions to initiate the transport of dopamine (Reith et al., 1997). By blocking DAT, cocaine causes an increase in synaptic DA leading to an increase in DA binding to postsynaptic receptors (Nestler, 2005; Koob and Volkow, 2010; Dürsteler et al., 2015). Cocaine also binds to the transporters for other neurotransmitters including norepinephrine and 5HT (Nestler, 2005). While cocaine does target these other neurotransmitter systems, the action on the DA system is hypothesized to mediate the addictive nature of cocaine.

Cocaine is metabolized in the liver into two major metabolites: benzoylecgonine (BE) and ecgonine methyl ester (EME) which together represent 95% of urinary excretion products (Valente et al., 2012). The liver is responsible for metabolizing cocaine to BE by the enzyme carboxylesterase type 1 and EME is formed by carboxylesterase type 2 (Kolbrich et al., 2006; Valente et al., 2012). Afterwards, BE is further oxidized to m-hydroxybenzoylecgonine, p-hydroxybenzoylecgonine (mOHBE and pOHBE) (Kolbrich et al., 2006).

Approximately 5% of cocaine in the liver undergoes cytochrome P450 (CYP450)-mediated activation and is converted to norcocaine (NCOC) (Kloss et al., 1983; Valente et al., 2012). NCOC is metabolized further to other oxidative metabolites including norcocaine nitroxide (NCOC-NO) which plays an active role in inducing liver damage (heptatoxicity) (Kloss et al., 1983). Different cocaine N-

oxidative metabolites which result in reactive oxygen species are considered the leading contributors to cellular impairments and subsequently cellular damage to bodily organs like the liver (Kloss et al., 1983).

BE has a longer half-life than EME and cocaine and can be detected in human plasma 32 hours following administration of both low and high doses of cocaine (Kolbrich et al., 2006). Even 48 hours following cocaine administration, BE was detected in 85.7% of human plasma samples treated with a low dose of cocaine and 100% of plasma samples treated with a high dose of cocaine (Kolbrich et al., 2006).

These cellular effects and cocaine metabolites can have very toxic effects on the body and different organs especially in chronic cocaine abusers. Some of the toxic effects associated with chronic cocaine abuse include: myocardial ischemia and infarction, brain damage, pulmonary complications, liver and kidney damage (Valente et al., 2012).

Treatment Approaches for Addiction

Current treatments for almost all cocaine dependent patients center around cognitive behavioural therapy (CBT) and relapse prevention (Devoto et al., 2015; Nuijten et al., 2015). Vast amounts of time and money have been invested into investigating the therapeutic possibilities which arise from activating and blocking different neurotransmitter receptors in different brain regions. The development of DA receptor antagonists and agonists for the treatment of addiction has gained much interest (Nestler, 2002). The overall goal

is to develop agents that are capable of regulating the process of addiction across several drugs of abuse (Nestler, 2002). Activating opioid, cholinergic, or cannabinoid receptors increases dopaminergic transmission in the NAc (Nestler, 2002) suggesting potential targets for treatment.

For cocaine addiction, an effective treatment should be capable of reducing or eliminating the euphoric effects as well as the craving which persists during cocaine withdrawal (Koob et al., 1998). DA receptor antagonists are believed to be capable of inhibiting DA-enhancing drug effects and therefore have the therapeutic potential to limit drug use because of their ability to competitively bind to DA receptors (Koob et al., 1998). Although DA receptor antagonists can block acute drug effects, there is no evidence suggesting their ability to reduce drug craving or self-administration (Nestler, 2002). Unfortunately, this limits the therapeutic potential of DA receptor antagonists because of their inability to alleviate drug craving (Nestler, 2002). DA antagonists may also be responsible for increasing sensitivity to drugs of abuse by causing adaptive changes in the receptor signaling efficiency (Nestler, 2002). DA receptor agonists, on the other hand, are believed to mimic drug effects and could potentially be more effective in the treatment of addiction, similar to replacement therapy in heroin addiction (Nestler, 2002). Studies have shown promise for the use of D1 receptor agonists and D2 receptor partial agonists as their overall effect has been a decrease in cocaine craving and relapse in animal models (Pulvirenti and Koob, 1994).

Currently there remains no approved pharmacological treatment for cocaine dependence. Advances in understanding the neurobiology of cocaine dependence has led to the discovery of a growing number of promising pharmacological agents to prevent relapse. These pharmacological agents include antipsychotics, anticonvulsants, antidepressants, psychostimulants, other dopamine agonists, and anti-cocaine vaccines (TA-CD) (Devoto et al., 2015; Nuijten et al., 2015). Some of the most promising pharmacological treatments currently under investigation include disulfiram, modafinil, methylphenidate, and TA-CD vaccine.

Disulfiram (Antabuse) is an aversively acting agent which induces negative states by inhibiting aldehyde dehydrogenase (Haile et al., 2012). Disulfiram has been used in patients abusing both alcohol and cocaine as it was thought to deter alcohol consumption and eliminate the alcohol priming effect on cocaine use. In the presence of alcohol, two enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are involved in the breakdown of alcohol molecules. ADH metabolizes alcohol to acetaldehyde, a highly toxic substance and known carcinogen. Acetaldehyde is further metabolized by ALDH to a less active byproduct, acetic acid that can be further broken down to water and carbon dioxide for easy elimination from the body (Haile et al., 2012; Soyka and Mutschler, 2015). Disulfiram acts by inhibiting ALDH causing a build-up of acetaldehyde that results in extreme nausea and sickness (Haile et al., 2012). This sickness is caused by accumulation of acetaldehyde (due to disulfiram) and

induces a negative consequence of extreme sickness following alcohol consumption leading to abstinence from alcohol.

In addition to reducing alcohol consumption, disulfiram has been found to be effective in reducing the incidence and amount of cocaine use, even in non-alcoholic patients. It has been reported that disulfiram inhibits brain dopamine-beta-hydroxylase levels and subsequent monoaminergic modulation in the brain making it a promising candidate for the treatment of cocaine addiction (Soyka and Mutschler, 2015). DA-beta hydroxylase is the enzyme responsible for converting DA to norepinephrine (NE) and has more recently become a target for cocaine addiction (Devoto et al., 2015). Inhibiting dopamine-beta-hydroxylase increases the levels of DA in the brain and reduces the synthesis of NE in both humans and animals (Oliveto et al., 2011; Haile et al., 2012). This decrease in dopamine beta hydroxylase is suggested to be necessary for reducing drug seeking behaviours (Haile et al., 2012). In both animal and human studies it was found that high doses of disulfiram (250mg/day) are able to reduce cocaine seeking behaviour but low doses (62.5 – 125 mg/ day) increased cocaine use and ultimately the reinforcing effects of cocaine (Oliveto et al., 2011; Haile et al., 2012). Since high doses of disulfiram have resulted in reduced cocaine seeking in human participants, it may show promise as a treatment for cocaine dependence.

Modafinil is an agent commonly used to treat narcolepsy because it promotes wakefulness (Nuijten et al., 2015). Modafinil acts by weakly inhibiting DA reuptake enhancing extracellular DA in the NAc. This produces stimulating effects and may reduce substance use by serving as a substitute for cocaine (Herin et al., 2010; Nuijten et al., 2015). It has been found that modafinil possesses subtle stimulant-like effects and several clinical studies support its use as an agonist-like medication (Herin et al., 2010). Modafinil has modest stimulant and cognitive enhancing effects which may improve cocaine withdrawal symptoms (Schmitz et al., 2014). It is believed to function by promoting abstinence because of improved cognitive function or possibly by blunting the euphoric effects of cocaine (Schmitz et al., 2014). Modafinil (400 mg/ day) has shown promising effects in reducing cocaine use and craving in cocaine dependent patients thereby increasing cocaine abstinence (Dackis et al., 2005). Unfortunately, individuals with alcohol and cocaine codependence have not shown significant improvements in abstaining from cocaine use (Canavan et al., 2014). Modafinil has shown some promise for some target populations but there remains a need to design therapeutics for individuals suffering from multiple substance dependencies (Canavan et al., 2014).

Current therapeutics have used immunotherapy strategies to develop a cocaine vaccine (called TA-CD) which stimulates the production of cocaine specific antibodies. Anti-drug vaccines are considered active immunizations, upon administration of the vaccine an immunological response is triggered against the

agent leading to the production of an antigen specific immunoglobulin G (IgG) mediated antibody (Kosten et al., 2014). The body develops anti-drug antibodies which are recognized by the body as being a foreign antigen initiating an immune response (Kosten et al., 2014). These antibodies play a primary role in stopping cocaine addiction by preventing the user from experiencing the euphoric effects associated with cocaine use (Jupp and Lawrence, 2010). The vaccine was developed by linking a cocaine derivative, succinyl norcocaine (SNC) with an immunogenic carrier protein, cholera toxin B subunit, and combining the conjugate with the human adjuvant alum (Ramakrishnan et al., 2014).When cocaine is consumed or administered, the antibodies sequester cocaine molecules in the peripheral circulation and prevent them from crossing the blood brain barrier (BBB) (Jupp and Lawrence, 2010). The TA-CD vaccine inhibits the rewarding effects of cocaine which ultimately can promote abstinence by preventing drug-induced relapse.

The vaccine has demonstrated promising effects for reducing cocaine induced sensitization, self-administration, and relapse in preclinical studies (Kosten et al., 2002). Early clinical trials have supported these positive findings in terms of tolerance, persistence of antibodies following immunization and reduction in cocaine use in patients with high antibody concentration (Kosten et al., 2002). Phase I and Phase II clinical trials were completed on the TA-CD vaccine and some subjects did develop anti-cocaine antibodies without any significant side effects. Unfortunately, only about one third of patients produced

the levels of anti-cocaine antibody required to demonstrate cocaine free urine samples when the vaccine antibodies were circulating through the body (Ramakrishnan et al., 2014). These lackluster results have led to the re-evaluation of the design of the vaccine itself.

There are major obstacles associated with this vaccination approach. The individual must be motivated to quit as the treatment can be overridden by simply increasing the amount of cocaine consumed (Jupp and Lawrence, 2010). The vaccine is only effective on cocaine allowing individuals to switch to a different and potentially more harmful illicit substance. Furthermore, ethical issues exist regarding whether the vaccine should be used to prevent addiction or only as a treatment option (Jupp and Lawrence, 2010). Although the vaccine may be a solution to cocaine abuse and addiction, the underlying ethical controversy is a major limitation to its effectiveness.

Methylphenidate (Ritalin) is a stimulant and is the most commonly prescribed medication for children suffering from ADHD. Methylphenidate (MPH) is prescribed in more than 90% of ADHD cases in the United States (Guerriero et al., 2006). Despite its widespread use, the specific pharmacological mechanism of MPH remains unclear (Guerriero et al., 2006). MPH and cocaine are both known to block the DA transporter, inhibiting DA reuptake in the synapse leading to an increase in extracellular DA (Seeman and Madras, 2002; Guerriero et al., 2006). MPH at higher doses produces the same high in cocaine abusers as

cocaine does (Volkow et al., 1999). MPH and cocaine elicit increases in locomotor activity following acute exposure and continual increases in chronic exposure paradigms (Meririnne et al., 2001) and primates will self-administer MPH (Guerriero et al., 2006).

Due to a similar pharmacokinetic mechanism as cocaine, MPH has been recommended as a potential treatment of cocaine addiction using the replacement therapy approach (Dürsteler et al., 2015; Soyka and Mutschler, 2015). The approach suggests that cocaine dependence can be treated by substituting cocaine with a compound that alleviates the withdrawal symptoms by a similar mechanism of action, inhibiting the presynaptic DA transporters (Dürsteler et al., 2015; Soyka and Mutschler, 2015). In order to assess the reinforcing nature of drugs, a progressive-ratio procedure is used where the number of responses required for drug delivery increases following repeated injections until the subject stops responding (Panlilio and Goldberg, 2007). In this procedure, the breakpoint is classified as being the highest response made by subjects at a specific dose of a drug (Panlilio and Goldberg, 2007). It has been found that MPH and cocaine both have similar breakpoints suggesting that the motivation to take either drug is comparable (Calipari et al., 2014).

Cocaine self-administration has been shown to reduce DAT levels and the rate of DA uptake whereas MPH self-administration increased MPH DAT levels, DA uptake rates, and DA release in rats suggesting that there are different

pharmacodynamics underlying the two drugs (Calipari et al., 2014). Following repeated self-administration of cocaine, a decrease in potency was revealed whereas the potency of MPH following repeated self-administration remained unchanged (Calipari et al., 2014). There remains a need to evaluate whether MPH reduces craving in cocaine dependent individuals in order to evaluate the therapeutic potential for MPH as a treatment for cocaine dependence. However, given its similar pharmacokinetic properties, similar breakpoints to cocaine, MPH may be a promising replacement therapy approach and help cocaine dependent individuals abstain from using cocaine.

Although all of the aforementioned treatments may offer promising approaches for cocaine dependence, none of the treatments have shown a high degree of success in treating relapse to drug taking. There have been multiple advances in the field of nanotechnology resulting in the development of highly specific macromolecules with countless applications. One promising breakthrough in nanotechnology and potential applications to addiction treatment is the development and utility of aptamers.

Aptamers as a therapeutic intervention for addiction

Aptamers are DNA or RNA ligands that can form tertiary structures with binding pockets to selectively recognize various targets such as small molecules, peptides, proteins, whole cells, antibiotics, viruses, even metal ions (Banerjee and Nilsen-Hamilton, 2013a; Jin et al., 2016; Seok Kim et al., 2016). They offer

multiple advantages compared to antibodies as they are relatively inexpensive, easy to chemically modify, have multiple analytical abilities, and can have multiple targets (Seok Kim et al., 2016). Aptamers have high binding affinity and selectivity to their targets (Guo et al., 2008). Aptamers are designed to bind to their target using van der Waals, hydrogen bonding, salt bridges, hydrophobic, and electrostatic interactions (Banerjee and Nilsen-Hamilton, 2013b). Aptamers are relatively easy to synthesize and typically are 10 – 80 base pairs in length (Yan et al., 2016). Aptamers have been used widely in a variety of fields including diagnostics, therapeutics, biosensors, and bio-analytical fields (Seok Kim et al., 2016).

Designing Aptamers

Current technology allows for the development of highly selective DNA or RNA aptamer ligands generated using a method called SELEX (Systematic evolution of ligands by exponential enrichment)(Guo et al., 2008). The process consists of a series of repetitive selection and amplification steps following introduction and exposure to the target cell type or ligand (Sun et al., 2016). The process of synthesizing a specific, selective aptamer begins with an oligonucleotide library where approximately 10^{15} DNA molecules are generated using chemical synthesis and are amplified by polymerase chain reaction (PCR) (Proske et al., 2005). Next, binding and non-binding ligands are separated using a specific target binding strategy, any non-binding nucleic acids are removed and the process continues (Proske et al., 2005). The small number of aptamers which

contain the preferred binding ability are then amplified by real time – PCR (RT-PCR). These binding aptamers are then exposed to the target once again. The selection and amplification process is repeated until target binding sequences dominate.

The SELEX process itself at times has a low success rate and takes anywhere from 8-20 rounds of repetitive steps (Sun et al., 2016). The entire SELEX process can take anywhere from weeks to months, from the early stages of generating an oligonucleotide pool, incubation, partitioning, amplifying, and even sequencing the resulting aptamer sequence (Sun et al., 2016). There have been some substantial modifications made to the SELEX procedure itself in order to increase the success rate by more than 80% while also reducing the number of repetitive rounds required to select an effective aptamer (Sun et al., 2016). Early SELEX used RNA libraries resulting in the generation of RNA aptamers. More recent work utilizes single stranded DNA (ssDNA) to develop aptamers. The use of ssDNA has resulted in more stable aptamer ligands and the improved the selection process (Darmostuk et al., 2015).

In 2004 the, FDA approved the first aptamer drug, pegaptanib sodium also known as "Macugen" (by Pfizer and Eyetech) serving as a major breakthrough for the clinical application of aptamers (Proske et al., 2005). Macugen is a vascular endothelial growth factor (VEGF)-binding aptamer for the treatment of age-related macular degeneration, the leading cause of blindness in adults over 50 years old (Banerjee and Nilsen-Hamilton, 2013b; Santarelli et al.,

2016). Macugen has a high affinity and specificity to bind to a specific extracellular VEGF isoform, VEGF-165 (Santarelli et al., 2016). Patients received a 0.3mg, 1.0mg, 3.0mg, or sham intravitreous injection of Macugen into one eye every 6 weeks for a period of 48 weeks in total (Zampros et al., 2012). By binding to VEGF-165 Macugen reduced the risk of progression to legal blindness and promoted visual stability in some patients (Zampros et al., 2012).

Since the original development of aptamers, there has been increased support for aptamers and their potential to act as novel therapeutic agents suggesting that aptamer development may be of great clinical relevance. There have been multiple other aptamers undergoing clinical development to treat multiple different diseases and illnesses. For example there has been an aptamer that acts to inhibit HIV replication (Kohn et al., 1999) and an anti-thrombin aptamer developed as an anticoagulant/antithrombotic for use during coronary bypass surgery (Proske et al., 2005). Rusconi *et al.* were first to find that aptamers against coagulation factor IXa serve as potent anticoagulants (Rusconi et al., 2002). Complementary oligonucleotides to these aptamers act like antidotes which can efficiently reverse the anticoagulant aptamer actions in the plasma of patients and healthy individuals who cannot tolerate heparin (Rusconi et al., 2002). This ability to safely control anticoagulant activity of heparin enables safer treatment for patients undergoing medical procedures requiring a high level of anticoagulation at which point the risk of hemorrhage is high (Rusconi et al., 2002). This strategy for creating a drug-antidote pair can be used

for developing a wide range of aptamer-based therapeutics (Rusconi et al., 2002; Proske et al., 2005).

The use of Aptamers and Nanocarriers in Research

One of the major benefits of aptamer advancement has been the design and discovery of functioning aptamer-based biosensors. Antibody-based immunoassays have been developed for years and two of the most recognized methods ELISA (enzyme-linked immunosorbent assay) and LFIA (lateral flow immunoassay) require multistep washing and have low sensitivity (Yan et al., 2016). Due to the basic properties of aptamers being nucleic acids with a flexible structure, it allowed for the development of aptasensors (Fiore et al., 2015). Aptasensors are sandwich based assays that have made significant breakthroughs in the development of homogenous and highly sensitive assays (Yan et al., 2016). The sandwich based assay requires that two elements bind to the protein target simultaneously, making them by nature very specific (Fiore et al., 2015). Some approaches to designing these sandwich based assays simply split the functioning oligonucleotide in two components. Unfortunately, this approach can result in reduced sensitivity because the ternary complex stability can be reduced (Fiore et al., 2015). Ahirwar and Nahar successfully developed a gold nanoparticle (AuNP)-based aptasensor for estrogen receptor alpha (Ahirwar and Nahar, 2016). Estrogen receptor alpha is a crucial biomarker for breast cancer diagnosis and targeted therapeutics (Ali and Coombes, 2000). AuNP-

based aptasensors absorb light at different wavelengths upon detection of the target this colour can be detected immediately allowing for real time detection of aptamer specific targets (Liu et al., 2014). Overall, aptasensors have made a significant impact on science and can be designed to be more sensitive and homogenous than current antibody-based immunoassays.

One of the major limitations of clinically relevant aptamers in neuroscience is that synthetic DNA or RNA is unable to cross the BBB. Several techniques have been studied to in order to move drugs and aptamers across the BBB. One technique to deliver aptamers across the BBB is the use of liposomes as a nanocarrier molecule.

Nanotechnology has played an active role in the discovery and development of many aspects related to delivery vehicles. Importantly, nanoparticles have shown great potential for targeted drug delivery because of their enhanced permeability and retention (Zhu et al., 2014). Nanotechnology has driven widespread support of more specific novel targeting agents like aptamers, short peptides, and other small molecules (Zhu et al., 2014). One of the most promising drug delivery strategies is aptamer-mediated nanovehicles (AMNVs) which may show use as a possible cancer therapy, targeting cancer epitopes (Zhu et al., 2014; Darmostuk et al., 2015).

Cancer therapy approaches using AMNVs are focused on developing dual functioning complexes consisting of an outstanding aptamer that targets the extracellular region of a tumor-specific surface biomarker and a cargo portion

which carries a cytotoxic agent (Zhang et al., 2013b; Zhu et al., 2014). The anthracycline class of anti-cancer drugs, like doxorubicin, preferentially bind to CG repeats inserting itself into the sequence leading to a physical conjugate formation (Zhang et al., 2013b). In this particular scenario, the aptamer acts as both a detector for the cancer epitope and as the cargo for drug delivery (Zhang et al., 2013b). It is possible to have different aptamers contained within the nanocarrier and separate aptamers bound to the outer surface. Furthermore, there are multiple nanocarrier options which can hold an aptamer as its internal cargo, such as: nanoparticles, quantum dots, liposomes, carbon tubes etc. (Darmostuk et al., 2015).

The discovery of lipid nanoparticles also termed "liposomes" occurred in the 1960s when it was observed that dry lipid film formed spherical vesicles (Lian and Ho, 2001). These spherical vesicles resembled miniature cellular organelles containing lipid bilayers (Lian and Ho, 2001). Since that discovery, liposome systems have gained major attention and support as drug carriers and cancer therapy treatments (Ho Jin et al., 2015). Lipid nanoparticles are currently the leading delivery systems enabling the delivery of small interfering RNA in peripheral cells (Rungta et al., 2013a). Liposome products have been approved for clinical use by the FDA for over two decades (Xing et al., 2016). However, in order to increase the half-life and bio-availability of peptide aptamers, chemical modifications are applied like a polyethylene glycol (PEG) conjugation to the surface of the nanocarrier (Kadioglu and Efferth, 2016a). In order to increase

circulation time for liposomes, PEGylated formations are added to their surface (Ho Jin et al., 2015; Kadioglu and Efferth, 2016b).

Liposome delivery

Liposome nanoparticle delivery of small inferring RNA to neurons in the CNS is unknown in the literature due to the fact that liposome nanoparticles are unable to cross the BBB (Rungta et al., 2013b). Lipsome nanoparticles have been used as a drug carrier system to deliver specific small interfering RNA (siRNA) to the liver as a novel Hepatitus C treatment approach (Duan et al., 2016). Liposome nanoparticles have also been used to deliver siRNA to the brain by intracortical or intracerebroventricular (ICV) injections resulting in silencing of target genes in close proximity of the injection site as well as in widespread areas (following ICV injection) with no apparent toxicity or immune reactions as a result of the liposome nanoparticle system (Rungta et al., 2013b).

It has been speculated that the liposome nanoparticles bind with their targets and merge with the cell membrane releasing their contents (Joshi et al., 2016). The underlying mechanisms regarding the release of liposomal contents is poorly understood but the method by which gold nanoparticles react with their environment is well documented. It has been found that gold nanoparticles have been used as a light triggering component where light activates the release of the internal contents from liposomal nanoparticles (Lajunen et al., 2016a). Gold nanoparticles convert light energy into heat thereby penetrating the liposomal

membrane allowing for the release of the liposomal contents into the surrounding tissue (Paasonen et al., 2007, 2010; Lajunen et al., 2016b). Not all liposome nanoparticles function in this manner and many of the specific underlying mechanisms regarding liposomal nanoparticle release remains unclear.

One of the major challenges of using liposomes is that they have slow and reduced drug release (Rungta et al., 2013b; Ho Jin et al., 2015). Current strategies for designing more improved liposomes are directed at increasing the sensitivity of the liposome by making them more sensitive to pH, temperature, and even high intensity focused ultrasound can be used to improve drug release from liposomes (Ho Jin et al., 2015). A specific class of liposome systems, cell penetrating peptides (CPP), have proven to be capable of delivering drugs and even molecules into cells (Kang et al., 2015). CPPs are short peptides, usually shorter than 30 amino acids in length which carry a net positive charge that can translocate across the plasma membrane of eukaryotic cells enabling the delivery of nanomolecules, drugs, or molecular therapeutics into cells (Kang et al., 2015). CPPs show promising abilities in transporting macromolecules across bio-membranes, including the BBB, gastroenteric mucosa, and skin dermis (Zhang et al., 2016).

In the present work, liposomes were chosen as the delivery carrier/vehicle system due to their well characterized and demonstrated biocompatibility and biodegradability (Simão et al., 2015). Ease of synthesis, stability, and batch-to-

batch reproducibility make liposomes an ideal complement to aptamers for the development of targeted drug delivery systems. The FDA has approved several liposome-based drug delivery systems for clinical use to treat disease (Jiang et al., 2015). PEGylated liposomes were chosen as PEG inclusion has shown to increase circulation time yielding a cost effective, easily mass produced, and effective delivery modality (Allen and Cullis, 2013). Studies performed by Yang and Fu showed that oxaliplatin (an anti-cancer chemotherapy drug for cancer) liposomes were encapsulated at ~30% efficiency whereas long circulating liposomes modified by PEG (polyethylene-glycol) had ~58% encapsulation efficiency (Yang and Fu, 2014). It is for this reason PEGylated liposomes were used as the delivery system in the present study.

Many ways of detecting aptamers have been discovered and are now applied to the development of new aptamer sequences. A novel and sensitive phase assay uses aptamers to generate fluorescent signals upon structural rearrangement, termed aptamer beacons (Hamaguchi et al., 2001; Proske et al., 2005). In the absence of the target, a fluorophore attached to the DNA aptamer is quenched by the complementary DNA fragment carrying a quencher dye, resulting in no fluorescent signal (Hamaguchi et al., 2001; Proske et al., 2005). However, when the system is exposed to the target, the aptamer binds to it and the quencher-labeled DNA is released (Hamaguchi et al., 2001; Proske et al., 2005). Since the target-binding event generates a fluorescent signal, it serves as

an effective method for monitoring aptamer target recognition in real time (Hamaguchi et al., 2001).

Quantitative polymerase chain reaction (qPCR) has been recently used to evaluate the pharmacokinetics of a specific aptamer in mice (Perschbacher et al., 2015). Different multiple organs, tissues, blood, and brain samples were analyzed to evaluate the concentration of aptamer at various time points following a single i.p. injection of the DNA aptamer (Perschbacher et al., 2015). The DNA aptamer used showed broad and rapid tissue delivery with the highest accumulation found in the liver and spleen and approximately 1000 fold less found in the central nervous system in healthy and MS-like animals (Perschbacher et al., 2015). Furthermore, the DNA aptamer distribution peaked within minutes of the i.p. injection compared to radiolabeled immunoglobin M antibodies which peaked approximately 24 hours later. This was the first time qPCR had been used to demonstrate the presence of an aptamer after intraperitoneal administration of a guanosine-rich DNA aptamer shown to mediate re-myelination in a mouse model of MS (Perschbacher et al., 2015).

Present Study

The present study injected a novel single stranded DNA aptamer with high binding affinity for dopamine (DA) and norepinephrine (NE) into mice exposed to cocaine to determine its effect on cocaine induced hyper-locomotor activity. The aptamer was selected intentionally as it has been administered into the NAc of rats previously and was shown to significantly reduce cognitive impairments in a

rat model of schizophrenia (Holahan et al., 2011). Specifically, upon administration of MK-801, rats could not extinguish a bar pressing response (Holahan et al., 2011). If rats were treated with the aptamer, the cognitive deficits were reversed and rats were able to extinguish this bar pressing behaviour (Holahan et al., 2011). The aptamer acted like an antagonist of the dopaminergic/ noradrenergic systems as it was hypothesized to bind directly to DA and NE preventing the interaction of the neurotransmitters with post-synaptic receptors. The overall goal of the present set of experiments was to investigate the effect of acute and repeated administration of this aptamer systemically by injecting it in the periphery and monitoring its interaction with cocaine-induced brain and behavioral changes.

Prior to creating the DNA based aptamer, an RNA version had been well characterized and studied *in vitro*. This RNA homolog was shown to bind to dopamine with high affinity and specificity with a dissociation constant (K_d) of 1.6μM (Walsh and DeRosa, 2009). The dissociation constant is a measurement of the tendency of a larger object to separate reversibly into smaller components. The DNA version of the aptamer showed improved affinity (K_d=0.7μM) for DA and NE (K_d=0.4μM) while maintaining similar specificity as the RNA homolog (Walsh and DeRosa, 2009). The aptamer sequence forms two hairpin loops which are essential for target binding. Using hydrogen bonding, these hairpin loops will fold to bind and hold the target neurotransmitters in a pocket until dissociation. Walsh *et al.,* revealed that binding can only be achieved by the

precise arrangement of five specific nucleotides of each hairpin loop which comprise the binding pocket (Walsh and DeRosa, 2009).

For the current study, the aptamer was encapsulated in a liposome and the outer surface of the liposome contained a transferrin receptor aptamer. This novel modification was made to optimize the delivery and transport of the aptamer across the BBB. The hypothesized manner by which the aptamer functions is by binding to transferrin receptors on the surface of BBB. Once this occurs, it is speculated that the liposome will merge with the BBB and the liposomal contents (aptamer) will be transported across the BBB. To ensure the aptamer was working in the CNS, multiple chemical manipulations that altered the functionality were also tested.

The specific goals of this study were to assess the *in vivo* utility of the aptamer designed to cross the BBB. Our first experiment focused on investigating the effects of repeated administration of the aptamer to verify that the aptamer did not result in neuronal degeneration or long-term motor deficits. Our subsequent experiment investigated the locomotor effects of the aptamer when mice were administered different concentrations of cocaine. Next, we evaluated the locomotor behaviour of mice when administered a modified, more concentrated aptamer after having received cocaine at different concentrations. We also examined brain homogenate and performed qPCR to evaluate whether the aptamer was present in brain tissue. Our control experiment investigated multiple chemical manipulations of the aptamer to determine which components

were required to facilitate entry of the aptamer into the brain. Finally, we compared the distribution of rhodamine fluorescence based in the NAc on the presence or absence of the transferrin receptor aptamer on the surface of the rhodamine-tagged liposomes.

Materials and Methods

i) *Repeated Administration Experiment*

Animals received their respective treatments for 5 consecutive days followed by a 2 day rest period and a final test day. Mice were administered a single 0.1mL i.p. injection for 5 consecutive days to evaluate the safety and toxicity of the 1X aptamer.

Animals

Male CD-1 mice (n=20) were purchased from Charles River (St. Constant, Quebec) and were housed individually in 27 x 21 x 24 cm plastic cages. The room temperature was maintained at 21°C and lighting was kept on a 12 hour light/dark cycle. Mice were fed with Purina mice chow and food and water were available *ad libitum*. All animal procedures were approved by the Canadian Council on Animal Care (CCAC) and Carleton University Ethics Board (Ottawa, Ontario).

Acclimation

Upon arrival at the Carleton University Vivarium facility, all mice were housed individually for a 5-7 day period to acclimate to the facility. No studies or tests were carried out during this time.

Habituation

Following the acclimation period, all mice underwent a 3 day habituation period for 30 minutes each day. Animals were transported by cart to a windowless test room and were placed individually in 24 x 48 x 20 cm enclosed locomotor boxes for 30 minutes. All habituation trials began between 08:00 and 10:00 am. Locomotor activity levels during the three 30-minute habituation trials were recorded to obtain baseline motor activity recordings. Locomotor activity was recorded by 16 sensors separated by 2.5cm along the bottom of the box. A horizontal movement is detected and counted when a break in the beam sensor is experienced serving as an indication that the mouse had moved from a specific location to another. The sensors on the bottom of the locomotor box transmitted the horizontal activity counts to a computer running the Fusion HC software system. The software setting were arranged to record the horizontal motor activity of the mice every 5 minutes for the full 30 minute trial.

Locomotor Testing

Identical to the habituation phase, mice were transported by cart to a windowless test room and were individually placed in the same locomotor boxes used during the habituation phase. The test phase was 30 minutes.

i) *Repeated Administration experiment*

The repeated administration experiment involved 20 male CD-1 mice. The mice were divided into 3 treatment groups: repeated aptamer (n=5), repeated liposome (n=10), and a no injection (n=5) treatment groups. All mice underwent an acclimation and habituation period. The acclimation period allowed the mice to settle following transportation from the breeding facilities. During the habituation period mice were placed in the locomotor boxes for 30 minutes for three consecutive days to collect baseline motor activity recordings. Mice received a 0.1mL intraperitoneal (i.p.) injection every day over the course of 5 days, a 2 day rest period, followed by a final test day. Recordings were obtained after mice were administered a 0.1mL IP injection of: DAL-TRAM (the aptamer housed in a liposome), TRAM (an empty liposome without the aptamer), or no injection. Following the treatment all animals were placed in a locomotor box for 30 minutes where horizontal activity was recorded every 5 minutes. The cumulative horizontal activity was calculated for all animals on the final day of treatment. This repeated administration procedure was used to evaluate the safety of the aptamer and delivery system. Brain tissue was analyzed using the fluorojade-B stain.

Treatments

Animals were given a single 0.1mL I.P injection for 6 days. All aptamer and control manipulations were prepared, stored, and obtained from the De Rosa Laboratory in the Department of Chemistry in Carleton University.

Refer to Table 1 for details regarding abbreviations and aptamer/delivery components.

i) DA/NE aptamer (DAL-TRAM) – a DNA based aptamer built from a 58 base pair DNA sequence loaded into a liposome. There is a transferrin receptor (TfR) aptamer on the outer surface of the liposome that allows for the aptamer to be delivered across the blood brain barrier (BBB). The DA/NE aptamer was designed at a 1X concentration.

ii) Liposome (TRAM) – an empty liposome that does not contain the aptamer in the liposome. The TfR aptamer is attached to the outer surface of the liposome allowing the liposome to cross the BBB. Since the liposome does not contain the aptamer this serves as a control manipulation of the DA/NE aptamer.

iii) No injection – animals did not receive an injection.

Injections

All injections were administered intraperitoneally. Animals in the aptamer and liposome treatment group all received a single 0.1mL injection. All injections were performed using a BD 1mL TB syringe with 26G 3/8 tip. Immediately

following the injections, the mice were placed individually in their respective locomotor boxes and motor activity was recorded over a 30-minute trial.

Flurojade-B Staining

Brains were extracted and post fixed in a solution of 30% sucrose in 4% paraformaldehyde (PFA), were flash frozen, and cryostat sliced into 35 µm saggital sections. Frozen sections were mounted and once dry were washed 6 times for 10 minutes in 0.1M phosphate buffer (pH 7.4). Subsequently tissue was immersed in 100% ETOH (3 mins), 70% ETOH (2 mins), 30% ETOH (2 mins), followed by distilled H_2O (2 mins). Sections were then incubated in filtered 0.06% $KMnO_4$ for 15 minutes and were rinsed with distilled H_2O for 2 minutes. Next, sections were incubated in 0.001% Fluorojade-B with 0.1% acetic acid solution for 30 minutes (Chemicon cat# AG310) and were briefly rinsed in distilled H_2O. Immediately after, sections were dried using a drier set on high for 2 minutes, all excess water was removed from the slides. Sections were then placed in clearene for 2 minutes and were cover slipped using DPX.

ii) *Acute experiments*

a) Acute Experiment 1

Mice received two 0.1mL i.p. injections on test day to evaluate the effectiveness of a 1X aptamer in the presence of cocaine to determine whether aptamers offer potential as a new therapeutic avenue for cocaine dependence.

b) Acute Experiment 2

Mice received two 0.1mL i.p. injections on test day to evaluate the effectiveness of a 2X aptamer in the presence of cocaine to determine whether a 2X aptamer is a more effective therapeutic option than the 1X aptamer.

c) Acute Control Experiment

Investigate multiple chemical manipulations of the aptamer (at 1X) to determine whether different aspects of the delivery system impact the motor activity of the mice.

Animals

Male CD-1 mice (n=148, total for all acute experiments) were purchased from Charles River (St. Constant, Quebec) and were housed individually in 27 x 21 x 24 cm plastic cages. The room temperature was maintained at 21°C and lighting was kept on a 12 hour light/dark cycle. Mice were fed with Purina mice chow and food and water were available *ad libitum*. All animal procedures were approved

by the Canadian Council on Animal Care (CCAC) and Carleton University Ethics Board (Ottawa, Ontario).

Acclimation

Upon arrival at the Carleton University Vivarium facility, all mice were housed individually for a 5-7 day period to acclimate to the facility. No studies or tests were carried out during this time.

Habituation

Following the acclimation period, all mice underwent a 3 day habituation period for 30 minutes each day. Animals were transported by cart to a windowless test room and were placed individually in 24 x 48 x 20 cm enclosed locomotor boxes for 30 minutes. All habituation trials began between 08:00 and 10:00 am. Activity levels during the three 30-minute habituation trials were recorded to obtain baseline motor activity recordings. Locomotor activity was recorded by 16 sensors separated by 2.5cm along the bottom of the box. A horizontal movement is detected and counted when a break in the beam sensor is experienced serving as an indication that the mouse had moved from a specific location to another. The sensors on the bottom of the locomotor box transmitted the horizontal activity counts to a computer running the Fusion HC software system. The software settings were arranged to record the horizontal motor activity of the mice every 5 minutes for the full 30 minute trial.

Locomotor Testing

Identical to the habituation phase, mice were transported by cart to a windowless test room and were individually placed in the same locomotor boxes used during the habituation phase. The test phase was 30 minutes.

Drugs

Each animal was administered two 0.1mL I.P injections on test day. All drugs were prepared, stored, and obtained from the De Rosa Laboratory in the Department of Chemistry in Carleton University.

a) Injection 1:

Refer to Table 1 for details regarding abbreviations and aptamer/delivery components.

i) DA/NE aptamer (DAL-TRAM) – a DNA based aptamer with high affinity for DA and NE loaded into a liposome. On the outer surface of the liposome is a transferrin receptor (TfR) aptamer that allows for the aptamer to be delivered across the blood brain barrier (BBB). The aptamer was administered at both the 1X and 2X concentration.

ii) Liposome (TRAM) – an empty liposome that does not contain the DA/NE aptamer in the liposome. The TfR aptamer is attached to the outer surface of the liposome allowing the liposome to cross the BBB. Since the liposome does not contain the DA/NE aptamer this serves as a control manipulation of the aptamer.

iii) Random Oligonucleotide (ROL-TRAM) – A random 58 base pair sequence of DNA loaded into a liposome. The outer surface of the liposome contains TfR aptamers allowing for the random oligonucleotide sequence to be delivered across the BBB. This treatment serves as a control for the DA/NE aptamer and can demonstrate the novel abilities of the aptamer.

iv) Substituted Aptamer (Sub-TRAM) – A specific 58 base pair sequence of DNA very similar to DAL-TRAM that has 4 base pairs altered so that the aptamer is no longer able to bind to the target DA/NE. This serves as a control treatment for the aptamer.

v) No Transferrin (DAL) – 58 base pair aptamer housed inside of a liposome. The outer membrane of the liposome does not have the TfR aptamer which limits the ability of the aptamer to cross the BBB. This treatment serves as a control for the aptamer as it is able to reveal the novel effects of the aptamer when it is not designed to cross the BBB.

vi) Saline – 0.9% sodium chloride. The saline injection served as a control which demonstrated the direct effect of the aptamer in comparison to a single injection of saline.

b) Injection 2:

i) Cocaine – Ecgonine methyl ester benzoate HCl was administered in three different concentrations (1mg/mL, 5mg/mL, 10mg/mL).

ii) Saline – 0.9% sodium chloride. The saline injection acted as a control to the cocaine injection as it does not cause the build-up of dopamine in synapses. The vehicle injection revealed how the aptamer functioned in the absence of excess dopamine release.

Injections

All injections were administered intraperitoneally. All animals received two 0.1mL i.p. injections, chemicals injected were determined based on treatment group. All injections were performed using a BD 1mL TB syringe with 26G 3/8mm tip. Immediately following the 2 injections, the mice were placed individually in their respective locomotor boxes and motor activity was recorded over a 30-minute trial.

Behavioural Design Details

a) Acute Experiment 1:

The experiment involved 53 male CD-1 mice separated into 7 treatment groups: Sub-TRAM + 10 mg/mL of cocaine (n=8), DAL-TRAM + 10mg/mL of cocaine (n=8), DAL-TRAM + 5 mg/mL of cocaine (n=8), DAL-TRAM + 1 mg/mL of cocaine (n=7), TRAM + saline (n=8), and DAL-TRAM + saline (n=8), and saline + saline (n=6). All mice endured an acclimation and habituation phase to the locomotor boxes on three consecutive days for 30 minutes to collect baseline motor activity data. On the test day, mice were given a 0.1mL pre-treatment by

i.p. injection of substituted aptamer loaded into a liposome, aptamer loaded into a liposome, or an empty liposome without the aptamer. Following the pre-treatment mice were i.p. injected with 0.1mL of saline, 1 mg/mL, 5 mg/mL, or 10 mg/mL of cocaine. All mice were then placed in the locomotor boxes for 30 minutes where horizontal activity was recorded every 5 minutes.

b) Acute Experiment 2:

The behavioural procedure mimics that of Experiment 2 however the animals were treated with a more concentrated aptamer treatment (2X DA/NE aptamer). The experiment involved 41 CD-1 mice divided into 6 treatment groups: 2X DAL-TRAM + 10mg/mL cocaine (n=10), 2X DAL-TRAM + 5mg/mL of cocaine (n=4), 2X DAL-TRAM + 1mg/mL of cocaine (n=3), 2X DAL-TRAM + saline (n=10), TRAM + saline (n=8), and saline + saline (n=6). Identical to experiment 2 all mice experienced both an acclimation period and a 3 day habituation period. Following habituation mice were tested by receiving a 0.1mL i.p. injection as a pre-treatment.

c) Acute Control Experiment:

The behavioural procedure mimics that of Experiment 2 & 3 however the animals were treated with chemical manipulations of aptamer treatment instead. The experiment involved 54 male CD-1 mice divided into 8 treatment groups: Sub-TRAM + 10 mg/mL cocaine (n=8), DAL + 10mg/mL of cocaine (n=9), ROL-TRAM

+ 10mg/mL of cocaine (n=7), TRAM + 10 mg/mL cocaine (n=8), DAL + 10mg/mL cocaine (n=8), TRAM + saline (n=8), and saline + saline (n=6). Identical to experiment 2 & 3 all mice experienced both and acclimation period and a 3 day habituation period. Following habituation mice were tested by receiving a 0.1mL i.p. injection as a pre-treatment of: substituted aptamer loaded into a liposome (Sub-TRAM), aptamer loaded into a liposome without transferrin- receptor aptamer (DAL), random non-binding aptamer sequence loaded into a liposome (ROL-TRAM), empty liposome without the aptamer (TRAM), aptamer loaded into a liposome (DAL-TRAM), or saline. Following the pre-treatment mice were i.p. injected with 0.1mL of 10 mg/mL of cocaine or saline. All mice were then placed in the locomotor boxes for 30 minutes where horizontal activity was recorded every 5 minutes.

Real Time Polymerase Chain Reaction (RT- PCR)

Mice were rapidly decapitated without anesthesia 30 minutes following treatment. Brains from the following groups; 2X DAL-TRAM + 10 mg/kg cocaine (n=5), 2X DAL-TRAM + saline (n=4) and Sub-TRAM + 10mg/kg cocaine (n=5) were extracted within 30 seconds of sacrifice and tissue punches from nucleus accumbens (NAc), ventral tegmental area (VTA), and prefrontal cortex (PFC) were collected in a sterile manner and flash frozen on dry ice within the next 60 seconds. Samples were stored at -80°C until ready for extraction.

Aptamer Extraction

Tissue was homogenized in Trizol and extracted using the Purelink RNA micro kit (Invitrogen Cat no. 12183-016) following the manufacturers protocol then stored at -80°C until use.

PCR

The presence of the aptamer was determined via standard PCR performed on the brain extractions using the following forward and reverse primers: 5' CTAGACTAGAAGCTGAGCTGCTAGACTAGAAGCTGAGCTGGTCTCTGTGTGCGCCAGA and 5' ACGTTACGTTATGACATGACACGTTACGTTATGACATGACGGGCCTCATTCTG-TGCTG. PCR was performed on a Bio-Rad CFX Connect Real Time System using 20 uL reactions with 5uL of extracted DNA, 2.66 uL of 200nM combined primers, 2.34uL of Milli-Q Ultrapure H_2O and 10 uL of Bio-Rad Green Supermix Master Mix. Reaction was performed in accordance with the following thermal profile: 95°C for 3 mins; 40 two-step cycles [95°C for 10 secs; 58°C for 30 secs] 95°C for 10 seconds. Once completed a melt curve from 60°C to 95°C was run in increments of 0.5°C. Melt curve and quantification data were used as the primary detection method and an electrophoresis gel was run to confirm findings.

Gel Electrophoresis

Amplified DNA produced during the qPCR was run on a standard 2% agarose gel containing 0.01% Sybr safe DNA dye. 17uLs of sample and 3uL of 0.25% bromothymal blue loading buffer were placed in each well and run beside a well

containing 5uLs of Invitrogen Tackit 50bp DNA ladder. Gels were run at 120V for 60 minutes and imaged using a Biorad GelDoc.

iii) *Fluorescent Detection Experiment*

Animals

Male CD-1 mice (n=9) were purchased from Charles River (St. Constant, Quebec) and were housed individually in 27 x 21 x 24 cm plastic cages. The room temperature was maintained at 21°C and lighting was kept on a 12 hour light/dark cycle. Mice were fed with Purina mice chow and food and water were available *ad libitum*. All animal procedures were approved by the Canadian Council on Animal Care (CCAC) and Carleton University Ethics Board (Ottawa, Ontario).

Acclimation

Upon arrival at the Carleton University Vivarium facility, all mice were housed individually for a 5-7 day period to acclimate to the facility. No studies or tests were carried out during this time.

Drugs

Each animal was administered a 0.1mL i.p. injection on test day. All drugs were prepared, stored, and obtained from the De Rosa Laboratory in the Department of Chemistry in Carleton University.

a) Injections:

Refer to Table 1 for details regarding abbreviations and aptamer/delivery components.

i) Aptamer (DAL-TRAM) – a 58 base pair DNA based aptamer loaded into a liposome. The outer surface of the liposome is labeled with rhodamine dye and has transferrin receptor (TfR) aptamers allowing the aptamer to bind to transferrin receptors on the BBB in turn enabling the delivery of the aptamer into the central nervous system.

ii) No Transferrin (DAL) – 58 base pair aptamer housed inside of a liposome. The outer membrane is labeled with rhodamine dye however, the liposome does not have the TfR aptamers on its surface limiting the ability of the aptamer to cross the BBB. This treatment served as a control for the aptamer in order to reveal effects of the aptamer in the absence of the transferrin receptor aptamer.

iii) Saline – 0.9% sodium chloride. The saline injection serves as a control that demonstrated the direct effects of the aptamer in comparison to a single injection of saline.

Injections

All injections were administered intraperitoneally. All injections were performed using a BD 1mL TB syringe with 26G 3/8mm tip. Five minutes following the injection, mice were euthanized by rapid decapitation.

Fluorescent Aptamer Detection

Mice were euthanized by rapid decapitation 5 minutes after receiving a single 0.1mL i.p. injection. Brains were removed and fixed in a 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) solution. All tissue was stored at 4° for 24 hours prior to being switched and stored in 30% sucrose/ 0.1 M phosphate (pH 7.4) at 4° for a minimum of 72 hours.

To provide an initial, qualitative assessment of aptamer entry into the brain, 3 mice were injected with the Rhodamine-labeled aptamer (DAL-TRAM), 3 mice were injected with the Rhoadmine-labeled liposome containing the aptamer without the transferrin-receptor aptamer on the surface of the liposome (DAL), and 3 mice were injected with saline.

Brains were sectioned on a cryostat at 35 µm through the nucleus accumbens (anterior posterior plane of the Paxinos and Watson atlas: 2.20 – 1.0 mm from bregma). Tissue sections were mounted on glass slides and coverslipped with Fluromount. Using an Olympus BX61 microscope (Olympus Canada, ON), digital images of the nucleus accumbens were obtained (20x). Using ImageJ software, digital images were converted to greyscale and threshold levels were normalized across all brain sections. Densitometric analyses were carried out to provide an intensity measure for all tissue and treatment groups.

Integrated Fluorescent Density

Using an Olympus BX61 microscope (Olympus Canada, ON), digital images of the nucleus accumbens core and shell regions were obtained (20x, NAc 0.4; InVitro version 3.2.2; Media Cybernetics, MD) using the same exposure time to reduce photobleaching and equalize intensities across subjects. Pixel intensity maps were generated for rhodamine staining using the Image-Pro Analyzer version 6.2.1.491 (Media Cybernetics, MD). Briefly, 5 horizontal lines were generated across the entire nucleus accumbens region and intensity measures were collected. An average raw pixel intensity profile was generated for each section. For quantification, intensity measures were normalized to intensity measures in the anterior commissure. Statistical analyses between groups were made using these normalized intensities.

Table 1: Description of all Abbreviations and Components for the Aptamer, multi-aptamer delivery system, and control modifications

Abbreviation	**Aptamer details**
Sub	Non-binding/base substituted aptamer. The sequence is identical to the aptamer with the exception of 4 specific point mutations, the presence of which eliminates aptamer binding (Walsh and DeRosa, 2009)
ROL	Random oligonucleotide of comparable length to the aptamer previously shown not to affect dopamine-related behavior (Holahan et al., 2011)
Abbreviation	**Component details**
DAL-TRAM	Aptamer loaded-transferrin receptor aptamer modified liposome (Transferrin Receptor Aptamer (TRA)-positive liposome)
DAL	Aptamer loaded liposome (TRA-negative liposome)
TRAM	Transferrin receptor aptamer modified liposome (no oligonucleotide payload)
Sub-TRAM	Non-binding/base substituted aptamer loaded – transferrin receptor aptamer modified liposome
ROL-TRAM	Random oligonucleotide loaded-transferrin receptor aptamer modified liposome

Table 2: Aptamer sequences used in the novel drug delivery system design

Aptamer	Sequence (5'→3')
DA/NE aptamer	**GTCTCTGTGTGCGCCAGA**GACACTGGGGCAGATATGGGC**CA GCACAGAATGAGGCCC**
Non-binding base substituted aptamer (Sub)*	**GTCTCTGTG<u>CCAAA</u>CAGA**GACACTGGGGCAGATATGGGC**C<u>C</u> GCACAGAAT<u>CC</u>GGCCC**
Random oligonucleotide (ROL)	AGAATCTGTCGGGCTATGTCACTAATACTTTCCAAACGCCCCGT ACCGATGCTGAACA
Transferrin receptor aptamer (TRA)	GAATTCCGCGTGTGCACACGGTCACAGTTAGTATCGCTACGTTC TTTGGTAGTCCGTTCGGGAT

* Base substitutions are underlined. Primer regions are bolded.

Results

Repeated Administration Experiment:

Behavioural Analysis

The purpose of the first experiment was to evaluate any detrimental effects of the aptamer following repeated injections. Three groups of mice received either 6 injections of the aptamer, the liposome control, or no injections over 6 days. Horizontal motor activity was recorded for 30 minutes on each day and after the last day, brains were removed.

Average locomotor activity was calculated for the 30 minute trials following each day of treatment and the data were analyzed using IBM SPSS 19 software. The average locomotor activity for each treatment group throughout the repeated administration procedure was calculated and is plotted in Figure 1A below.

Average locomotor activity for each treatment group on each day was analyzed with a three way ANOVA (5 min bin and day as the within factors and treatment as the between factor; Figure 1A) and average locomotor activity was analyzed on the last test day with a one-way ANOVA (Figure 1B). A three way ANOVA (treatment condition by day by 5 min time bin) revealed no main effect of treatment condition ($F_{(2,12)} = 1.35$), no main effect of day ($F_{(5,10)} = 1.71$) and no interaction between treatment condition and day ($F_{(10,60)} < 1.0$). There was a main effect of 5-min time bin ($F_{(5,10)} = 14.18$, $p < 0.001$) and a significant interaction between 5-min time bin and treatment condition ($F_{(10,60)} = 2.99$, $p <$

0.01). Finally, there was an interaction between day and 5-min time bin ($F_{(25,50)}$ = 2.00, $p < 0.01$) and a three way interaction between treatment condition, day and 5-min time bin ($F_{(50,300)}$ = 1.65, $p < 0.01$). Because of the lack of a main effect of treatment condition, no further analyses were carried out.

Mice were treated for 6 days with aptamer, (DAL-TRAM, n= 5), liposome control (TRAM, n= 5) or no injection (n= 5). Horizontal movement was recorded on each of the treatment days. The horizontal movement recorded following the final treatment day is shown in Figure 1B. A one-way ANOVA on the total locomotor activity revealed no main effect of group ($F_{(3,24)}$=0.949, p=0.433.

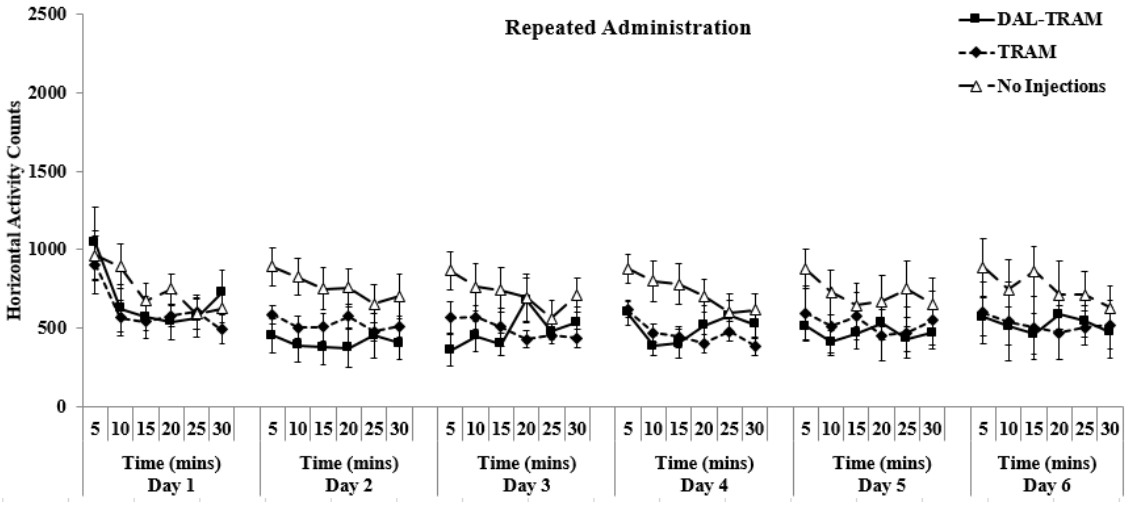

Figure 1A: Repeated administration of the aptamer (DAL-TRAM), the empty liposome (TRAM) and, no injections did not result in significantly different locomotor activity between treatment groups

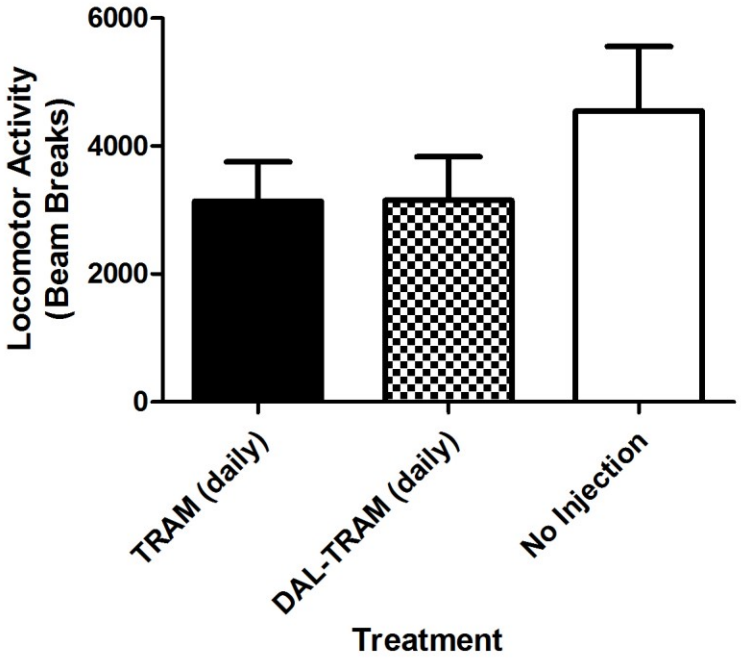

Figure 1B: Repeated administration of DAL-TRAM in the absence of cocaine had no effect on locomotor behaviour. The total horizontal activity recorded during the final 30 min session of the experiment is shown. One-way ANOVA revealed no significant group effect ($F_{(3,24)}$=0.949, p=0.433).

Fluorojade-B Assessment

Forty eight hours after the last locomotor assessment, all mice were euthanized and brain tissue was processed for Fluorojade B staining to examine whether repeated systemic administration of either DAL-TRAM or TRAM caused damage at the cellular level (Schmued and Hopkins, 2000). Images of tissue procured from the DAL-TRAM, TRAM, and no injection groups (from Repeated Administration Experiment) were compared to control tissue where damage had been caused by implantation of a chronic, indwelling cannulae (Figure 2A) from a published report (Wartman et al., 2014). In the positive control, there is extensive Fluorojade B staining near the cannula tip and extending outward, likely reflecting damaged neurons and reactive astrocytes or gliosis. White arrows highlight Fluorojade B stained damaged neurons in the positive control (A) of Figure 2. In comparison, Fluorojade B stained tissue following repeated administration of the DAL-TRAM (B) and the TRAM (C) showed no evidence of degeneration, appearing similarly stained to the negative, no injection control (D).

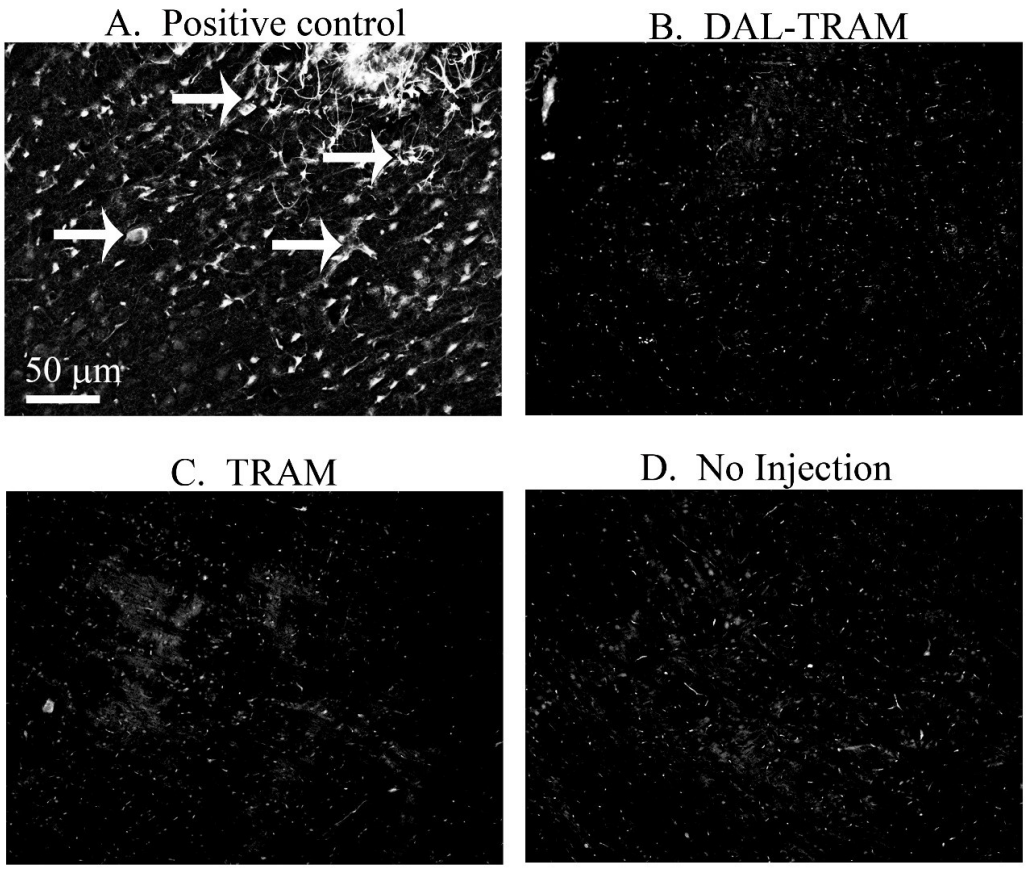

Figure 2: Fluorojade B staining revealed no indication of neuronal degeneration in animals that had been repeatedly treated with DAL-TRAM or TRAM. White arrows (A) indicate regions of neural tissue damage. Magnification is at 20X.

Acute Experiment 1

The purpose of the second experiment was to evaluate the effectiveness of an acute treatment of the DA/NE aptamer (DAL-TRAM) in reducing hyperlocomotion resulting from different doses (1mg/mL, 5mg/mL, and 10mg/mL) of cocaine. Mice were acclimated to the laboratory environment and were habituated on the micromax equipment for 30 minutes on 3 separate days. Each mouse received an i.p. injection of either DA/NE aptamer housed in a liposome, a substituted (non-binding) aptamer, an empty liposome, or saline followed by a 0.1mL injection of 10mg/mL, 5mg/mL, 1mg/mL of cocaine, or saline. Horizontal motor activity was recorded every 5 minutes for a 30 minute trial period.

Cumulative locomotor activity was calculated for the 30 minute trial following treatment and the data was analyzed using IBM SPSS 19 software. The average locomotor activity for each treatment group was calculated and this data is plotted in Figure 3 below.

Statistical analysis using a one way ANOVA revealed a Main effect of Treatment ($F_{(6,46)}$= 22.708, $p<0.001$). Fishers LSD post-hoc analyses revealed that mice in the substituted aptamer treatment group had significantly different locomotor activity than all other treatment groups ($p<0.001$). Fishers LSD post-hoc analyses also revealed that DAL-TRAM + 10mg/mL cocaine and DAL-TRAM + 5mg/mL cocaine treatment groups did not reveal a significant difference in locomotor activity ($p=0.76$). However, both treatment groups (DAL-TRAM +

10mg/mL cocaine & DAL-TRAM + 5mg/mL cocaine) revealed a significant difference in locomotor activity when compared to all other treatment groups ($p<0.05$).

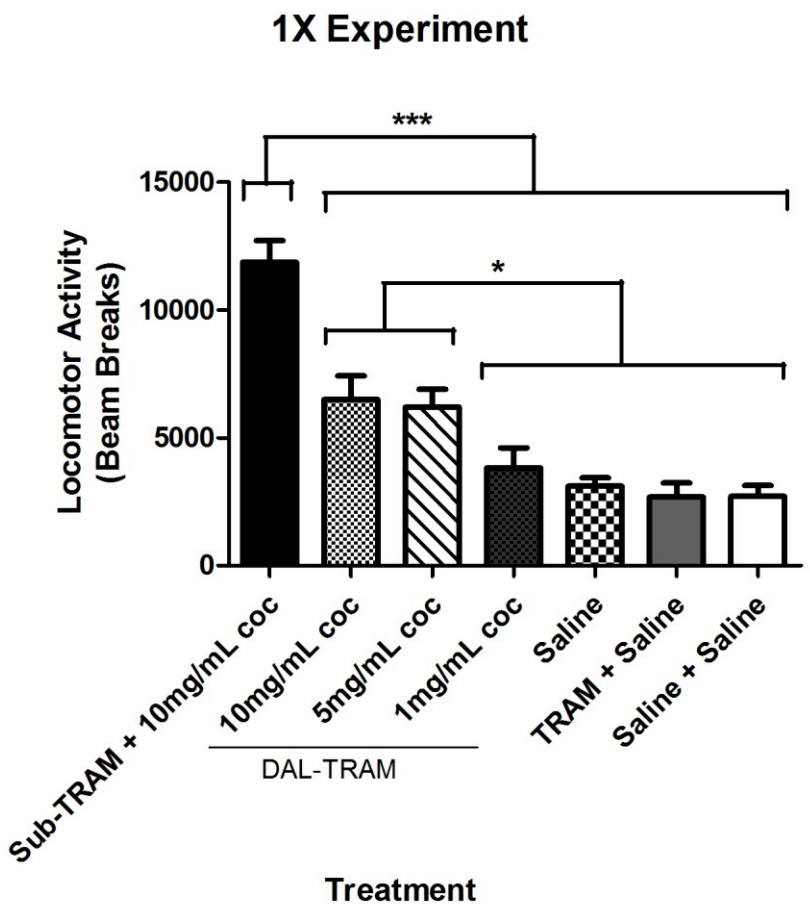

Figure 3: Ability of DAL-TRAM to reduce cocaine-induced hyperlocomotion. Total horizontal activity over 30 min is shown. ANOVA revealed a significant effect of group ($F_{(6,46)} = 22.71$, $p<0.001$). Fisher's LSD post-hoc analyses revealed significant differences between treatment conditions as indicated.

(***) $p<0.001$

(*) $p<0.05$

Acute Experiment 2

The purpose of the third experiment was to investigate the effectiveness of the aptamer when designed at a higher concentration and administered in the presence of different doses (10mg/mL, 5mg/mL, and 1mg/mL) cocaine. Mice were acclimated to the laboratory environment and were habituated on the micromax equipment for 30 minutes on 3 separate days. On the test day, each mouse received an i.p. injection of either the 2X aptamer housed in a liposome, a substituted (non-binding) aptamer, an empty liposome, or saline followed by a 0.1mL injection of 10mg/mL, 5mg/mL, 1mg/mL of cocaine, or saline. Horizontal motor activity was recorded every 5 minutes for a 30 minute trial period.

Cumulative horizontal activity was calculated following the trial and the data was analyzed using the IBM SPSS 19 software. The average locomotor activity for each treatment group was calculated and this data is plotted in Figure 4 below.

Statistical analysis using a one way ANOVA revealed a Main effect of Treatment ($F_{(6,42)}$= 24.525, $p<0.001$). Fishers LSD post-hoc analyses revealed that mice in the Sub-TRAM + 10mg/mL cocaine and 2X DAL-TRAM + 10mg/mL cocaine treatment groups did not reveal a significant difference in locomotor activity ($p=0.273$). However, Sub-TRAM + 10 mg/mL cocaine showed significantly different locomotor activity when compared to 2X DAL-TRAM + 5mg/mL cocaine and 2X DAL-TRAM + 1mg/mL treated animals ($p<0.05$). Furthermore, Fishers LSD post-hoc analyses revealed that saline treated animals

(2X aptamer + saline, Liposome + saline, saline + saline) showed a significant difference in locomotor activity compared to cocaine treated animals (Sub-TRAM + 10mg/mL cocaine, 2X DAL-TRAM + 10mg/mL, 2X DAL-TRAM+ 5mg/mL cocaine, 2X DAL-TRAM + 1mg/mL) ($p<0.05$).

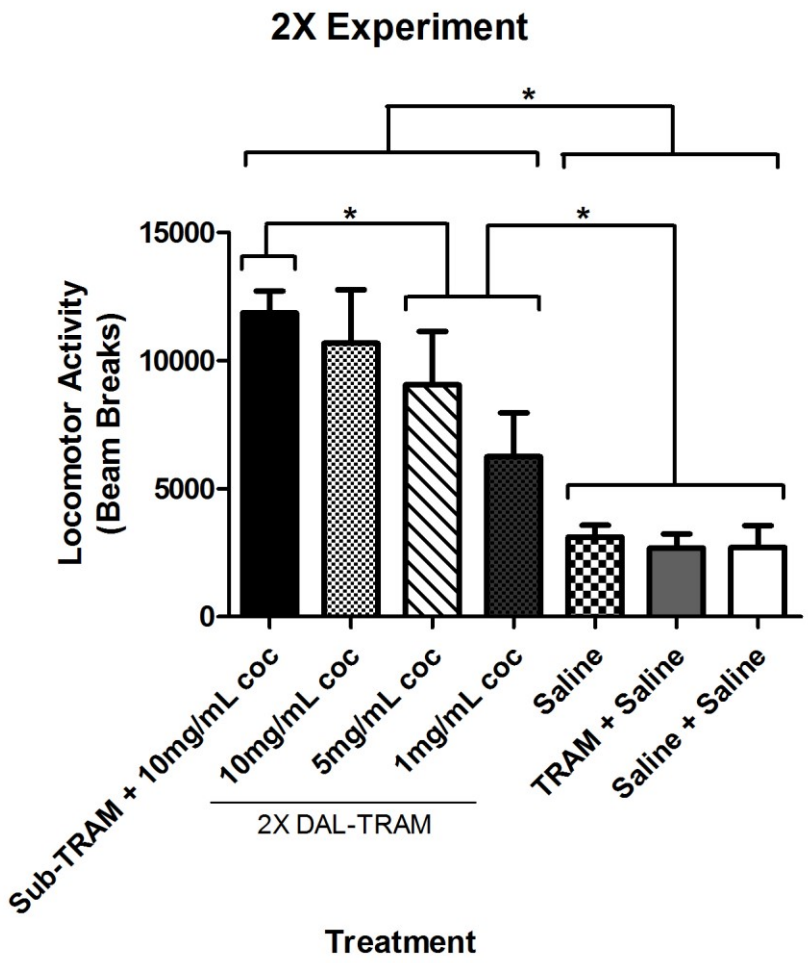

Figure 4: Cumulative Locomotor activity counts (±SEM) of mice during a 30 minute trial following an i.p. injection of Sub-TRAM, 2X DAL-TRAM, TRAM, or saline following administration of 10 mg/mL, 5 mg/mL, 1 mg/mL cocaine, or saline.

(*) indicates a significant difference in locomotor activity among treatment groups ($p<0.05$)

PCR analysis

The delivery of aptamer into the central nervous system was assessed by RT-PCR. Tissue was extracted from animals treated with 2X DAL-TRAM+ saline or 2X DAL-TRAM + 10mg/mL cocaine and Sub-TRAM + 10mg/mL cocaine. Since the DA/NE aptamer is a small RT-PCR target the forward and reverse primers were extended by 39 base pair tails at the 5' –end, a strategy used by Perschbacher *et al.,* to successfully amplify an aptamer sequence extracted from brain tissue (Perschbacher et al., 2015). Tissue from 2X DAL-TRAM + saline treated animals were first to be extracted and were compared to non-template controls (n=2). Amplification of the DA/NE aptamer was successful and confirmed by gel electrophoresis (Figure 5). A representation of the amplification and the melt curves for the DA/NE aptamer extracted nucleus accumbens tissue are shown in Figure 6 below. Figure 7 is a representation of the amplication and melt curves for the Sub sequence extracted from the NAc of animals treated with Sub-TRAM + 10mg/mL cocaine.

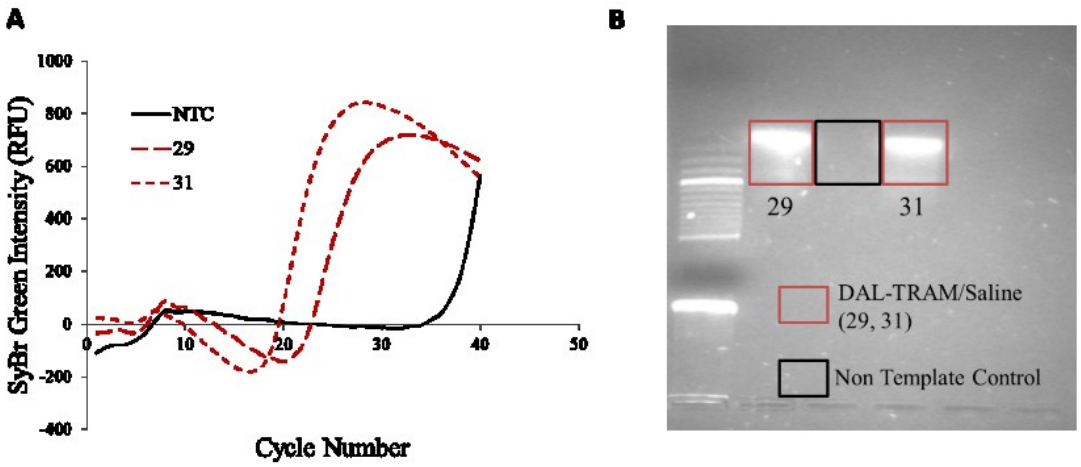

Figure 5: RT-PCR revealed specific amplification of the aptamer from 2X DAL-TRAM+ saline treated animals in the NAc compared to a non-template control (NTC). Amplification curves for the DA/NE aptamer from DAL-TRAM + saline treated animals 29 and 31 shown in (A). Agarose Gel Electrophoresis for the DA/NE aptamer (DAL-TRAM + saline) product compared to a NTC (shown in B) Lanes from left to right: DNA ladder (1), animal 29 (2), NTC (3) and animal 31 (4).

The average quantitation cycle (Cq) from the DA/NE aptamer (2X DAL-TRAM + saline) treated samples (n=8) was 18.9 ± 1.4. The average melting temperature (T_m) of the DA/NE aptamer was 80.9 ± 0.2 °C. Analysis by gel electrophoresis showed the amplified product appears at a similar cycle for both the DA/NE aptamer and the substituted control (Figure 6 and Figure 7). The amplified product extracted from animals treated with DAL-TRAM is consistent with the expected size of the DA/NE aptamer based on the migration is shown on the 2% agarose gel (Figure 5B).

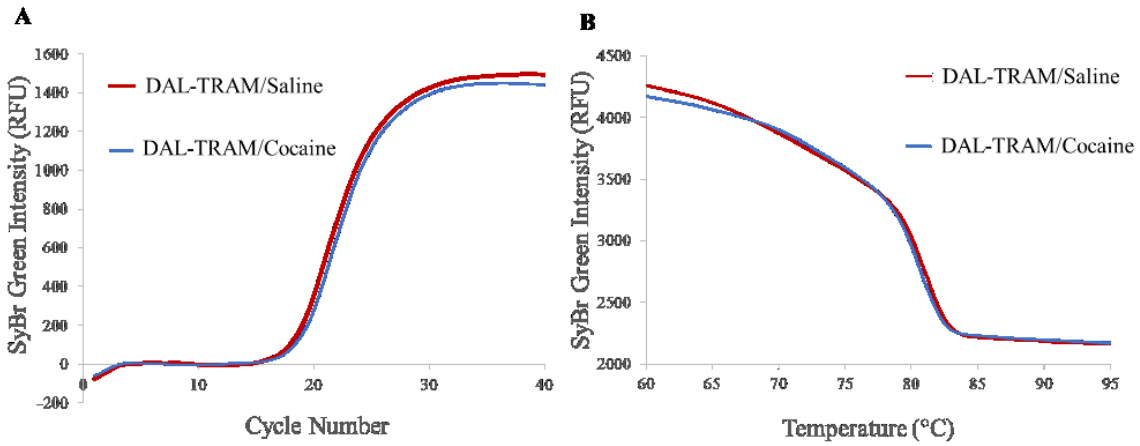

Figure 6: Representation of RT-PCR amplification (A) and melt curves (B) for DA/NE aptamer sequence delivered to the brain in a liposome tagged with transferrin receptor aptamers following isolation from NAc tissue.

RT-PCR was used to detect the delivery of Sub sequence to the central nervous system. Tissue was extracted and prepared as described in the *Materials and Methods* section for RT-PCR detection of DA/NE aptamer. The same forward and reverse primers were used to amplify the substituted aptamer sequence, given the similarity of the Sub sequence to the DA/NE aptamer. The Cq values for the Sub control sequence (Sub-TRAM, n=2) were 24.4 and 23.9. The T_m for the substituted samples were 81.0 and 81.5°C. The average quantitation cycle was lower for the Sub sequence compared to the aptamer (18.9 ±1.4) however the T_m was the same.

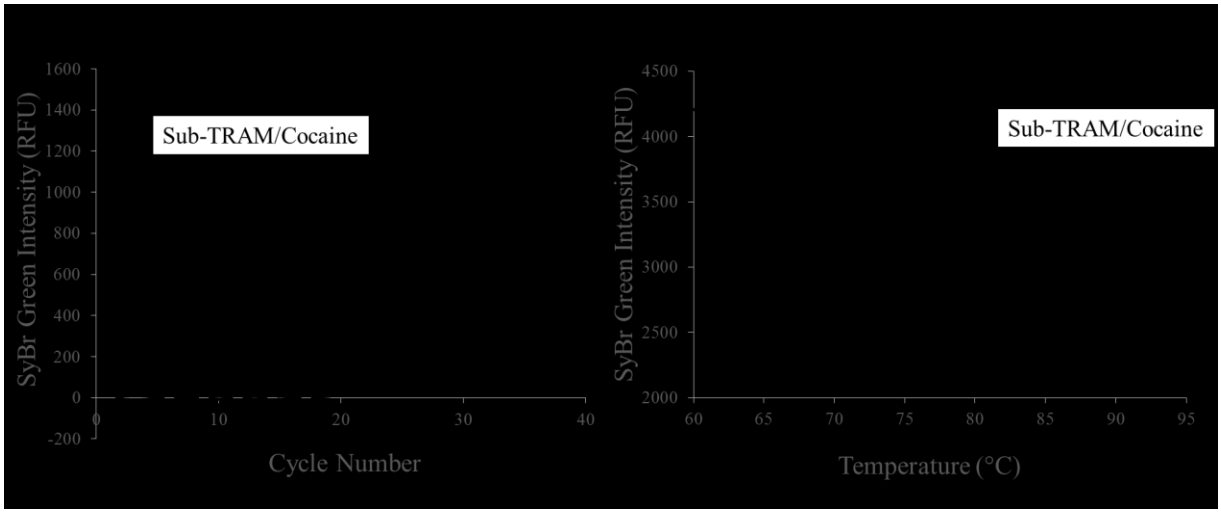

Figure 7: Representation of RT-PCR amplification (A) and melt curves (B) for Sub sequence was observed when delivered to the brain in a liposome tagged with transferrin receptor aptamers following isolation from NAc tissue

Acute Control Experiment

The purpose of this control experiment was to evaluate multiple chemical manipulations of the aptamer to verify that no aspect of the delivery system impacted the behavioural activity of mice. Mice were acclimated to the laboratory environment and were habituated on the micromax equipment for 30 minutes on 3 separate days. On the test day, each mouse received an i.p. injection of Sub-TRAM, DAL-TRAM, TRAM, ROL-TRAM, DAL, or saline followed by a 0.1mL injection of 10mg/mL or saline. Horizontal motor activity was recorded every 5 minutes for a 30 minute trial period.

Cumulative horizontal activity was calculated following the trial and the data was analyzed using the IBM SPSS 19 software. The average locomotor activity for each treatment group was calculated and this data is plotted in Figure 8 below.

Statistical analysis using a one way ANOVA revealed a Main effect of Treatment ($F_{(7,54)}$= 14.216, $p<0.001$). Fishers LSD post-hoc analyses revealed that mice in the DAL-TRAM + 10mg/mL cocaine treatment group had significantly different locomotor activity than all other treatment groups ($p<0.05$). Fishers LSD post-hoc analyses also revealed that animals treated with saline (TRAM + saline, DAL-TRAM+ saline, and saline + saline) showed significantly different locomotor activity compared to animals treated with 10mg/mL cocaine (DAL-TRAM+ 10mg/mL cocaine, DAL + 10mg/mL cocaine, ROL + 10mg/mL cocaine, or TRAM + 10mg/mL cocaine) ($p<0.05$).

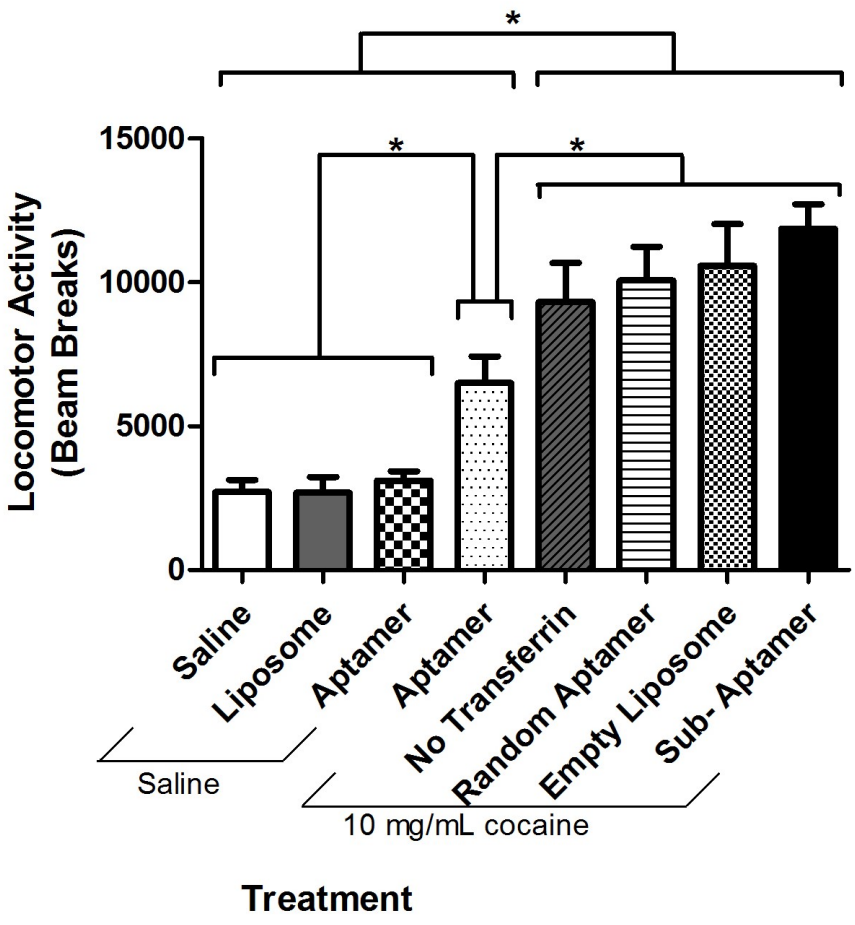

Figure 8: Variations of DAL-TRAM were examined to determine the specific efficacy of the multi-aptamer payload/targeting system in cocaine-induced hyper-locomotion. Cumulative horizontal activity is shown. One-way ANOVA revealed a significant group effect ($F_{(7,54)} = 14.22$, $p < 0.001$). Post-hoc analyses by Fisher's LSD revealed a significant difference between groups.

(*) indicates a significant difference in locomotor activity among treatment groups ($p<0.05$).

Fluorescent Detection Experiment

In order to assess the distribution of the aptamer in the brain, animals were treated with saline (n=3), rhodamine labeled liposome without transferrin receptors on the liposomal surface (DAL, n=3), or rhodamine labeled aptamer (DAL-TRAM, n=3). The rhodamine found on the outer surface of the liposome is a fluorescent marker which can reveal the distribution of the DA/NE aptamer in the presence and absence of the transferrin receptor aptamer in the central nervous system. Figure 9 shows that the rhodamine fluorescence is predominately found surrounding capillaries and does not appear in the interstitial space for animals treated with the no transferrin receptor aptamer on the liposomal surface (DAL). Whereas, the DAL-TRAM treated animals show a far more diffuse spread in rhodamine fluorescence suggesting that the aptamer can travel throughout the central nervous system and is not trapped within capillaries. The contrast between cell bodies and rhodamine fluorescence is more apparent in the brain tissue of DAL-TRAM treated animals in comparison to the brain tissue of DAL treated animals.

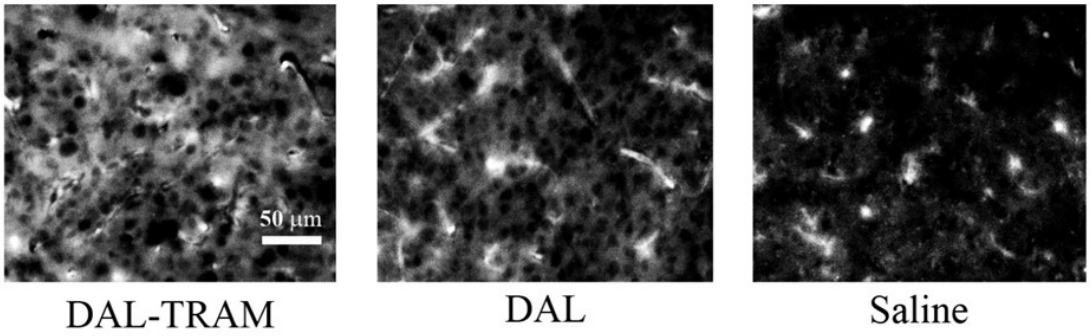

Figure 9: Distribution of rhodamine fluorescence in a coronal section (35 µm thickness) of the nucleus accumbens as imaged using fluorescence microscopy. Mice were injected with TRA-positive liposomes (DAL-TRAM, n=3), TRA-negative liposomes (DAL, n=3) or Saline (n = 3) and euthanized 10 min later. Digital fluorescence images were obtained at 20X magnification. Scale bar as indicated in DAL-TRAM.

In order to evaluate differences among treatment groups integrated fluorescent density was compared for all tissue using ImageJ software as described in the *Methods* section above. There was an apparent difference in the distribution of rhodamine depending on whether the transferrin receptor aptamer is present or absent on the surface of the liposome. Both the DAL-TRAM and the DAL treated animals had significantly different fluorescent intensity when compared to saline treated animals ($F_{(2,7)=}$ 9.943, $p<0.01$). Figure 10 demonstrates the differences in fluorescent intensities among the different treatment groups.

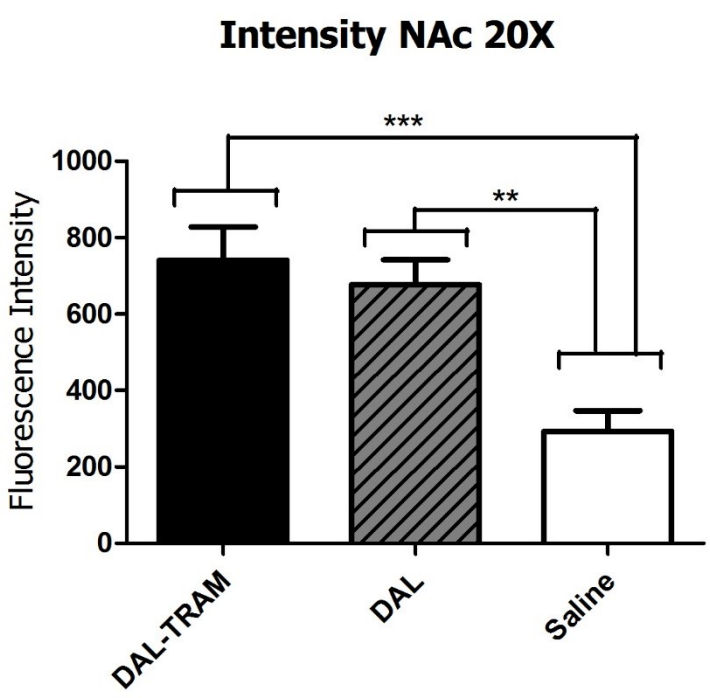

Figure 10: Integrated Fluorescent density as an indication of rhodamine fluorescence in brain tissue. One-way ANOVA revealed a main effect of group ($F_{(2,7)}$= 9.94, $p<0.01$). Post-hoc analysis revealed a significant difference between the DAL-TRAM and saline treatment groups as well as a significant difference between DAL and saline treatment groups.

(**) indicates a significant difference in fluorescent intensity where $p<0.05$

(***) indicates a significant difference in fluorescent intensity where $p<0.01$

Discussion

Repeated Administration Experiment

Repeated administration of the aptamer and an empty liposome resulted in similar locomotor activity as animals that did not receive any treatment (as shown in Figure 1). These results suggest that there was no gross locomotor changes associated with repeated administration of aptamer

It was also shown that repeated administration of the aptamer produced no adverse neural degeneration following 6 days of treatment with the aptamer (DAL-TRAM) or an empty unloaded liposome (TRAM). Following repeated administration of these treatments, mice did not show any Fluorojade B positive staining compared to the positive control. It was extremely important to verify that the aptamer itself did not induce neural damage. If the aptamer were ever to be used as a treatment for cocaine dependence or craving, it is imperative that the sequence itself does not induce neural degeneration or have behavioural consequences (e.g., alterations in motor activity).

The results of the repeated administration of the aptamer suggest that this novel drug delivery system is safe and does not induce any gross behavioural changes or neural degeneration, as assessed with Fluorojade, following repeated administration. While this experiment was valuable in establishing preliminary "safety" evidence, it could be improved by staining for multiple cytokines or inflammatory markers to ensure the aptamer is not inducing a detrimental inflammatory response. It would also be valuable to

increase the length of the administration procedure to better reflect the stages of human addiction (possible, months). The repeated experimental procedure included 6 injections of cocaine one after the other. A more valid model might include periods of chronic use, separated by periods of abstinence, and then a chronic re-exposure period to the addictive drug. Zhang *et al.,* used a chronic cocaine model of 14 day cocaine administration/ 14 day withdrawal period (of no injections) / and a 14 day cocaine re-exposure period to mimic the relapsing nature of addiction (Zhang et al., 2013a). This design might provide a more valid cocaine addiction model to evaluate the utility of the aptamer on long-term effects.

An additional output measure that should be included in future experiments would be the assessment of neurochemical changes in the brain. The use of high performance liquid chromatography (HPLC) could be used to analyze tissue from repeatedly treated animals to determine whether there are any long-term changes in DA or NE concentration. It will be important to determine whether the DAL-TRAM, and TRAM treated animals show any changes in neurochemical concentration following repeated treatment to assure that no long term complications result from the DAL-TRAM treatment. For example, dopamine and dopamine receptors play a crucial role in the brain but also in peripheral tissues where they have important regulatory functions (Labandeira-Garcia et al., 2012). Notably, dopamine and the renin-angiotensin systems (RAS) directly counter regulate each other and abnormal counter regulatory

interactions play a major role in renal changes and hypertension. Research by Labandeira-Garcia *et al.,* showed that RAS hyperactivation exacerbated oxidative stress and neuroinflammation, which in turn contributed to dopaminergic degeneration (Labandeira-Garcia et al., 2012).

Acute Experiment 1

The purpose of acute experiment 1 was to evaluate the aptamer (DAL-TRAM) *in vivo* following cocaine administration. Cocaine has been shown to increase DA activity and result in elevated locomotion (Tortorelli et al., 2015). Pretreatment with the aptamer reduced this cocaine-induced hyperactivity but did not change locomotor activity in the presence of saline or at the lower dose of cocaine. Furthermore, all animals treated with saline showed similar locomotor activity despite whether they were treated with liposome (TRAM), aptamer (DAL-TRAM), or saline. These results suggest that the DA/NE aptamer reduced cocaine-induced hyper-locomotion but only in the presence of elevated DA levels as would happen following cocaine administration.

The results from acute experiment 1 suggested that there is a threshold when the DA/NE aptamer functions. At basal DA levels, the aptamer had no impact on locomotor activity. Cocaine acts by blocking DA reuptake and is known to increase the extracellular concentration and physiological effects of DA (Pettit et al., 1990). Experiments by Pettit *et al.,* showed that extracellular DA concentration in the NAc increases in a dose-dependent manner following a single intravenous infusion of cocaine in anesthetized animals (Pettit and Justice,

1989). A single intravenous infusion of cocaine (at 0.25, 0.75, and 1.25 mg/kg) produced a maximum increase in the extracellular concentration of DA within 10 minutes where DA concentration increased to 800% of basal levels (Pettit and Justice, 1989). Pettit *et al.*, noted that this increase in DA concentration lasted for 60 minutes before returning to basal levels (Pettit and Justice, 1989). Similarly, experiments performed by Andrews and Lucki showed that systemic administration of cocaine (10, 18, and 25 mg/kg, IP) dose-dependently increased extracellular DA and 5-HT levels in the NAc (Andrews and Lucki, 2001).

Our acute experiment 1 showed that 1mg/mL cocaine did not induce a hyperactive response as locomotor activity was not significantly different from saline treated animals. The 5mg/mL and 10 mg/mL of cocaine treatment groups showed significantly elevated locomotor activity compared to saline treated animals. Narasimhaiah *et* al., showed that cocaine treatments of 3mg/kg and higher are required to induce a conditioned place preference response (Narasimhaiah et al., 2009). Kalivas *et al.,* showed the highest concentration of extracellular DA in the ventral striatum occurred with a 15 mg/kg cocaine injection (Kalivas and Duffy, 1993). This rapid increase in extracellular DA was observed between 20-40 minutes after injection and returned to basal DA levels 80 minutes following injection (Kalivas and Duffy, 1993). This suggests that the aptamer under investigation in this study may become active when extracellular DA concentration increases by at least 180% in the ventral striatum, or 800% in the NAc (Pettit and Justice, 1989; Kalivas and Duffy, 1993). The aptamer does

not appear to be active under basal DA concentrations which suggests that natural DA homeostasis can continue and the aptamer might only be active when there is an abnormally high DA level.

These implications are clinically relevant as they suggest that the aptamer may only be active during periods of elevated DA levels. Under normal conditions (e.g., with no drug exposure), there are fluctuations in DA concentration and homeostatic mechanisms at work maintaining normal DA concentrations. Normal, daily fluctuations in DA levels are important for people to experience daily pleasures. If the aptamer interfered with DA under all circumstances, individuals taking the aptamer would have significantly less DA receptor activation and in turn, users would experience limited pleasure, motivation and, potentially motor deficits. This would likely translate into a depressive-like state in those using the aptamer, resulting in an entirely new set of symptoms. Instead, the aptamer is hypothesized to be active during abnormal increases in DA concentrations like those experienced during craving (Volkow et al., 2006). Volkow *et al.,* linked DA increases in the dorsal striatum and cocaine craving in humans showing that there is an increase in the caudate and putamen during instances of craving.

Acute Experiment 2

Acute experiment 2 examined a more concentrated version of the aptamer to determine whether a more concentrated dose of the DA/NE aptamer would further reduce cocaine-induced hyper-locomotor activity. Overall, the data

suggest that the 2X DA/NE aptamer was less effective at reducing locomotor activity in cocaine treated animals.

The results from acute experiment 2 were not as expected because the 2X aptamer treatment should have been more concentrated (i.e., a higher dose) and neutralized more dopamine thereby resulting in a more pronounced decrease in cocaine-induced locomotion. There are several factors that could have impacted the experimental results. First, it is often a challenge working with liposomes, due to their limited long term stability (Surapaneni et al., 2012). We speculate that the overall stability of the liposomes was sacrificed which in turn could have caused liposomes to aggregate or fuse together. Aggregation is the formation of larger units of liposomal material (Yadav et al., 2011). The presence of even mild aggregation may accelerate the process of coalescence of the liposomes (Yadav et al., 2011). Coalescence is the merging and formation of colloidal structures which are generally larger in size. This is an irreversible process where original liposomes cannot be retrieved (Yadav et al., 2011). After doubling the internal concentration of the loaded aptamer, it is hypothesized that the liposomes became thermodynamically unstable resulting in liposomal aggregation and possible colloid structure formation. This in turn impacted the experimental results rendering 2X aptamer less effective than the 1X aptamer treatment.

Glycisaminoglycans (GAGs) are linear polysaccharides containing disaccharide units of amino sugar and uronic acid have been shown to induce

the aggregation of liposomes (Nyren-Erickson et al., 2012). The interaction between the liposome charge and the GAG concentration is a leading contributor to the aggregation of liposomes (Nyren-Erickson et al., 2012). This suggests that the concentration of the aptamer may be too high resulting in the aggregation of the liposomes. Aggregation increases the overall size of the liposome which may in turn have made the aptamer too large to cross the BBB. In this case, less aptamer would have been able to cross the BBB resulting in less available aptamer to bind to the elevated DA present in cocaine treated animals.

There was variation in our baseline recordings from the acute experiment 1 to acute experiment 2. Mice in acute experiment 2 were noticeably more active than mice in experiment 1 which could have contributed to our unpredicted findings as they were simply more active during baseline recordings. It would be valuable to evaluate 1X and 2X aptamer in a single experimental procedure to determine whether or not the 2X aptamer is significantly less effective at reducing locomotor activity of mice exposed to cocaine. This would serve as a more direct way to compare the two concentrations (1X and 2X aptamer) and their respective effectiveness in reducing cocaine induced hyper-locomotor activity.

Acute Control Experiment

Our acute control experiment was performed to evaluate different manipulations of the aptamer to assess the importance of the liposome-aptamer design in crossing the BBB and producing its effects. The substituted aptamer

control manipulation (Sub-TRAM) revealed that the unique sequence of the DA/NE aptamer was required to significantly reduce locomotor activity. This is shown by the elevated locomotor activity in both the Sub-TRAM + 10mg/mL cocaine and the ROL-TRAM + 10mg/mL cocaine treatment groups compared to the DAL-TRAM + 10 mg/mL cocaine treatment group. The Sub-TRAM sequence has 4 base pairs changed from the optimal aptamer sequence yet this minor modification in the DNA sequence yields a significant change in function. This elevated activity suggests that the Sub-TRAM treatment is not binding to DA, although further experiments that measure DA concentration are required to confirm this hypothesis. The ROL-TRAM treatment also showed significantly higher locomotor activity and previous experiments by Holahan *et al.,* confirmed that this sequence does not bind to DA *in vivo* (Holahan et al., 2011). Future experiments will use RT-PCR to detect the ROL-TRAM treatment in tissue from the central nervous system. Using RT-PCR will allow confirmation that this sequence is in the central nervous system. These control manipulations were imperative to confirm that the unique sequence of DAL-TRAM was required to significantly reduce locomotor activity and suggests that only the specific 58 base pair sequence is able to produce the behavioral effect.

Another manipulation was the absence of the transferrin receptor aptamer on the surface of the liposome. The transferrin receptor aptamer is located on the outer surface of the liposome and appears to be an important component of the delivery system. The importance of the transferrin receptor aptamer is

demonstrated by the elevated locomotor activity in the DAL + 10 mg/kg cocaine treatment group compared to the locomotor activity of the DAL-TRAM + 10mg/kg cocaine treatment group. The DAL treated animals show reduced locomotor activity than Sub-TRAM treated animals although locomotor activity was not significantly different between groups. These manipulations confirm that the unique design of the delivery system and inclusion of the transferrin receptor aptamer was required to significantly reduce the locomotor activity of mice administered 10mg/mL of cocaine.

One of the major targets of this study is hijacking the transferrin receptors located on endothelial cells, components of the BBB and the blood cerebrospinal fluid barrier (Deane et al., 2004; Cheng et al., 2013) . The transferrin receptor aptamer conjugated to the surface of aptamer-loaded liposomes in this study, was previously selected by Chen *et al.,* to bind to the extracellular domain of the mouse transferrin receptor (Chen et al., 2008). This transferrin receptor aptamer was selected because of its ability to induce endocytosis across cellular membranes (Chen et al., 2008). Aptamers were conjugated to the surface of liposomes for the effective and specific delivery of drugs to their target, like anti-cancer drugs, which has been shown to improve cellular uptake (Jiang et al., 2015). This approach of targeting transferrin receptors was first applied to tumors cells as overexpression of transferrin receptors is a feature of cancerous tumors (Kobayashi et al., 2007). Transferrin receptors are over expressed

because of their role in iron homeostasis and as a result, have been the target for specific drug delivery into cancer cells (Sriraman et al., 2015).

This approach of hijacking the transferrin receptor raises concerns regarding the homeostatic consequences following long term use. When transport of iron is required there is an up-regulation of transferrin receptors leading to an increase in receptors and ultimately an increase in iron. The primary pathway for acquiring iron into different tissues and organs is by the receptor mediated uptake of transferrin bound iron (Qian et al., 2002). Iron bound to transferrin then binds to transferrin receptors and is transported into the cytoplasm by endocytosis (Qian et al., 2002). Iron concentrations in body tissues must be tightly regulated because excessive iron results in tissue damage because of free radicals (Qian et al., 2002). There are 3 possible outcomes associated with altering the function of the transferrin receptors on the endothelial cells of the BBB 1) no increase in transferrin levels (in turn transferrin receptors) leading to a decrease in iron 2) increases transferrin (increases in transferrin receptors) leading to normal iron levels or 3) increases in transferrin (increases in transferrin receptors) resulting in an increase in iron levels. Extreme decreases in iron have been linked to iron deficiencies like anemia, whereas, increases in iron can lead to increases in oxidative stress (Hare et al., 2013). However, in both cases this requires a large swing in iron concentration which is unlikely to be observed in acute models. Further chronic studies should evaluate blood iron levels, as well as transferrin receptor expression.

In order to fully evaluate the long term effects of the aptamer and the delivery system in general, it would be beneficial to stain or fluorescently tag brain tissue for transferrin receptors to compare differences in transferrin receptors following vehicle, acute, and chronic administration of the aptamer. This would be a good indication as to whether there are any changes in the number of transferrin receptors following use of this novel delivery system.

RT-PCR was used to confirm aptamer presence in brain tissue. PCR allowed us to identify the aptamer in brain tissue and provided strong evidence that the aptamer could in fact be found in brain tissue. Tissue punches from the NAc were examined following intraperitoneal injection of the aptamer. The aptamer was amplified from the DAL-TRAM + 10mg/mL (n=5) as well as the DAL-TRAM + Saline (n=5) treated animals, whereas the non-template control showed no amplification of a product (as shown in Figure 5). In addition, RT-PCR was used to amplify the substituted aptamer control sequence from Sub-TRAM + 10mg/mL cocaine treated animals. Amplification of the substituted sequence was observed, albeit after a slightly higher quantitation cycle number (Cq) compared to the aptamer sequence. The difference between the Cq values of the aptamer and substituted oligonucleotide extracted sequences is not surprising given the 4 base substitutions in the non-binding substitute sequence fall within the RT-PCR primer regions, and therefore the initial amplification would not have been as efficient. Nevertheless, gel electrophoresis of the samples against a nucleic acid ladder confirmed that the RT-PCR product was of the expected length (as shown

in Figure 5), confirming that the liposome designed with transferrin receptor aptamers on the surface are successful and efficient vehicles for delivery of the DA/NE aptamer and substituted oligonucleotide sequence across the BBB.

In order to improve our RT-PCR analysis, it would be beneficial to modify the delivery components to provide more conclusive results. RT-PCR could assess a liposome without the transferrin receptor aptamer or the random oligonucleotide sequence as the RT-PCR control. This would provide definitive evidence as to whether or not the DA/NE aptamer crossed the BBB without the transferrin receptor aptamers and using the same primer sequences for all samples should demonstrate that the DA/NE aptamer sequence is not found in the random oligonucleotide treated animals.

Fluorescent Detection Experiment

Our final experiment used fluorescence detection to assess the distribution of the aptamer in the brain. The transferrin receptor aptamer appeared to be important for the delivery of the aptamer across the BBB where widespread rhodamine fluorescence following DAL-TRAM injection was observed. Figure 9 suggests that the aptamer has ubiquitous distribution in the central nervous system and is not restricted to DA pathways or regions of higher DA concentration. In comparison, DAL treated animals show restricted rhodamine fluorescence that is localized around blood vessels following transferrin negative liposome administration. This suggests that the aptamer crossed the BBB and was widely distributed throughout the brain when the transferrin receptor

aptamer was included. Without the transferrin receptor aptamer, delivery of the aptamer was impaired at crossing the BBB but not halted entirely.

Statistical analysis of the fluorescent intensities measured from the different treatment groups revealed the DAL and DAL-TRAM treated animals were significantly different from saline treated animals. However, the fluorescent intensities of aptamer and no transferrin treatment groups were not significantly different. This suggests that liposomes may be able to cross the BBB even in the absence of the transferrin receptor aptamer on the outer surface. The rhodamine fluorescence observed by DAL treated animals is hypothesized to use endogenous capillary receptors, which promote the passage of large molecules by receptor-mediated transcytosis across the BBB (Re et al., 2011; Pardridge, 2012). The gaps between capillary endothelial cells in most areas of the brain are sealed by tight junctions granting limited permeability to compounds and molecules to the BBB (Liu et al., 2011; Pardridge, 2012). It is speculated that liposomes can diffuse across the BBB due to their small size and lipophilic nature (Pardridge, 2012). The delivery of aptamer appeared to be improved when the transferrin receptor aptamer was included on the surface of the liposome. From this it was found that when targeting chemicals in the central nervous system including the transferrin receptor aptamer as a component of the delivery system ensures widespread delivery across the BBB and also improves the overall delivery of the aptamer.

Limitations

There were a few limitations associated with this study and additional work might increase the overall understanding of aptamer utility *in vivo*. First, there were no measurements of DA or DA receptor activity following administration of the aptamer making it difficult to understand exactly how effective the aptamer was in altering DA activity. It would also be beneficial to examine DA and NE receptor activity to compare and concretely demonstrate how the aptamer is acting on both DA and NE neurotransmitter systems. By using RT-PCR, a comparison between DA rich (VTA, NAc) and NE rich (locus coeruleus) regions could be completed to determine whether the aptamer concentration differed *in vivo*. For the RT-PCR experiment, an additional manipulation should have been included, a transferrin negative liposome (DAL) treatment group. This would use the same forward and reverse primers to detect how much aptamer is found in brain tissue compared to the DAL-TRAM treated animals. It is speculated that very different quantities of aptamer would be observed among the different treatments. This control would provide a stronger understanding of the importance of the transferrin receptor aptamer on the outer surface of the liposome and how significant of a role it plays in the transcytosis of the aptamer across the BBB.

Applications and Future Directions

Prior to considering long term use of the aptamer as a human treatment, the molecular impacts associated with the regulation of transferrin receptors and

iron need to be fully investigated as well as any other potentially dangerous side effects. If the delivery system does not lead to negative outcomes, this aptamer could be used for the treatment of craving in addicts. A future direction would be to measure craving in an animal model and assess the impact of the aptamer. One model of craving in animals is to assess the strength of bar pressing during an extinction session. Rats will readily self-administer cocaine in an operant chamber and when the drug is removed the rodents will continue to bar press repeatedly; this continual pressing behaviour is used as a measure of drug craving (Pickens et al., 2011) . To assess craving, rodents would be placed in an operant chamber and an intravenous drug self-administration procedure will be used. The aptamer will be administered during the extinction phase prior to entering the operant chamber. Craving can be measured by comparing the bar presses of saline and aptamer treated animals when the drug is no longer available. If the aptamer is successful at reducing the number of bar presses recorded during the extinction or craving phase, it might suggest that the aptamer could serve as a treatment approach during craving. This is a highly important treatment period because approximately 80% of addicts relapse during the first year of abstinence and only 20% of users will successfully achieve permanent sobriety (Tjepkema, 2004). Thus, producing a treatment to reduce the craving towards drugs of abuse serves as a major breakthrough.

Conclusions

The overall goal of this study was to achieve a proof of concept to determine whether this novel drug delivery system could successfully deliver a substance across the BBB and whether a DNA based aptamer could successfully bind and function *in vivo.* The PCR analysis suggested that the aptamer successfully crossed the BBB as the aptamer sequence was extracted from the brain tissue of mice administered the modified aptamer. Our novel delivery system was able to successfully deliver a DA/NE binding aptamer to the central nervous system and reduce cocaine-induced hyperactivity suggesting it could be prepared and loaded to deliver various compounds or drugs across the BBB. This study serves as a major advancement in drug delivery systems and offers abundant support for aptamers as novel therapeutic agents.

This study has demonstrated a new approach for drug delivery across the BBB and revealed that synthetic aptamers can be administered by i.p. injection and successfully cross the blood brain barrier. This experiment serves as a breakthrough in drug delivery approaches and given the nature of the behavioural procedure, suggests that DA/NE aptamers could act as a novel treatment for cocaine dependence and other substance addictions where patients experience severe DA dependent craving. In the absence of excess DA, the aptamer does not impact behavioural activity suggesting that the aptamer would only show an effect during instances of elevated DA, such as times of extreme cocaine use or craving.

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