Intravenous delivery of a D1-D2 interfering peptide with antidepressant-like effects

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

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Abstract

Depressive disorders are common, debilitating diseases. Current treatments on the market are unsuccessful for many patients, have high rates of remission, and many side effects. After finding that the D1-D2 dopamine receptor complex is upregulated in post-mortem patients that suffered from major depressive disorder, our lab developed a peptide drug that interferes with this complex to serve as an antidepressant drug. Our lab has previously tested the efficacy of the D1-D2 interfering peptide through intracranial administration and intranasal administration. A major component of preclinical drug testing is finding the optimal method of administration. In this study, we find that the peptide has antidepressant-like effects compared to saline in rats in the forced swimming test at doses greater than 1.5nmol/g. These findings are an important step in the preclinical research of a peptide with antidepressant-like effects.
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List of Abbreviations

5-HT - Serotonin
5-HTT - Serotonin transporter
AC - Adenylyl cyclase
ATP - Adenosine triphosphate
BBB - Blood-brain barrier
cAMP - Cyclic adenosine monophosphate
CNS - Central nervous system
Co-IP - Co-immunoprecipitation
CPP - Cell-penetrating peptide
D(1-5)R - Dopamine D(1-5) receptor
DAG - Diacylglycerol
DSM-5 - Diagnostic and Statistical Manual of Mental Disorders, fifth edition
ECT - Electroconvulsive Therapy
FRET - Fluorescence resonance energy transfer
FST - Forced swim test
GDP - Guanosine diphosphate
GPCR - G protein-coupled receptor
GTP - Guanosine triphosphate
IC - Intracranial
IN - Intranasal
IP3 - Inositol 1,4,5-triphosphate
IP - Intraperitoneal
IV - Intravenous
LH - Learned helplessness
MAOI - Monoamine oxidase inhibitor
MDD - Major Depressive Disorder
NE - Norepinephrine
ORN - Olfactory receptor neuron
PFC - Prefrontal cortex
PIP2 - Phosphatidylinositol 4,5-bisphosphate
PKA - Protein Kinase A
PKC - Protein Kinase C
SNRI - Selective norepinephrine reuptake inhibitor
SSRI - Selective serotonin reuptake inhibitor
TAT-D1-D2-I Pep - TAT-linked D1-D2 receptor complex interfering peptide
TAT-Pep - TAT peptide
TCA - Tricyclic antidepressant
1 Introduction

1.1 Background

Depressive disorders are common, debilitating disorders characterized by the presence of sad, empty, or irritable mood, accompanied by somatic and cognitive changes that significantly affect the individual’s capacity to function. There are several different types of depressive disorders, including disruptive mood dysregulation disorder, major depressive disorder (MDD), persistent depressive disorder (dysthymia), and others (American Psychiatric Association, 2013). They are the leading cause for years lost to disability worldwide (WHO, 2013) and the many treatments available today are incapable of achieving remission in many patients, and the relapse rates in patients that do achieve remission are unacceptably high (Andrews, 2015; Ferrari, 2013; Gaynes, 2008). MDD represents the classic condition of the depressive disorders, and is characterized by discrete episodes of at least 2 weeks’ duration involving changes in affect, cognition, and neurovegetative functions and inter-episode remissions (American Psychiatric Association, 2013). Drug treatments in the past have predominantly targeted the serotonergic and norepinephrine pathways as a result of the early discoveries that iproniazid, a monoamine oxidase inhibitor originally used as a treatment for tuberculosis, and imipramine, a monoamine reuptake inhibitor originally used as a treatment for schizophrenia, both reduced depressive symptoms. These drugs each share the pharmacological property that seemingly results in an increase in monoamines at the synapse (Andrews, 2015). Further, it was shown that
administration of tryptophan along with a monoamine oxidase inhibitor resulted in the potentiation of antidepressive effects. This information led to what is now known as the low serotonin hypothesis, stating that depression stems from a reduced amount of serotonin at the synapse, and consequently has led to a focus on the serotonergic system in the majority of research on depression and antidepressants (Andrews, 2015). More recently, however, studies are beginning to show that other neurotransmitters, like dopamine, may also play a significant role in the pathophysiology and treatment of depression.

The dopaminergic system is one system that many studies have shown may be a key player in depressive disorders. Evidence suggests that dopaminergic neurotransmission may be reduced in major depression, either from diminished release at the presynaptic terminal or due to changes in receptor number or function and/or altered intracellular signal processing (Dunlop, 2015). There is data to support each of these mechanisms and it is still unclear exactly how the dopaminergic system affects depression (Dunlop, 2015). Dopamine has 5 different receptors, titled D1 through D5 (Beaulieu, 2011). These receptors are capable of coming together to form dimers to serve different functions than the individual receptors themselves. For example, the D1 and D2 receptors dimerize in the brain to form a D1-D2 complex, which uses a different G-protein than either of the individual D1 or D2 receptors (George, 2007; Maggio, 2009; Hasbi 2011). In 2010, Pei et al. found that the D1-D2 receptor was upregulated in a group of brains taken from post-mortem depressed patients. They proposed that disrupting the interaction between the D1 and D2 receptor might exert antidepressant-like effects. After
creating a TAT-linked peptide that interferes with the D1-D2 complex (TAT-D1-D2-I Pep), they injected it directly into the brains of rats and found that it had antidepressant-like effects during the forced swim test (FST). Since intracranial injection is not a clinically viable route of administration, Brown and Liu followed up this study in 2014 by testing the TAT-D1-D2-I Pep using a pressurized olfactory device to administer the peptide intranasally. This study revealed a clinically viable method of successfully administering TAT-D1-D2-I Pep into the prefrontal cortex (PFC) whilst having an antidepressant-like effect in an animal model (Brown et al. 2014). In order for a drug to pass the preclinical trial stage, the optimal method of administration must be identified.

The purpose of this thesis is to test whether TAT-D1-D2-I Pep can be effectively delivered to the prefrontal cortex through intravenous (IV) injection. IV delivery is a clinically relevant, widely used method of drug delivery that delivers drugs through direct injection into the blood stream. Current drugs that are delivered through IV injection include drugs that need to be delivered fast in emergency situations or for pain relief, such as morphine. IV injection is also widely used for peptide drugs delivered through drug delivery systems, like liposome and lipid based carriers. Products delivered this way that are currently on the market include AmBisome® and Doxil® (Allen, 2013). This project aims to: 1) show that TAT-D1-D2-I Pep reaches the PFC following IV injection, 2) show that TAT-D1-D2-I Pep exhibits antidepressant-like effects in rats in the FST following IV injection and 3) assure that TAT-D1-D2-I Pep disrupts the interaction of the D1-D2 receptor complex.

In the introduction, I will begin by discussing Major Depressive Disorder
(MDD), its neurobiology, symptoms, and the currently used anti-depressive treatments and their pharmacological targets. I will then discuss the role of dopamine transmission in the mammalian brain and specifically focus on how it affects depression. I will continue by discussing the advantages and disadvantages of peptide drugs. Next, I will discuss drug delivery, specifically to the central nervous system (CNS), and discuss its advantages and disadvantages compared to other forms of drug administration. Finally I will give my rationale and hypothesis for my project.

1.2 Major Depressive Disorder

1.2.1 A History of Depression and Antidepressants

The history of antidepressants and subsequent hypotheses on the etiology of depression involve a series of discoveries, beginning with that of serotonin. In the mid-twentieth century, a major area of research involved the action of the hallucinogen lysergic acid diethylamide (LSD) on the body (Woolley, 1954). It was known that LSD is a structural relative to serotonin and an antagonist to serotonin receptors in smooth muscle. At the same time, the researchers also knew that LSD causes mental disturbances, and this led to a spark in research on serotonin and its role in mental disorders (Woolley, 1954; Hirschfeld, 2000). Further evidence for the role of serotonin in mood and mental disorders came with a few serendipitous discoveries of substances that affected mood. Reserpine, one of the first agents for hypertensive patients was first seen to trigger depressive states in hypertensive patients (Muller, 1955). It was noted that reserpine interferes with vesicular storage of serotonin and norepinephrine in the brain, decreasing the levels of serotonin and
norepinephrine released at the synapse (Muller, 1955; Hirschfeld, 2000). Iproniazid, an agent used for tuberculosis patients, was found to improve mood in tuberculosis patients with depression (Crane, 1956). Research led to the discovery that iproniazid acts as a monamine oxidase inhibitor (MAOI), which is the mitochondrial enzyme that is responsible for degrading monoamines - dopamine, serotonin and norepinephrine - in the presynaptic terminal. Therefore, iproniazid causes monoamines to be left in the synapse for a longer period of time, resulting in an increase in extracellular serotonin and norepinephrine. This led to the development of monoamine oxidase inhibitors specifically for the treatment of depression (Hirschfeld, 2000; Crane, 1956). Finally, the drug imipramine was developed around the same time as an anxiolytic for the use in agitated psychotic patients. Although it was unsuccessful in this regard, it proved to have an effect on patients with symptoms of depression (Kuhn, 1958). Studies showed that imipramine, a tricyclic antidepressant - named because of the three rings formed in the chemical structure - acted by inhibiting the reuptake of norepinephrine and serotonin at the synapse. Like the MAOIs, this would result in a higher concentration of extracellular monoamines at the synapse. Following these discoveries of substances that affected monoamines in the brain as well as mental states and mood disorders, a hypothesis on the etiology of depression began to form (Hirschfeld, 2000).

1.2.2 The Monoamine-Deficiency Hypothesis

By 1965, the building evidence on drugs which seemed to induce (reserpine) or correct (iproniazid and imipramine) mood disorders led to the catecholamine hypothesis of affective disorders posited by Schildkraut. This hypothesis stated that
“some, if not all, depressions are associated with an absolute or relative decrease in catecholamines, particularly norepinephrine, available at central adrenergic receptor sites. Elation, conversely, may be associated with an excess of such amines” (Schildkraut, 1965). Norepinephrine (NE) and serotonin (5-HT) are the two most common monoamines implicated in depression, due to reserpine, iproniazid and imipramine predominantly affecting extracellular levels of NE and 5-HT. In the 1970’s, the introduction and rapid success of selective serotonin reuptake inhibitors lent a lot of support to serotonin being the major monoamine in depressive disorders (Slattery, 2004).

This hypothesis, however, continues to be modified as research continues to produce more findings. The hypothesis first implied that neurotransmitter levels were to blame for MDD, but has since been altered to imply that receptor regulation and second messenger signalling is a more likely cause (Anderson, 2015). This is because the relief of depressive symptoms doesn’t occur until weeks to months after the patient begins a regular schedule of drug treatment even though the initial effect of antidepressants (e.g. inhibiting the re-uptake of serotonin) occurs almost immediately after the drug is taken (Anderson, 2015). Therefore, it is now believed that monoamine levels are a factor upstream of the actual primary causes of MDD, and a change in monoamine levels induces a change somewhere further down along the signalling cascade that is responsible for the antidepressant effects (Zarate, 2006; Anderson, 2015). There is a large interplay between monoamines, so the focus on serotonin and norepinephrine may be unwarranted. Many depressive patients have difficulty achieving remission, even after multiple rigorous trials of
antidepressant treatment, and this could be, in part, due to the heterogeneous nature of MDD (Friend, 2015; Duman, 2014; Gaynes, 2008). Recent evidence implicates dopamine as playing a larger role in MDD than previously thought (Finan, 2013; Hayes, 2013; Hamilton, 2012), so antidepressant drugs that target the dopaminergic system may be an important line of treatment for patients that are unresponsive to classical drugs that target the serotonin and norepinephrine systems (Craighead, 2014; Dunlop, 2007). Therefore, further research on drugs with an antidepressant effect that impacts the dopaminergic system must be explored.

1.2.3 Anatomically relevant areas in the pathophysiology of Depression

Although the main focus of depression and antidepressant treatment has been on neurochemical imbalance, animal studies and human brain imaging studies have reported structural alterations in certain areas (Duman, 2014). A functional depression circuit has been proposed that consists of reduced connectivity of the prefrontal cortex (PFC) with other limbic and subcortical structures (amygdala, hippocampus, ventral striatum). This model is supported by functional imaging studies in humans, post-mortem studies of patients that suffered from depression and animal studies (Duman, 2014). The areas important in the depression circuit consistently show a change in cell volume. In animals, repeated stress, an animal model of depression, reportedly causes cell atrophy, and decreased cellular proliferation in the PFC and hippocampus. In human brain imaging studies, there is a reported decrease in the volume of the hippocampus in patients with depression. There is also a reported decrease in volume in the prefrontal cortex and a decrease in the number of neurons and glia. Meanwhile, stress and depression seem to cause
hypertrophy in the amygdala (Duman, 2014). These changes in cell volume correlate with reduced connectivity between these areas, supported by brain imaging and post-mortem studies. The PFC negatively controls the amygdala, and a decrease in function in the PFC leads to an increase in function in the amygdala which results in decreased mood, decreased control of emotions and increased fear, anxiety and HPA axis reactivity. A strong relationship between stress and depression is well known, and the HPA axis has a well-documented importance for our response to stress. Antidepressant treatment is reported to block the atrophy of neurons in the hippocampus as well as upregulate cellular proliferation. This response was dependent on chronic treatment, consistent with the time course for symptom relief from antidepressant treatment in humans (Duman, 2014).

1.2.4 Symptomatology and clinical diagnosis of depression

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association, 2013) states the following on the diagnostic criteria of MDD: the patient must experience at least 5 of the following symptoms in the same period of at least 2 weeks or more, with at least one of the 5 symptoms being number 1 or 2.

1. Depressed mood most of the day, nearly every day

2. Diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day

3. Significant change in weight and/or appetite without dieting

4. Insomnia or hypersomnia nearly every day

5. Psychomotor agitation or retardation nearly every day
6. Fatigue or loss of energy nearly every day
7. Feelings of worthlessness or excessive or inappropriate guilt nearly every day
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day
9. Recurrent thoughts of death, or suicide, or a plan or attempt for committing suicide

The symptoms cause distress in important areas of functioning, such as social and occupational, and cannot be attributed to the physiological effects of a substance or to another medical condition. Fatigue and sleep disturbance are present in a high proportion of cases, while psychomotor disturbances and delusional guilt are less common but are indicative of greater overall severity.

1.2.5 Treatment of depression

As reviewed in Section 1.2.1, the oldest antidepressants are the MAOI’s and the TCA’s. Although effective, they are no longer the first choice antidepressants because of their often-dangerous side effect profiles. MAOI’s can have serious adverse effects, while TCAs have a high affinity for several receptors, including adrenergic, serotonergic, histaminic and muscarinic acetylcholine receptors, resulting in a large side effect profile (O’Leary, 2015; Gelenberg, 2010; Slattery, 2004; Ferguson, 2001; Craighead, 2014). Currently, the most popularly prescribed antidepressants are the selective serotonin reuptake inhibitors and the selective norepinephrine reuptake inhibitors, which were designed to increase the selectivity
towards their receptors in order to decrease the side effect profile and increase potency (Gelenberg, 2010; Craighead, 2014; Hirschfeld, 2000).

1.2.5.1 SSRI’s

Selective serotonin reuptake inhibitors (SSRIs) are the most widely prescribed antidepressants on the market due to their greater safety and tolerability and more favourable adverse effect profile (Gelenberg, 2010; Walker, 2013). There are currently 7 SSRIs on the market with approval from the FDA: citalopram, escitalopram, fluoxetine, paroxetine, sertraline, fluvoxamine and vilazadone (Gelenberg, 2010). The mechanism of action of SSRIs is as the name suggests: they inhibit the reuptake of 5-HT at the synapse by binding to the 5-HT transporter (5-HTT) and blocking its function. This results in a slight increase in extracellular 5-HT levels at the synapse that engages a negative feedback loop involving the 5-HT1A autoreceptors located on the presynaptic neuron (Walker, 2014). Persistent SSRI treatment leads to desensitization of these autoreceptors, reducing the level of negative feedback and in turn leading to an overall increase of 5-HT release and an increase in extracellular 5-HT levels at the synapse (Walker, 2014).

Compared to TCAs, SSRI’s have a similar efficacy and were originally thought of as almost side effect-free. However, after wide spread use, side effects that initially were thought to be uncommon were beginning to be reported (Ferguson, 2001). One of the most common side effects reported, sexual dysfunction, was initially reported to occur at a rate of about 2% after the original clinical trials of fluoxetine, the first SSRI on the market, but was eventually reported to occur at a rate as high as 75% (Ferguson, 2001; Gelenberg, 2010). Other side effects include gastrointestinal
issues, insomnia, headaches, weight gain and serotonin syndrome (caused by excess of serotonergic activity in CNS). This side effect profile, however, is still advantageous over TCAs and MAOIs.

### 1.2.5.2 Other classes of currently used antidepressants

The next most popular class of antidepressants currently on the market is selective norepinephrine reuptake inhibitors (SNRIs) (Gelenberg, 2010). As the name suggests, their role is similar to that of SSRIs, with their action taking place on the norepinephrine transporter as opposed to the 5-HTT, resulting in an increase in norepinephrine in the synapse. There are currently 3 SNRIs on the market: duloxetine, venlafaxine, and desvenlafaxine (Gelenberg, 2010). The most common antidepressant outside of the SSRI and SNRI classes are bupropion (a norepinephrine and dopamine reuptake inhibitor) (Khan, 2016) and mirtazapine (a drug that increases serotonin and norepinephrine concentrations through other mechanisms of action. i.e. is not a reuptake inhibitor) (Watanabe, 2011). Other classes include serotonin modulators, and norepinephrine serotonin modulators (Gelenberg, 2010).

### 1.2.5.3 Limitations of currently used antidepressant medication

Even with the multitude of antidepressant medication currently on the market, a large population of patients suffering from depression are still unable to remit, even after using multiple different treatment methods. The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study was done in 2008 and evaluated the current treatment strategies on improving clinical outcome for patients with depression (Gaynes, 2008). It is the largest study done of its kind. The study
consisted of 4 levels of augmented treatment; if the patient failed to achieve remission after a given level of treatment that persisted for up to 14 weeks, the clinicians would move them to the next level of treatment. The study found that after the first level of treatment 33% of patients were able to achieve remission, 57% achieved remission after the second level, 63% after the third level and 67% after the fourth. Therefore, even after 4 rigorous levels of treatment, 33% of patients are still unable to achieve remission. This is one of the current major problems with current antidepressant treatments and why further research on novel antidepressant treatments is important (Gaynes, 2008).

A second limitation with current antidepressant treatment is the delayed onset between the beginning of drug administration and the actual relief of depressive symptoms (Zarate, 2006; Walker, 2014; O’Leary, 2015; Dunlop, 2007). The effect of antidepressants is not manifested until potentially weeks or months after the start of treatment and the reason for this is not completely understood. This period between the start of treatment and the relief of symptoms results in considerable morbidity and high risk of suicidal behaviour, especially in the first 9 days after starting treatment (Zarate, 2006; Walker, 2014; O’Leary, 2015; Dunlop, 2007).

A third limitation with current antidepressant treatment is the rate and severity of side effects that occur. Studies estimate that 15% to 30% of patients discontinue use of SSRI’s because of side effects. Side effects can often cause patients to cease treatment before the drug is able to achieve the desired effect (Anderson, 2012).

For these reasons, the continued search for novel, more efficacious, faster acting and less harmful antidepressants is very important.
1.2.5.4 Cognitive Therapy

Another form of therapy for patients suffering from depression is cognitive therapy. Cognitive therapies are short-term, typically comprising 16-20 sessions over 12-16 weeks that consist of professional aid in an individual, in-person setting, although differing formats are being explored (Craighead, 2014). The evidence-based therapies are Beck’s cognitive behavioural therapy (CBT) (Beck et al. 1979), interpersonal therapy (IPT) (Klerman et al. 1984), and behavioural activation (Jacobson et al. 2001) (Craighead, 2014).

1.2.5.5 Deep Brain Stimulation/Electroconvulsive Therapy

Electroconvulsive therapy (ECT) for the treatment of depression is generally a last course of action in patients where antidepressant treatment has not been effective. It is, however, beneficial to be used as first-line treatment for patients suffering from severe MDD when it is coupled with psychotic features, catatonia (abnormal movement), suicide risk, food refusal, or any other circumstances requiring a rapid treatment response (Gelenberg, 2010). It involves the application of electricity to the scalp to induce seizure activity in the brain. This results in a range of effects on the neurobiological features of depression: it increases cortical GABA concentrations and enhances serotonergic functions, it affects the HPA axis, it alters functional brain activation, and it appears to influence neuronal structure and synaptic plasticity, showing an increase in cell proliferation (Krishnan, 2016). The exact reasons why ECT is effective are still not completely understood, similar to antidepressant medication, but many different studies since the beginning of its use
in the 1930s have proven its efficacy in patients suffering from depression (Krishnan, 2016; Lisanby, 2007; UK ECT Review Group, 2003).

**1.2.5.6 Current guidelines for treatment**

Current guidelines for treatment suggested by the American Psychiatric Association (Gelenberg, 2010) suggest that treatment be initiated with an SSRI, SNRI, mirtazapine or buproprion. If little to no improvement is seen after 8 weeks, switching to a different antidepressant or augmentation with another antidepressant from a different class, lithium, thyroid hormone or a second-generation antipsychotic is recommended. If there continues to be no response, electroconvulsive therapy is recommended (Gelenberg, 2010; Wong, 2012).

**1.2.6 Animal models of depression: the forced swim test**

Currently, there are no ideal animal models for depression that completely meet all 3 major criteria for an animal model of human disease. The 3 major criteria are: face validity (behavioural phenotype of animal resembles the clinical symptom profile), predictive validity (symptoms improve with clinically effective antidepressant treatments and fail to improve with clinically ineffective treatments) and construct validity (similar neurobiological underpinnings) (Czeh, 2016).

Current animal models for depression include genetic mouse models, stress models and other physiological or pharmacological manipulation models (Czeh, 2016). The important model for this study is the forced swim test (FST), a type of stress model.

The FST is one of the most widely used animal models to test the efficacy of antidepressant medication, largely due to its ability to test a large number of animals, ease of use, predictive validity and inter-laboratory reliability and
specificity (Slattery, 2012). The idea is based off of the observation that exposing rats to water results in an initial escape-directed behaviour and is then followed by a passive immobile state. The immobile state is believed to represent behavioural despair (unwillingness to continue escape directed behaviour following stress). The test measure is the amount of time the rat spends in an immobile state. Many antidepressants have been shown to reduce the amount of time spent in the immobile state, while a number of stressors have been shown to increase this time. These bi-directional results support the predictive validity of the FST. (Slattery et al. 2012; Czeh et al. 2016).

1.3 Dopamine

1.3.1 Dopamine neurotransmission in the mammalian brain

Dopamine is one of the principal neurotransmitters in action in the mammalian brain. It is a catechol (a benzene ring with a hydroxyl group at both carbons 3 and 4) with an amine side chain, putting it in the catecholamine group of neurotransmitters (norepinephrine, epinephrine and dopamine) (Seamans, 2004). It is considered a neuromodulator, meaning it is neither excitatory nor inhibitory but modulates neuronal activity. It plays a very important role in movement, cognition, motivation, sleep regulation, attention, pleasure, and reward (Beaulieu, 2015). Dopamine originates from dopamine-producing neurons that have specific cellular machinery capable of producing dopamine from the amino acid tyrosine. These neurons are located in cell groups in the midbrain part of the brainstem or in the hypothalamus. The cell groups originating in the midbrain include the substantia nigra pars compacta, the ventral tegmental area, and a region of the midbrain tegmentum,
while the dopaminergic cell group in the hypothalamus originates in the arcuate nucleus (Kandell, 2013). Signalling in the mammalian brain follows one of four projection pathways that originate from one of these cell groups. The four pathways are: the nigrostriatal pathway, the mesocortical pathway, and the mesolimbic pathway (all 3 of which originate from the cell groups in the midbrain) and the tuberoinfundibular pathway (originates from the arcuate nucleus in the hypothalamus) (Beaulieu 2011).

The nigrostriatal pathway originates from the substantia nigra pars compacta and projects to the dorsal striatum (the caudate and the putamen; areas important in movement). This pathway plays an important role in the planning and execution of motor activities, as well as cognition. A progressive loss of the dopaminergic neurons in the substantia nigra pars compacta leads to Parkinson’s disease, a disease characterized by resting tremor, bradykinesia, rigidity and an impairment in the ability to initiate and sustain movements (Dunlop, 2007; Kandell, 2013).

The mesocortical pathway originates from the ventral tegmental area (VTA) and projects to the cortex. Specifically, it projects to the anterior cingulate, the entorhinal cortex and the prefrontal cortex. This pathway is believed to be most important for concentration and executive functions, such as working memory. Deficits in signalling along this pathway are believed to contribute to the cognitive deficits seen in schizophrenia (Dunlop et al. 2007; Kandell, 2013).

The mesolimbic pathway also originates from the VTA but instead projects to the areas of the limbic circuit, including the ventral striatum, the bed nucleus of the stria terminalis, hippocampus, amygdala and septum. This pathway is important for
motivation, pleasure, reward, and is also implicated in addiction. Addictive drugs increase extracellular dopamine levels in the nucleus accumbens, leading to an increased activation of the reward circuitry (Dunlop, 2007; Kandell, 2013).

The tuberoinfundibular pathway originates in the arcuate nucleus of the hypothalamus and projects to the median eminence of the hypothalamus, which then sends the signal to the anterior pituitary gland. The anterior pituitary gland is responsible for releasing hormones to keep our body in homeostasis. Dopamine release in the median eminence acts to inhibit the release of the hormone prolactin from the anterior pituitary gland. Prolactin plays an important role in coordinating actions that facilitate caring for offspring, such as lactation, maternal behaviours, temporal spacing of pregnancies and weight gain (Lyons, 2012; Kandell, 2013; Dunlop, 2007).

1.3.2 Dopamine receptors

Release of dopamine from the presynaptic terminal activates any of five members of the dopamine receptor family, simply named D1 through D5. These receptors all belong to G protein-coupled receptor (GPCR) family and all have a very similar structure and amino acid sequence that consists of seven transmembrane domains. They transmit their downstream signals through second messenger systems, resulting in slower but longer lasting results. These five receptors are separated into two classes: the D1-class receptors and the D2-class receptors (Beaulieu, 2011; Beaulieu, 2015).
different types of G proteins have been identified, G proteins are heterotrimers, consisting of α-, β-, and γ-subunits. Approximately 20 different types of G proteins have been identified, three of which will be discussed.
here, due to their importance for dopamine receptors: $G_s$, $G_i/G_o$, and $G_q$ (Kandell et al. 2013; Beaulieu et al. 2011).

The most well characterized $G$ protein-signalling cascade is the cyclic AMP (cAMP)-signalling cascade. This cascade is activated when a neurotransmitter binds to a GPCR that is coupled to a $G_s$ protein. Once the neurotransmitter binds to the receptor, a guanosine diphosphate (GDP) bound to the $G$ protein is exchanged for a guanosine triphosphate (GTP), activating the $G$ protein. Once activated, the $G_s$ protein activates the enzyme adenylyl cyclase (AC) to convert adenosine triphosphate (ATP) into cAMP, which then targets protein kinase A (PKA) to switch it to its active state. In its active state, PKA can phosphorylate many different targets downstream in a neuron, such as gene transcription factors, voltage- and ligand-gated ion channels, and synaptic vesicle proteins. This can lead to many long-term changes in a neuron (Kandell et al. 2013; Beaulieu et al. 2011).

The $G_i/G_o$ protein acts in the opposite way of the $G_s$ protein and inhibits AC and therefore inhibits the cAMP-signalling cascade, thereby decreasing the downstream effects of PKA. $G_i/G_o$ protein-coupled receptors, however, also have several other downstream effects. They are known to activate mitogen-activated protein kinases (MAPKs), including extracellular-signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (Chen, 2012). They are also known to stimulate inositol monophosphate turnover, the potentiation of arachidonic acid release, and inwardly rectifying $K^+$ and $Ca^{2+}$ channels (Wong, 2012).

The $G_q$ protein-signalling cascade has a completely different second messenger system than $G_s$ and $G_i/G_o$. When activated, the $G_q$ protein activates phospholipase C,
leading to the hydrolysis of PIP$_2$. Hydrolyzing PIP$_2$ results in two second
messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$). These
second messengers can then phosphorylate many membrane-associated and
cytoplasmic protein substrates in the cell. For example, IP$_3$ releases intracellular
Ca$^{2+}$ stores, while DAG activates protein kinase C (PKC). The reduction in
intracellular PIP$_2$ also has an effect on the activity of some ion channels (Kandell,
2013; Beaulieu, 2011).

1.3.2.2 D1-type dopamine receptors

The separation of D1-type and D2-type dopamine receptors came from the
discovery that there are two distinct types of dopamine receptors and only one type
stimulates AC activity; this type was classified as the D1-type receptors (Garau,
1978). The D1-type dopamine receptor class is comprised of the D1 and D5
receptors. These receptors are believed to primarily act through the G$_s$ protein,
stimulating activity of the cAMP second messenger cascade (Beaulieu, 2011;
Beaulieu, 2015). In some types of neurons, like in the striatum, an area of the brain
with the densest dopamine innervation and the highest expression of the D1
receptor, D1 receptors are believed to be coupled to the G$_{olf}$ protein, which is
another type of G protein that activates AC to stimulate the cAMP-signalling cascade
(Neve, 2004; Beaulieu, 2011; Beaulieu, 2015). Therefore, in most cases, the
downstream effect of dopamine signalling on D1-type receptors is to activate the
cAMP signalling-cascade.

The D1 receptor is found exclusively on dopamine-receptive postsynaptic cells. It
is densely expressed in the nigrostriatal, mesocortical and mesolimbic pathways.
This includes the striatum, the nucleus accumbens, the substantia nigra, the olfactory bulb, the amygdala and the frontal cortex. It is also expressed at lower levels in the hippocampus, cerebellum, thalamic areas and hypothalamic areas. The D5 receptor is less common in the brain, expressed at low levels in pyramidal neurons of the prefrontal cortex (PFC), the premotor cortex, the cingulated cortex, the entorhinal cortex, substantia nigra, hypothalamus, hippocampus, and the dentate gyrus (Beaulieu, 2011; Beaulieu, 2015).

1.3.2.3 D2-type dopamine receptors

The D2-type dopamine receptor class was determined to be the class of dopamine receptors that does not stimulate AC activity (Garau, 1978). These receptors include the D2, D3 and D4 dopamine receptors. These receptors are coupled with the $G_i/G_o$ protein, meaning they inhibit AC activity, and therefore inhibit the cAMP-signalling cascade. D2 receptors have a more complex signalling regimen, however, than D1 receptors, due to the variety of $G_i/G_o$ protein targets (Chen, 2012; Beaulieu, 2011). The downstream targets of the $G_i/G_o$ protein (and the D2-type dopamine receptors) are explained in Section 1.3.2.1. The D2 receptor has two different splicing variants: D$_2$-short (D2S) and D$_2$-long (D2L). The two variants differ by a 29-amino acid sequence that gives them distinct physiological, signalling and pharmacological properties. D2L receptors are more abundant than D2S in most brain regions. D2L receptors are considered to be post-synaptic receptors while D2S are considered to be pre-synaptic. Although they each have different receptor properties, treatment with a D2 agonist on each isoform resulted in a
similar inhibition of AC activity (Chen 2012; Beaulieu et al. 2011; Beaulieu et al. 2015).

The D2-type dopamine receptors are expressed on both dopamine-receptive postsynaptic cells and dopaminergic presynaptic neurons. The highest density of D2 receptor expression is in the striatum, the nucleus accumbens and the olfactory tubercle. It is also expressed at high levels in the substantia nigra, the VTA, the hypothalamus, cortical areas, the septum, the amygdala and the hippocampus (Beaulieu, 2011; Beaulieu, 2015). It has been determined that medium spiny neurons (MSNs) in the striatum and nucleus accumbens selectively express the D1 or D2 receptor based on their projection site. MSNs that project to the medial globus pallidus and the substantia nigra pars reticulata express D1 receptors while MSNs that project to the lateral globus pallidus selectively express D2 receptors. There is also a separate group of MSNs in the dorsal striatum that express both D1 and D2 receptors, however the percentage of MSNs that do so is relatively low (5-10%) (Beaulieu et al. 2011; Hasbi et al. 2011). Importantly for this study, Zhang observed 20-25% of pyramidal neurons in the prefrontal cortex of mice express both D1 and D2 receptors (Zhang, 2010).

1.3.2.4 D1-D2 dopamine receptor complex

In 1983, Gershanik et al. showed that many actions of dopamine required both D1 and D2 to be stimulated. Many follow up studies supported this D1-D2 synergism by showing that certain dopamine-mediated processes, such as that in motor behaviour or reward processes, are likely the result of co-activation of D1 and
D2 and these processes do not occur under the stimulation of only one receptor (Dziedzicka-Wasylewska et al. 2004; Hasbi et al. 2011).

Following the primary studies supporting D1-D2 synergism, studies began to emerge on how D1 and D2 interact within the cell, and evidence of D1-D2 heterodimers began to surface. In 2004, Lee et al. determined that D1 and D2 are co-localized in the human caudate nucleus, the rat frontal cortex and the neonatal rat striatum using immunohistochemistry. Secondly, they showed that agonist co-activation of the D1 and D2 receptors in cells expressing both receptors increased intracellular calcium signalling through a pathway that is not activated by either receptor alone. Thirdly, they demonstrated that the D1 and D2 receptors could be co-immunoprecipitated from cells where they are both expressed. These findings were supported by studies performing fluorescence resonance energy transfer (FRET), showing that D1 and D2 do, indeed, exist within the same oligomeric complex in the plasma membrane as a D1-D2 receptor complex (So et al. 2005; Dziedzicka-Wasylewska et al. 2006). In 2007, Rashid showed that the intracellular calcium rise from the activation of both receptors is likely due to the D1-D2 receptor complex coupling to the G_q protein. They used human embryonic kidney (HEK) cells that expressed both the D1 and D2 receptor and subjected them to D1 and D2 receptor agonists, which showed a robust increase in intracellular calcium. A PLC inhibitor, an antagonist of intracellular IP_3 and a G_q inhibitor all abolished this intracellular rise in calcium, suggesting that the D1-D2 oligomers are coupled to the G_q protein (Rashid, 2007).
1.3.3 Dopamine and depression

Since the discovery of tricyclic antidepressants (TCAs), research on depression and antidepressants has mostly focused on the noradrenergic and serotonergic systems. However, there is a large and increasing amount of evidence that supports a prominent role for dopamine in depression. For example, motivation, psychomotor speed, concentration and the ability to experience pleasure are all, at least in part, regulated by dopamine in the CNS, and depression commonly features impairment of some, or all, of these abilities (Russo, 2013; Argyropoulos, 2013; Rea, 2013). Further, animal models of depression, such as the FST, learned helplessness, and chronic stress models show altered dopamine signalling in areas such as the nucleus accumbens, and the dorsal and ventral striatum, which could be driven by the hyperactivity in the iLPFC (Dunlop, 2007; Grace, 2016).

The large amount of patients that are unable to remit from current antidepressant treatment (Gaynes, 2008) also leads one to believe that there are perhaps different subtypes of depression, such as a “dopaminergic subtype” (Fried, 2015). If subtypes exist, a significant proportion of patients may be unable to remit under current medications due to most current antidepressants targeting the serotonergic and norepinephrine systems and may benefit from antidepressants targeting the dopaminergic system (Dunlop, 2007). In 2010, Pei et al. linked a dopaminergic receptor complex with depression and developed a potential antidepressant targeting this receptor (Pei, 2010).
1.3.3.1 D1-D2 and depression

The D1-D2 receptor complex was first thought to play a role in depression after work done by Pei et al. in 2010 on post-mortem patients that suffered from depression. They performed co-immunoprecipitation (Co-IP) of the D1R by the D2R antibody in post-mortem control patients and post-mortem patients that suffered from depression and noticed a significant upregulation in patients that suffered from depression. This led them to believe that there must be a greater amount of D1-D2 coupling in patients suffering from depression, and this may play a role in the disease itself. This led to the idea that uncoupling the D1-D2 complex could potentially have antidepressant effects (Pei, 2010).

In the same study, Pei et al. used biochemical analyses to identify the regions of D1R and D2R that were important for D1-D2 coupling. They found that the two receptors interact at the carboxyl tail of the D1R (D1CT) and at the second region of the extra 29 amino acids of the long isoform of the D2R (D2LL3-29-2). They followed these findings up by developing a peptide consisting of the D2LIL3-29-2 sequence to interfere with the D1-D2 complex and found that it had antidepressant-like effects when infused intracranially in the prefrontal cortex in both the FST and the LH task. Peptide infusion into the hippocampus and nucleus accumbens did not have antidepressant-like effects (Pei, 2010).

Since intracranial administration is not a clinically viable method of administration for an antidepressant, Brown et al. performed a follow up study in 2014, administering the peptide intranasally (Brown, 2014). The results were promising: they reported that the peptide could reach the PFC, disrupt the D1-D2
receptor complex, and have an antidepressant-like effect in rats in the FST when administered intranasally (Brown, 2014).

Figure 1-2 Demonstration of D1-D2 complex and effect of TAT-D1-D2-I Pep

(A) Mechanism of action of D1-D2 receptor complex. Activation of the complex results in activation of phospholipase C, in turn activating IP3, resulting in a rise in the intracellular concentration of Ca^{2+}. (B) TAT-D1-D2-I Pep effect on D1-D2 complex. The peptide interferes with the complex, inhibiting the downstream signalling of the complex but not affecting the individual receptors themselves. Figure prepared with help from S. Chen, Liu Lab (Brown, 2014).
1.4 Drug delivery to the CNS

The interstitial fluid of the brain must be highly regulated for the brain to function properly. The brain must be protected from toxic substances and fluctuating levels of neurotransmitters that circulate in the bloodstream, such as norepinephrine and glutamate. The system in place to achieve this is called the blood-brain barrier (BBB), which is formed by endothelial cells of the cerebral microvasculature to regulate movement of molecules between the blood and the interstitial fluid (Kandell, 2013). Although important to protect the brain, the BBB can make it difficult to get therapeutic drugs into the brain. To overcome this barrier, many different routes of administration exist. The optimal route in terms of ease of delivery, bioavailability, and pharmacokinetic profile of the drug is different for every drug and must be identified at an early stage of drug development through experimentation.

1.4.1 Peptide Drugs

Most current antidepressants on the market are small molecule drugs, including SSRIs and SNRIs. This means they are less selective and can bind to multiple different receptors or other unintended targets, which increases their side effect profile. Peptide drugs are a part of a more recent class of drugs that are larger in size and consist of amino acid sequences that are typically used to selectively target a certain amino acid sequence on endogenous peptides such as a peptide receptor. This makes them more selective to their targets, giving them a significant advantage in side effect profile over small molecule drugs (Bruno, 2013). Peptide drugs that target a receptor complex and aim to disrupt the interaction between the receptors
do not affect the signalling of each individual receptor, which also diminishes their side effect profiles (Wong, 2012). Peptide drugs have been a very successful class of drugs, both economically and therapeutically. There are many peptide drugs currently on the market generating in the billions of dollars of sales for a range of different diseases including multiple sclerosis, cancer, rheumatoid arthritis, and more (Craik, 2013).

Peptide drugs, however, have significant disadvantages. These include in vivo stability, route of administration and BBB penetration. First, many peptides are cleared from the bloodstream within minutes to hours after administration from rapid renal clearance and/or enzymatic digestion. Altering the size of the peptide, modifying the site where it’s most commonly cleaved, modifying the N- and C-terminals and encapsulation of the peptide into liposomes are methods that have shown to increase the in vivo half-life of peptide drugs (McGonigle, 2012). Second, the route of administration is limited for peptide drugs. It is not possible to use oral administration for peptide drugs because peptides are poorly absorbed and rapidly degraded by digestive enzymes (McGonigle, 2012; Craik, 2013; Bruno, 2013). Many peptide drugs are currently administered subcutaneously using an injection pen. Other possible methods of delivery include intranasal (Brown et al. 2014), intravenous and transdermal delivery (McGonigle, 2012). Intravenous administration is the most common route of administration for liposome-encapsulation (Allen, 2013). Third, peptides generally do not cross cell membranes, so it is necessary to use a transport mechanism to get the peptide across the cell, like cell-penetrating peptides such as the TAT peptide. Targeting BBB receptors is
considered the most promising mechanism for delivering peptides to the brain. It involves coupling the peptide to a specific ligand or antibody for these transporters. It can also be done using liposome-mediated delivery by coupling the liposome to the ligand or antibody (McGonigle, 2012; Craik, 2013).

1.4.2 TAT peptide

Cell membranes, including the BBB, prevent proteins, peptides and nanoparticulate drug carriers from entering cells unless there is an active transport mechanism involved. Therefore, peptide drugs do not efficiently permeate the cell on their own (Kandell, 2013). One way to overcome this barrier is to bind the peptide with a cell-penetrating peptide (CPP), which is a class of peptides that are able to cross cell membranes. TAT peptide (TATp) is one such CPP that is important for this study. TATp is derived from the transcriptional activator protein encoded by human immunodeficiency virus type 1 (HIV-1). This protein is efficiently internalized by cells when introduced into the surrounding area due to the positive charge in a stretch of amino acids that make up the transduction domain of the peptide. Conjugating this peptide to a peptide drug has been shown to allow the drug to cross cell membranes, including the BBB (Banks, 2005; Torchilin, 2008; Schwarze, 1999; Cooper, 2012).

1.4.3 Routes of administration

The method of drug delivery is very important. Some drugs are unable to reach their target through certain methods of delivery, such as peptide drugs and oral administration. For drugs meant to target the CNS, more than 98% of small molecule drugs and nearly 100% of large molecules (like peptides) are incapable of
crossing the BBB without a delivery system (Dhuria, 2010). The drug also has to be able to be administered in a clinically viable method that optimizes patient convenience, adherence and comfort (McGonigle, 2012; Kaminsky, 2015). For these reasons, different drugs require different routes of administration. The FDA lists over 100 different routes of drug administration, of which the important ones for this study will be discussed (Routes of Administration, FDA).

1.4.3.1 Intracranial administration

Intracranial administration is the infusion of a drug directly into the cerebrum. This method gives a better estimation of the central effects of a drug because it is deposited directly into the brain and diffusion across the BBB is not required. However, it is extremely invasive and not clinically viable for administration of antidepressant drugs. It is primarily used on animals pre-clinically to test the effects of a drug in the brain (Haley 1956; Pei 2010). The D1-D2 interfering peptide had antidepressant-like effects when administered directly into the PFC in rats (Pei 2010).

1.4.3.2 Intranasal administration

Intranasal administration has been shown to get drugs into the brain without changing blood concentration levels (Dhuria et al. 2010; Hoekmann 2011). The exact mechanism of intranasal drug passage to the CNS is not completely understood, but there is a body of evidence that has led to proposed mechanisms. The olfactory nerve pathway leads from the nasal cavity directly to the CNS. The olfactory receptor neurons (ORNs) have dendrites in the mucous layer of the olfactory epithelium and extend their axons centrally through the cribriform plate,
the subarachnoid space containing CSF and terminate in the olfactory bulb. It is likely that drugs entering the nasal passage are transported to the CNS by traversing along these nerve endings in the nasal cavity, either through intracellular or extracellular means, which leads the drug directly to the CNS. It is more likely that drugs travel through extracellular transport because evidence shows that drugs taken through intranasal administration reach the CNS almost immediately after delivery (Dhuria, 2010). Due to the non-invasive and easy administration, the rapid uptake into the CNS and the ability to bypass the BBB, intranasal administration is an advantageous method of drug delivery to the CNS, especially for peptide drugs. Intranasal administration can still be improved in terms of delivery efficiency, and direct targeting to specific brain areas (Dhuria, 2010; Brown, 2014). Some drugs that are currently administered intranasally include fentanyl for the management of acute pain, midazolam for the treatment of acute seizures, and haloperidol as an antipsychotic (Corrigan et al., 2015). In 2014, Brown et al. successfully delivered the D1-D2 interfering peptide intranasally and found that it had antidepressant-like effects in rats in the FST when delivered this way (Brown et al. 2014).

1.4.3.3 Intravenous injection

Intravenous (IV) drug administration delivers the drug directly into the bloodstream, eliminating the need for drug absorption. IV administration is advantageous for its 100% bioavailability, fast-acting effect, its minimal discomfort for the patient, and the ability to give a more accurate dose of medication (Doyle, 2015). A disadvantage of IV administration, however, is that it must be done by a healthcare professional and is impractical for at home use, making it less appealing
for an antidepressant because patients suffering from depression are used to orally administered antidepressants. IV administration is the most common route of administration for drug delivery systems (DDS). DDS are particulate carriers that are designed to improve the pharmacokinetics and biodistribution of their associated drugs, or to function as drug reservoirs (i.e., as sustained release systems), or both (Allen, 2013; Allen 2004). DDS could be important to combat the difficulties and improve the delivery of peptide drugs to the CNS. There are many liposomal and lipid-based products approved and on the market that are given with intravenous administration, such as AmBisome (for fungal infections) and Doxil/Caelyx (for certain forms of cancer) (Allen, 2013). For more on DDS, see Section 4.2.1.1.

1.5 Rationale

Since the serendipitous findings of mood-altering drugs in the 1950s, the progress on depression and antidepressant drug research has been slow. Current antidepressant drugs are unable to achieve a high percentage of patient remission (STAR* D), have a delayed onset of action and have problematic side effects. For these reasons, research on depression and treatment of depression, and the development of alternative therapeutics for patients suffering from depression are very important.

The majority of research on antidepressant drugs has focused on the serotonergic and noradrenergic systems. However, studies have shown that dopamine may also be a key player in the pathology of depression, and could be an important system to target for antidepressant treatment.
In 2010, Pei et al. found that the D1-D2 receptor complex is upregulated in post-mortem patients that suffered from depression. This up-regulation could play a role in depression and its symptomatology. Pei et al. developed a peptide to interfere with this D1-D2 receptor complex to cause a subsequent down-regulation of the complex. They showed that the interfering peptide exerts antidepressant-like effects when administered to the PFC through intracranial injection.

In 2014, Brown et al. followed up the work done by Pei et al. by studying whether this peptide could exert antidepressant-like effects when administered through a clinically viable method. They showed that this peptide was able to exert antidepressant-like effects in rats in the FST when administered via intranasal administration. Although it was proven to be effective, intranasal administration is not necessarily the optimal route of administration for this peptide, so further research on the method of delivery of this peptide must be done. An important step in the preclinical development of novel drugs is determining the optimal method of delivery, which must be determined through experimentation and testing.

The previous studies done by Pei et al. in 2010 and by Brown et al. in 2014 have paved the way for a novel peptide with antidepressant-like effects. The purpose of this study is to discover the effects of TAT-D1-D2-I Pep when delivered through intravenous administration. We will test to see if TAT-D1-D2-I Pep can reach the PFC, disrupt the D1-D2 receptor complex, and whether it will have an antidepressant-like effect in the FST in rats after intravenous administration. We will also determine the minimum dose required for the peptide to have a significant
antidepressant effect in the FST, and the maximum amount of time the peptide
continues to have an effect following intravenous administration.

1.6 Hypothesis

We hypothesize that intravenous administration will be an effective route of
administration for TAT-D1-D2-IPep, and our peptide will have comparable
antidepressant effects when delivered this way as it had in our previous studies
when it was administered intracranially and intranasally (Pei et al. 2010; Brown et
al. 2014). We hypothesize that the peptide will reach the PFC when administered
intravenously, which will be visualized by using immunofluorescent imaging by
tagging the peptide. We hypothesize that cells that have been treated with TAT-D1-
D2-IPep will show reduced levels of D1-D2 co-localization, which will be visualized
by analyzing cultured neurons that co-express D1 and D2 and analyzing the co-
localization of the D1 and D2 receptors before and after treatment with TAT-D1-D2-
IPep. Finally, we hypothesize that rats that have been intravenously injected with
the D1-D2 interfering peptide will show antidepressant-like effects in the FST. We
hypothesize that the rats that have been injected with TAT-D1-D2-IPep will not
show a significant difference in immobility behaviour from our positive controls,
and will show significantly less immobility behaviour compared to our negative
controls. We predict that the peptide will have an antidepressant-like effect for up
to 2 hours following administration, similar to past experiments. The minimum dose
required to have an effect is less predictable, but based on previous data we will
start at 3nmol/g (Aarts, 2002).
2 Materials and Methods

2.1 Animals

All experiments (except the immunohistochemistry experiment) used adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). The rats were housed in pairs at a constant temperature (20-23°C) on a 12-hour light/dark cycle (7:00-19:00 light). Rats were given 1 week to acclimatize before injections and behavioural testing took place. Rats were handled for ~5 minutes each day the week before behavioural testing to reduce stress during the testing. All rats weighed 300-350g at the time of behavioural testing; the animals were food restricted if necessary to maintain this weight.

The mice used were housed in groups at a constant temperature (20-23°C) on a 12-hour light/dark cycle (7:00-19:00 light). Mice had >1 week to acclimatize before injections. All mice weighed 35-40g at the time of injection. The Animal Care Committee at the Centre for Addiction and Mental Health (Toronto, ON) approved all experimental procedures.

2.2 Intravenous administration procedures

2.2.1 Intravenous administration

Prior to injection, animals were anaesthetized in the anaesthetic chamber using 5% isoflurane, an inhalant anaesthetic (Benson Medical Industries, Inc.) approximately 90 seconds. Once fully anaesthetized, the rats were removed from the chamber and laid on the surgical table in a prone position with an anaesthetic hose covering their nose and mouth, emitting 2.5% isoflurane to keep them
anaesthetized. The tail was submerged in a water bath at 45-50°C, followed by immediate IV injection in the tail vein using an insulin needle. The needle was removed and pressure was applied to the area using a cloth to stop the bleeding. Animals were under close observation until they regained consciousness from anaesthesia.

2.2.2 Substances injected intravenously

Animals received 3 IV injections during each experiment over a 24-hour period at hour 0, hour 5 and hour 23. They received either filtered saline (0.9% 9-NaCl, 300μL/injection), the D1-D2 interfering peptide (TAT-D1-D2-I Pep) (300μL, varying concentration), a membrane permeable TAT peptide (TAT-Pep) (300μL, varying concentration) from the Human Immunodeficiency Virus 1 (HIV1) TAT protein, or D1-D2-FLAG tagged interfering peptide (TAT-D1-D2-FLAG-I Pep) (300μL, 3nmol/L). The TAT-peptide is a 9 amino acid sequence that allowed all peptides used to be cell-permeable. All peptides were custom synthesized by Gen Script (New Jersey, USA) and/or Biomatik, Inc (Cambridge, Ontario) and had purity levels between 95 and 99%. All peptides were dissolved in filtered saline at a concentration of 10mM and stored at -80°C.

2.3 Intra-peritoneal injection procedure

Animals in the positive control group received an intra-peritoneal (IP) injection of imipramine hydrochloride (15mg/mL, Sigma-Aldrich) at a dose of 15mg/kg into the abdominal cavity. These animals followed the same anaesthesia procedure as the animals receiving intravenous administration; the rats were anaesthetized in the anaesthetic chamber using 5% isoflurane for approximately 90 seconds.
Following this, they were removed from the chamber and placed on the surgical table with an anaesthetic hose covering their nose and mouth, emitting 2.5% isoflurane to keep them anaesthetized while the IP injection was being delivered.

2.4 The forced swimming test

2.4.1 FST procedure

The FST takes place over two days, involving a training period, an injection period and a testing period. On the first day, the animal is brought into the training room 10 minutes prior to the training period so it is given the time to acclimatize to the room. The training period then consists of the animal being placed in a plexiglass, vertical cylinder 60cm high and 20cm in diameter that is filled with 40cm of 25°C (±0.3°C) water for 15 minutes. To prevent hypothermia, the animal is dried off following removal from the cylinder and is placed under a heat lamp for 10 minutes. The injection period consists of 3 injections given to the animal at 30 minutes, 5 hours and 23 hours following the end of training. Rats then underwent the 5-minute FST 1 hour following the final injection (except during the timing experiments) in the same cylinder, in the same place of the same room in which they were tested. The test was filmed with a video camera and analyzed blindly at a later time. Rats were again placed under a heating lamp for 10 minutes following the test.

2.4.2 FST Scoring

The videos that were taken of the 5-minute FST were scored into 60 different 5-second blocks, each block scoring as 1 of 4 different behaviours: climbing, diving, swimming, or immobility. Climbing was characterized as vertical forepaw movement, usually against the side of the cylinder. The rat was deemed to be diving
when its head dipped below the water and below its body. Swimming was characterized as the rat making horizontal movements through the cylinder. Immobility was characterized as the rat floating in the water without struggling, and only making moves necessary to keep its head above the water. A circular clock was placed in the frame of view of the video camera to easily separate the behaviours into 5-second blocks.

Immobility counts across groups were analyzed using one-way independent groups analysis of variance (ANOVA). Differences across individual treatment groups were measured using post-hoc Tukey multiple comparisons tests. Outliers were common so results are reported with and without outliers. Outliers were considered to be rats >10 immobility counts away from the mean.

2.5 Experimental Design

2.5.1 Effect of the D1-D2 interfering peptide

We tested the antidepressant effect of the D1-D2 interfering peptide using the FST. The FST is a highly variable experiment so outliers were not rare. Results that both include and exclude outliers are included in this paper (n=* represents the number of animals that include all data points). Animals were randomly split up into four treatment groups: TAT-D1-D2-I Pep (IV, n=4 (n=7*), 3nmol/g), TAT-Pep (IV, n=4 (n=6*), 3nmol/g), saline (IV, n=6 (n=8*), Volume-controlled), or imipramine (IP, n=4 (n=6*), 15mg/kg). The procedure for all groups is outlined in Section 2.4.1. The video was analyzed as outlined in section 2.4.2.
Figure 2-1 Rat behaviour during the FST

(A) Climbing behaviour: the rat vigorously moving forepaws in a vertical motion against the side of the cylinder, as if attempting to climb out. (B) Diving behaviour: the rat’s head dips below its body in an attempt to get to the bottom of the cylinder. (C) Swimming behaviour: the rat’s body is in a more horizontal position and is swimming horizontally through the cylinder. (D) Immobility behaviour: the rat’s forepaws are immobile and the rat is only making movements necessary to keep its snout above water.
2.5.2 Effect of the D1-D2 interfering peptide at different intravenously administered doses

The minimum dose required for the TAT-D1-D2-I Pep to have an effect was determined by repeating the FST after administering decreasing doses to the animals. New animals were used for each dose. The first experiments were done starting at 3 nmol/g, then subsequent experiments tested the animals at 1.5 nmol/g (IV, n=7 (n=9*)) and 0.75 nmol/g (IV, n=8)), all while following the same FST procedure as outlined in Section 2.4.1. The immobility counts of each treatment dosage group were compared to TAT-Pep (IV, n=6 (n=10*), 1.5 nmol/g), saline (IV, n=6 (n=8*), Volume-controlled), and imipramine (IP, n=4 (n=6*), 15 mg/kg) using 1-way independent groups ANOVA, followed by post-hoc Tukey multiple comparison tests.

2.5.3 Time dependency of TAT-D1-D2-I Pep antidepressant-like effects

We tested the time dependency of the efficacy of the TAT-D1-D2-I Pep by changing the time at which the FST was conducted. In the original experiment, the 5-minute FST was done 1 hour following the third injection, so we also tested the efficacy of the TAT-D1-D2-I Pep by testing the rats in the FST at 2 hours and 4 hours following the third injection. The rest of the FST protocol was as described in Section 2.4.1. The immobility counts of each TAT-D1-D2-I Pep group (IV, n=5 (n=7*), 3 nmol/g, FST at 2 hours following injection) (IV, n=8, 3 nmol/g, FST at 4 hours following injection) during the FST were compared to TAT-Pep (IV, n=6 (n=10*), 1.5 nmol/g, FST at 1 hour following final injection) using an unpaired students’ t-test.
2.6 Locomotor activity test

We used a locomotor activity test to confirm that the TAT-D1-D2-I Pep was indeed having an antidepressant-like effect and not simply increasing motor activity. For this test, we used rats that had already done the FST, from the TAT-D1-D2-I Pep (IV, n=4, 3nmol/g), TAT-Pep (IV, n=4, 3nmol/g), saline (IV, n=7, 3nmol/g), and imipramine (IP, n=4, 15mg/kg) groups. We used the same injection protocol as that used in the FST: the rats received an injection at hour 0, hour 5 and hour 23, then the test was performed at hour 24.

The locomotor activity test was done using a custom-made locomotor activity recording system. Each animal had its own 20cm high x 20cm wide x 30cm long standard housing cage, placed in the locomotor activity recording system for 60 minutes. The system had 12 infrared photocells that were placed at the bottom, along the long axis of the cage. The locomotor activity of the animal was detected by counting the interruption of the infrared beams that transected through the cage. The total locomotor activity was recorded for the full 60 minutes. Total activity data was analyzed using 1-way independent groups ANOVA, followed by Tukey multiple comparisons tests. A baseline was done before injection for each of the 3 days leading up to the test day (i.e. baseline test on Day 1, Day 2 and Day 3, followed by first injections on Day 3 followed by test day on Day 4).

2.7 Co-localization

2.7.1 Primary neuron culture preparation

Cortical tissues from embryonic day 14 (E14) mouse brains were dissected out, incubated with 0.25% trypsin for 15 min at 37°C, and dissociated by mechanical
trituration. Neurons were then plated at a desired density onto glass coverslips previously coated with 0.1mg/ml poly-d-lysine, and grown in Neurobasal medium with 4mM L-glutamine, 1× B27, 100U/ml penicillin and 100μg/ml streptomycin in an incubator (37°C, 5% CO₂). Media was changed every 3-4 days. Cultured neurons at 10 days in vitro (DIV) were pretreated with 10µM SKF38393 and 10µM quinpirole for 20 min, followed by treatment with either 10µM TAT-control peptide or 10µM TAT-D1-D2 peptide for an hour.

2.7.2 Immunocytochemistry

Neurons were fixed in 4% PFA/4% sucrose, permeabilized with 0.1M PBS containing 0.1% Triton X-100 for 10 min, and blocked for 1 hour with 1% bovine serum albumin in PBS at room temperature. They were incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at room temperature. Primary antibodies include anti-D1R (1:200; Abcam) and anti-D2R (1:200; Abcam). All fluorescent images were captured using a confocal microscope (Olympus FluoView FV1200).

2.8 Immunohistochemistry

Adult mice injected with either saline or TAT-D1-D2-IPep were sacrificed by cervical dislocation. Brains were harvested, fixed in 4% paraformaldehyde (PFA) overnight, cryoprotected in 30% sucrose and frozen at -80°C before further processing. 10µm-thickness frozen coronal sections were cut using a microtome cryostat system (Bright Instrument Co. 5030). Free floating sections were initially blocked in 5% fetal bovine serum, 1% Triton X-100, 0.5% Tween 20 and 1% skim milk in 0.1M PBS for 2 hours at room temperature to reduce non-specific binding.
Afterwards, brain sections were incubated with anti-HIV1 tat (1:200; Abcam) overnight at 4°C, followed by fluorescent secondary antibodies conjugated to Alexa 488 (1:200; Invitrogen) for 2 hours in blocking solution at room temperature. DAPI was used as a counterstain.

2.9 Co-immunoprecipitation and western blot

For co-immunoprecipitation experiments, 500~1000 µg solubilized protein extracted from mouse whole brain tissue was incubated in the presence of primary antibody or control IgG (2~4 µg) for 4 hrs at 4°C, followed by the addition of 25 µl protein A/G plus agarose (Santa Cruz Biotechnology) for 12 hrs. Pellets were washed, boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE. 10~100 µg of tissue extracted protein was used as control in each experiment. Beads were washed, boiled for 5 min in SDS sample buffer and subjected to SDS-PAGE. After transfer of proteins into nitrocellulose, membranes were Western blotted with the primary antibodies. The intensity of protein level was quantified by densitometry (software: Image J, NIH). The antibodies used were D2R (Santa Cruz Biotechnology, goat) for immunoprecipitation; D2R (Santa Cruz Biotechnology, mouse) and D1R (Sigma Aldrich, rat) for Western blot.
3 Results

3.1 The TAT-D1-D2-IPep has an antidepressant-like effect in the FST

We compared immobility behaviour between the 4 different groups of rats, first removing outliers: TAT-D1-D2-IPep (IV, n=4, 3nmol/g), TAT-Pep (IV, n=4, 3nmol/g), Saline (IV, n=6, 3nmol/g), and imipramine (IP, n=5, 15mg/kg). A one-way independent groups ANOVA revealed that the immobility behaviour in the FST between groups was significantly different (p<0.0001). A post-hoc Tukey test revealed significantly less immobility in the TAT-D1-D2-IPep group compared to the saline group (p<0.05) but not the TAT-Pep group (p=0.0551). It also revealed that the TAT-D1-D2-IPep had significantly more immobility compared to the imipramine group (p<0.05). The imipramine group had significantly less immobility than both the TAT-Pep and saline groups as well (both p<0.001). The TAT-Pep and saline groups were not significantly different (p>0.9999).

When the immobility behaviour is compared across all data points (including outliers), a one-way independent groups ANOVA revealed that there is no significant difference in immobility in the FST across all groups (p=0.0971) (Figure 3-1).
Animals who received intravenous injections of TAT-D1-D2-IPep (3nmol/g) were compared to animals that received IV injections of saline (volume controlled) and TAT peptide (3nmol/g), and to animals that received IP injections of imipramine (15mg/kg). Immobility of each group was compared using a one-way ANOVA followed by a post-hoc Tukey test. Data was not significant.

A) Data excludes outliers. *p<0.05 compared to saline. #p<0.05 ##p<0.001 compared to imipramine. B) Includes all data points. Data was not significant.

Figure 3-1 TAT-D1-D2-IPep has an antidepressant effect when outliers are removed compared to saline
3.2 Immobility behaviour effect of TAT-D1-D2-I Pep in the FST at different intravenous doses

To fully understand the dose required for the TAT-D1-D2-I Pep to have an effect intravenously, we repeated the FST after administering decreasing doses until immobility in the FST was no longer observed to be significantly different from negative controls. After our first set of tests was done at 3nmol/g, we administered a dose of 1.5nmol/g in our next set. We compared the immobility behaviour between 4 different groups of rats, first with the removal of outliers: TAT-D1-D2-I Pep (IV, n=7, 1.5nmol/g), TAT-Pep (IV, n=6, 1.5nmol/g), saline (IV, n=6, volume-controlled), and imipramine (IP, n=4, 15mg/kg). A one way independent groups ANOVA revealed that immobility behaviour was significantly different across all groups (p<0.01). Post hoc Tukey multiple comparisons tests revealed that TAT-D1-D2-I Pep had significantly less immobility compared to saline (p<0.05), but not compared to TAT-Pep (p=0.0647). The TAT-D1-D2-I Pep group also had significantly more immobility than imipramine (p<0.0001). TAT and saline had no significant differences, and were both significantly different than imipramine (p<0.0001). The same test was done including outliers and the ANOVA failed to reach significance across all groups (p>0.05) (Figure 3-2).

We followed this up by repeating the FST for a third time with a dose of 0.75nmol/g for the TAT-D1-D2-I Pep group. We compared the immobility behaviour in the FST across 4 groups, removing all outliers: TAT-D1-D2-I Pep (IV, n=8, 0.75nmol/g), TAT-Pep (IV, n=6, 1.5nmol/g), saline (IV, n=6, volume-controlled) and imipramine (IP, n=4, 15mg/kg). A one way ANOVA revealed a significant difference across all groups (p<0.0001), however post hoc Tukey multiple comparisons tests
revealed no significant differences between TAT-D1-D2-IPep and saline (p>0.05) and TAT-D1-D2-IPep and TAT-Pep (p>0.05). It also revealed significantly less immobility in the imipramine group compared with TAT-D1-D2-IPep, saline and TAT (p<0.0001 for all comparisons). We repeated a one-way ANOVA with the inclusion of all data points: TAT-D1-D2-IPep (IV, n=8, 0.75nmol/g), TAT-Pep (IV, n=10, 1.5nmol/g), saline (IV, volume-controlled, n=8), and imipramine (IP, n=6, 15mg/kg). This test did not reach significance (p=0.0622) (Figure 3-3).

3.3 Effect of TAT-D1-D2-IPep over time

We tested the amount of time the TAT-D1-D2-IPep maintains an antidepressant-like effect in the FST by varying the time between the final injection and the 5-minute FST. The original experiments were done with 1-hour between the final injection and the FST, so we repeated the FST experiments at 2- and 4-hours following the final injection.

3.3.1 Effect of TAT-D1-D2-IPep 2-hours following intravenous injection

We compared immobility behaviour in the FST between 2 different groups of rats, first removing all outliers: TAT-D1-D2-IPep (IV, n=5, 3nmol/g, 2-hr before FST) and TAT-Pep (IV, n=6, 1.5nmol/g, 1hr before FST). A student’s t-test revealed that rats that were administered the TAT-D1-D2-IPep had significantly less immobility behaviour in the FST 2 hours following injection compared to rats that were administered TAT-Pep (p<0.05). When a student's t-test was repeated including all outliers, the comparison failed to reach significance (p>0.05) (Figure 3-4).
Figure 3-2 TAT-D1-D2-I Pep has an antidepressant effect at a dose of 1.5nmol/g when outliers are removed

Animals that received intravenous injections of TAT-D1-D2-I Pep (1.5nmol/g) were compared to animals that received IV injections of saline and TAT peptide (1.5nmol/g) and to animals that received IP injections of imipramine (15mg/kg). Immobility of each group was compared using a one-way ANOVA followed by a post-hoc Tukey test. A) Outliers were removed from data. * p<0.05 compared to saline. # p<0.0001 compared to imipramine. B) All data points were included. Data was not significant.
Animals that received intravenous injections of TAT-D1-D2-IPep (0.75nmol/g) were compared to animals that received IV injections of TAT peptide (1.5nmol/g) and saline (volume-controlled) and to animals that received IP injections of imipramine (15mg/kg). Immobility of each group was compared using a one-way ANOVA. A) Outliers were removed. # p<0.0001 from imipramine. B) All data points were included. Data was not significant.

Figure 3-3 TAT-D1-D2-IPep does not have an antidepressant effect at a dose of 0.75nmol/g
Figure 3-4 TAT-D1-D2-IPep has an antidepressant effect 2 hours following injection when outliers are removed

Animals that received intravenous injections of TAT-D1-D2-IPep (3nmol/g, 2 hours following injection) were compared to animals that received IV injections of TAT peptide 1 hour following injection. Immobility of each group was compared using an unpaired student’s t-test. (A) *p < 0.05 (B) p > 0.05.
Figure 3-5 TAT-D1-D2-IPep does not have an antidepressant effect 4 hours following injection when outliers were removed

Animals that received intravenous injections of TAT-D1-D2-IPep (3nmol/g, 4 hours following injection) were compared to animals that received IV injections of TAT peptide (1.5nmol/g, 1 hour following injection). Immobility of each group was compared using an unpaired student’s t-test. p=0.4813.
Summary of figure 3-4, 3-5, and 3-1 when all outliers were removed. Immobility counts for the different time points of TAT-D1-D2-IPep are shown along with TAT-Pep and imipramine for visual comparison. # p<0.05 compared to TAT-Pep. 2-hr and 4-hr time points were not compared to imipramine; imipramine is shown for comparison.

Figure 3-6 TAT-D1-D2-IPep dose response curve
3.3.2 Effect of TAT-D1-D2-IPep 4-hours following intravenous injection

We compared immobility behaviour in the FST between 2 different groups of rats: TAT-D1-D2-IPep (IV, n=8, 3nmol/g, 4-hr before FST) and TAT-Pep (IV, n=6, 1.5nmol/g, 1-hr before FST). A student's t-test revealed that there was no significant difference in immobility between groups in the FST, with or without outliers (p>0.05) (Figure 3-5).

3.4 Locomotor activity testing following TAT-D1-D2-IPep administration

In order to be sure that the immobility behaviour in the FST induced by administration of TAT-D1-D2-IPep isn’t due to an increase in locomotor activity, we compared general locomotor activity between 4 different groups: TAT-D1-D2-IPep (IV, n=4, 3nmol/g), TAT-Pep (IV, n=4, 3nmol/g), saline (IV, n=7, Volume-controlled), and imipramine (IP, n=4, 15mg/kg). A one-way independent groups ANOVA revealed that there was no significant difference in beam breaks across all groups (p>0.05) (Figure 3-7).

3.5 D1-D2 receptor co-localization is reduced after subjecting cells to the TAT-D1-D2-IPep

We analyzed whether TAT-D1-D2-IPep was actually interfering with the D1-D2 receptor complex by using cultured neurons co-expressing D1 and D2 then subjecting them to either TAT-D1-D2-IPep or TAT-Pep. We imaged the neurons 1-hour following application of the treatment. The images show significantly less co-localization in the neurons subjected to TAT-D1-D2-IPep compared to the TAT-Pep group (Figure 3-8).
3.6 TAT-D1-D2-I Pep is observed in the PFC following intravenous administration

After intravenously injecting mice with TAT-D1-D2-I Pep or saline and collecting the brains, immunohistochemistry was used to visualize TAT-D1-D2-I Pep in the frontal cortex. Images show a significant increase in fluorescence in the mice that were injected with TAT-D1-D2-I Pep compared to the negative controls (Figure 3-9).

3.7 A reduction in D1-D2 complex in the PFC in rats is observed following administration of TAT-D1-D2-I Pep

Co-IP of the D1-D2 complex in mice that were given an IV injection of either TAT-D1-D2-I Pep or TAT-Pep shows that TAT-D1-D2-I Pep, but not TAT-Pep, reaches the CNS and interferes with the D1-D2 receptor complex in the mouse brain. Densitometry showed that the level of Co-IP D1R, normalized by taking the ratio to the IP D2R, in mice that were administered TAT-D1-D2-I Pep was 0.416087 of the level of Co-IP D1R:IP D2R in mice that were administered TAT-Pep (p<0.05). The level of IP D2R between each group of mice was not significantly different (p>0.05).
Figure 3-7 TAT-D1-D2-IPep does not have an effect in the locomotor activity test

Animals that received IV injections of TAT-D1-D2-IPep (3nmol/g) were compared to animals that received IV injections of TAT peptide (3nmol/g) and saline (volume-controlled) and to animals that received IP injections of imipramine (15mg/kg). Locomotor activity of each group was compared using a one-way ANOVA. Data was not significant.
Figure 3-8 Application of TAT-D1-D2-IPep to cultured neurons reveals interference on D1-D2 receptor complex

Left column images show staining of D1 receptor (D1R), middle column images show staining of D2 receptor (D2R) and right column images show combined staining of D1R, D2R and the control DAPI. Top row is a cell that has been subjected to the agonist (10µM SKF38393 + 10µM Quinpirole) at 20 minutes. Middle row shows a cell that has been subjected to the agonist + TAT-Pep at 1 hour. Bottom row shows a cell that has been subjected to the agonist + TAT-D1-D2-IPep at 1 hour.
Mice were administered saline through IV injection. (B) Mice were administered TAT-D1-D2-IPep through IV injection. Immunohistochemistry using anti-HIV1 TAT reveals significantly more fluorescence in the mice that received TAT-D1-D2-IPep. **Blue:** DAPI **Green:** TAT.

Figure 3-9 TAT-D1-D2-IPep reaches the PFC
Figure 3-10 TAT-D1-D2-I Pep disrupts the D1-D2 receptor interaction

Mice were injected with either TAT-Pep or TAT-D1-D2-I Pep.  
A) Co-IP shows that TAT-D1-D2-I Pep, but not TAT, is able to disrupt the D1-D2 receptor complex in mouse whole brain. Western blot of D1R and D2R precipitated by D2R antibody. (B) The intensity of each protein band for D1R and D2R was quantified by densitometry. They level of Co-IP D1R (D1R Co-IP) was normalized after divided by the level of precipitated D2R (D2R IP). Results for each sample are presented as the percentage of the mean of the TAT samples on the same blot. **p<0.01 as compared to the TAT group, n=5 (TAT)/4 (TAT-D1-D2-I Pep), t-test. (C) The intensity of each protein band of D2R was quantified by densitometry. Results for each sample are presented as the percentage of the mean of the TAT samples on the same blot. n=5 (TAT)/4 (TAT-D1-D2-I Pep), t-test.
4 Discussion

The aim of this study was to see whether the TAT-D1-D2-I Pep could reach the PFC and have an antidepressant-like effect when delivered intravenously. To accomplish this, we separated rats into 4 different treatment groups, delivering a different drug to each group (the test group, 2 negative control groups and 1 positive control group). We tested the efficacy of each drug as an antidepressant by subjecting each rat to the forced swim test (FST), one of the most widely used models for assessing antidepressant-like activity in rats (Slattery, 2012). The negative control groups received saline (IV injection) and TAT-Pep (IV injection), the positive control group received imipramine (IP injection), a peptide that has a well-established antidepressant effect both in humans and in rats subjected to the FST (Slattery, 2012), and the test group TAT-D1-D2-I Pep (IV). It is also necessary to verify that the peptide is functioning as hypothesized, so we used cell cultures that co-expressed the D1 and D2 receptors and imaged the cells to determine the co-localization of D1 and D2 receptors before and after treatment with TAT-D1-D2-I Pep. We also performed co-immunoprecipitation (Co-IP) on the D1-D2 receptor complex to confirm that TAT-D1-D2-I Pep reached the CNS and disrupted the D1-D2 receptor complex in vivo. Finally, it is also necessary to be sure that the peptide reaches the PFC, as hypothesized, so we administered the TAT-D1-D2-I Pep intravenously to mice, collected the brain slices and used immunohistochemistry to detect the peptide in the PFC.
4.1 Antidepressant-like effect of TAT-D1-D2-I Pep when delivered intravenously

Our results demonstrate that TAT-D1-D2-I Pep has an antidepressant-like effect in rats in the FST following IV administration, but with the caveat that outliers had to be removed to reach significance. The FST is a highly variable experiment so outliers in the data were common and unfortunately, the FST is the best and most widely used animal model for testing the efficacy of antidepressants. Discovering a new animal model for the testing of antidepressant drugs is a future consideration to optimize the research on depression and its treatment. The requirement to remove outliers to reach significance may also denote that the amount of peptide that reaches the PFC after IV injection is more variable and less reliable compared to IN and IC administration.

Our primary experiments tested the peptide at a dose of 3nmol/g, which is in conjunction with other experiments involving IV injection of TAT-coupled peptides tested in our lab (Aarts, 2002). When all data points, including outliers, were included, the data was unable to reach significance following common statistical practices. However, when outliers were removed (both high and low outliers), the data showed that the TAT-D1-D2-I Pep had significantly reduced immobility behaviour in the FST compared to the negative controls (saline and TAT-Pep). However, the TAT-D1-D2-I Pep also had significantly more immobility behaviour compared to the positive control (imipramine) when outliers were removed.

There are multiple different conclusions that can be made from the data: the first is that the peptide has a comparable antidepressant-like effect when administered intravenously to intranasal administration, and the outliers can be disregarded. The
second is that the peptide does not have an antidepressant-like effect when administered intravenously, and the outliers cannot be disregarded. The third, and most likely conclusion that can be made from the data, is that the peptide does have an antidepressant-like effect when administered intravenously, but this effect is not comparable to the effect when the peptide is administered intranasally. This conclusion would be met by admitting that the data shows that the peptide appears to have an anti-immobility effect in the FST, even if the data doesn’t reach significance due to outliers. Conceptually, the most likely explanation for this conclusion is that the amount of TAT-D1-D2-I Pep that reached the PFC was more varied and less consistent amongst each rat and the average amount of peptide that reached the PFC was lower compared to IN or IC administration. This could be due to peptide degradation in circulation or due to difficulty crossing the BBB.

The immunofluorescence studies performed, although not quantifiable, confirm that a significant amount of peptide is reaching the PFC following intravenous administration. Further studies must be done to quantify the amount of peptide that reaches the PFC through both intranasal and intravenous administration, such as immunohistochemistry quantification (Taylor, 2006) in order to come to a better conclusion about the efficacy of intravenous administration of the TAT-D1-D2-I Pep. Our Co-IP experiment also showed that a significant amount of peptide is reaching the CNS and interfering with the D1-D2 receptor complex, however it was done in a mouse brain which is too small to extract the frontal cortex for Co-IP, so it had to be done on the whole brain. Since the previous studies done in our lab have shown that the D1-D2 receptor complex must be disrupted in the PFC, a future Co-IP or FRET
study showing that TAT-D1-D2-I Pep can separate the D1-D2 receptor complex in the PFC would have more power.

Neither Pei nor Brown reported difficulties reaching statistical significance in the FST, likely meaning their methods of delivery were more robust and reliable, and were able to deliver a higher concentration of TAT-D1-D2-I Pep to the PFC. Fortunately, there are ways to prevent degradation of peptide drugs and improve delivery from the bloodstream to the CNS across the BBB. The most notable of these methods is coupling the delivery of the peptide with a drug delivery system (DDS). DDSs are discussed in the future directions section.

4.1.1 Dose-dependent efficacy of TAT-D1-D2-I Pep

Testing for dose-dependent efficacy revealed that 1.5nmol/g is the lowest dose required for the TAT-D1-D2-I Pep to have an antidepressant-like effect in rats in the FST, when outliers were removed. 0.75nmol/g of TAT-D1-D2-I Pep through intravenous injection had no effect in the FST. This is comparable to the lowest dose required for an effect in the FST when TAT-D1-D2-I Pep was administered intranasally in rats, which was found to be 1.67nmol/g. Literature states that ~1-5% of drug reaches the CNS when administered intranasally (Dhuria, 2010; Brown, 2014), so we can conclude that, if outliers are not considered, intravenous administration has similar efficiency for the TAT-D1-D2-I Pep. The original experiment on the TAT-D1-D2-I Pep done by our lab found that 5nmol of peptide had to be infused intracranially to the PFC. 1.5nmol/g for a rat weighing 325g equates to 487.5nmol peptide. 487.5nmol of peptide is 97.5x more than 5nmol, which equates to about 2.5% efficiency of drug reaching the PFC after IV injection.
However, these are only approximations because we didn’t do any quantitative measurements to assess exactly how much peptide was delivered to the CNS, but this is a future consideration.

4.1.2 Time-dependent efficacy of TAT-D1-D2-IPep

Testing for time-dependent efficacy revealed that TAT-D1-D2-IPep stays active in the CNS for between 2-4 hours. When we performed the FST 2 hours following the final injection, there was still a significant anti-immobility effect in rats that received TAT-D1-D2-IPep when we removed outliers. However, testing the rats 4 hours following injection revealed no immobility effect. This result is consistent with IN administration of TAT-D1-D2-IPep, which confirmed that the peptide was active in the CNS at 2 hours, but not at 3 or 4 hours following final injection.

4.2 Intravenous delivery to the CNS

The immunohistochemistry experiment revealed that TAT-D1-D2-IPep reached the PFC following intravenous administration. This experiment, however, was not quantitative, so we are not sure how much peptide reached the PFC and how it compares with intranasal administration, we can only conclude that there was significantly more imaging signal in the PFC in the mice that were intravenously administered TAT-D1-D2-IPep compared to the mice that were intravenously administered saline.

4.2.1 Clinical relevance of IV delivery of TAT-D1-D2-IPep

Intravenous administration does not appear to be the most effective way of delivering TAT-D1-D2-IPep. It is also less clinically functional to use IV administration for an antidepressant because it is more difficult to do at home
compared to IN, it requires more attention from a medical professional and it is more invasive. It would also be significantly more difficult for patients suffering from depression, as they are used to taking antidepressant medication orally. Therefore, it is not the ideal administration method for this peptide. However, peptide drugs are unable to be delivered orally and IV can have advantages over IN. For example, IN administration is still not completely developed as a method for drug delivery and has the possibility of irritation to the nasal cavity (Dhuria, 2010). Another advantage of IV administration is that it is the preferred method of administration for Drug Delivery Systems (DDSs).

4.2.2 Drug delivery systems

Drug delivery systems (DDSs) are particulate carriers that are designed to improve the pharmacological properties of drugs. These particulate carriers are composed primarily of lipids and/or polymers that encapsulate the drug and function to carry it through the body to its target. They are designed to improve drug efficacy by improving the pharmacokinetic profile and biodistribution of the drug, or to function as drug reservoirs (i.e. sustained release systems). They also make it easier to actively target their desired destination (e.g. the BBB). Our lab is currently developing a liposome to encapsulate peptide drugs to facilitate the transport into the CNS. The liposome improves the stability and is conjugated with BBB targeting ligands to directly target passage across the BBB. The behavioural results of delivering TAT-D1-D2-IPep without liposome encapsulation are not promising: the antidepressant-like effects of TAT-D1-D2-IPep do not compare with imipramine and do not reach significance when compared with the negative
controls when all outliers are included. However, the use of liposome-mediated delivery could significantly improve the behavioural results in the FST. IV administration is the easiest and most popular way to get DDS into circulation, so this project is a stepping-stone to studying the effects of TAT-D1-D2-IPep in conjunction with liposome-mediated delivery (Allen, 2004; Unpublished data from Liu lab, 2015).

4.3 TAT-D1-D2-IPep

4.3.1 Mechanism of action

The co-localization and Co-IP studies showed that the peptide does, in fact, disrupt the D1-D2 complex. In conjunction with the results in the other experiments, this shows that separation of the D1-D2 receptor complex is the most likely mechanism of action for which our drug exerts antidepressant-like effects in rats in the FST. It is, however, difficult to hypothesize a mechanism of action for why disruption of the D1-D2 complex has antidepressant effects, given that the etiology of depression is unclear. It is possible, however, that an up-regulation of the D1-D2 complex, specifically in the PFC, contributes to depressive symptomatology, so interfering with this receptor complex reduces depressive symptoms.

4.3.2 Advantages of TAT-D1-D2-IPep

Targeting receptor-receptor interactions is a novel approach to treatment of neuropsychiatric disease. Current treatment mostly targets neurotransmitter receptors, transporters or metabolic pathways of neurotransmitters directly in an attempt to directly alter the extracellular availability of neurotransmitter or to alter synaptic transmission directly (Wong, 2012). If a change in regulation of receptor-
receptor interaction has a direct impact on the neuropsychiatric disease, targeting the interaction itself can have an impact on the symptoms of the disease, without impacting the functionality of the individual receptors themselves, and, in turn, having a symptom relieving effect on the disease while having a reduced side effect profile.

Peptide drugs are also more selective to their target compared to small molecule drugs. Being more selective to the target results in a reduced side effect profile. Side effects have long been one of the main problems with antidepressant drugs, so TAT-D1-D2-IPep could be a novel, impactful solution to this problem.

4.3.3 Disadvantages of TAT-D1-D2-IPep

TAT-D1-D2-IPep also has some significant disadvantages, mainly stemming from its being a peptide drug. First, the route of administration is non-ideal for peptide drugs; oral administration is the easiest and most commonly used route of administration for antidepressant drugs, however, peptide drugs are unable to be delivered orally because their bioavailability is severely limited by the digestive system. Therefore, the route of administration must be parenteral (subcutaneous, intranasal, intravenous, etc.). This is an immediate disadvantage compared to current antidepressants on the market. Subcutaneous and IV administration are more difficult for the patient and more invasive. IV administration also likely needs a DDS, which, although advantageous to allow the drug to reach the CNS, can be more costly and adds the risk of carrier toxicity (Allen, 2004; Allen, 2013). Intranasal administration is still not a completely developed method of administering drugs to the CNS, while adding the risk of irritating the nasal cavity
(Dhuria, 2010). Second, peptide drugs have difficulty crossing the BBB. Third, rapid renal clearance and enzyme digestion causes a low in vivo stability of peptide drugs. Again, difficulty crossing the BBB and low in vivo stability can potentially be overcome by using DDS, but DDS also carry their own disadvantages. Fourth, greater specificity of drugs acting on heterogeneous diseases, such as MDD, does not always lead to greater efficacy. The up-regulation of D1-D2 receptors is likely only one of many factors contributing to MDD, and therefore may not have as great an efficacy as some other small molecule drugs that bind to many targets. Therefore, due to all these factors, a small molecule drug with a D1-D2 interfering function may be better suited as an antidepressant over TAT-D1-D2-I Pep, in terms of route of administration, bioavailability, and pharmacokinetic profile (Wong, 2012). However, previous attempts of developing small molecule drugs to disrupt protein-protein interactions have not been successful (Wong, 2012). Our peptide could act as a positive control, however, to screen for such small molecule drugs in the future.

4.4 Limitations

A limitation to consider in this experiment is the nature of the FST. The FST is an acute test that tests the efficacy of an antidepressant within 1-4 hours following delivery of the drug, whereas antidepressant medication requires chronic treatment (weeks to months) before depressive symptoms improve. Therefore, the FST lacks face validity, but has high predictive validity, as past experiments have not only consistently demonstrated reduced immobility behaviour when animals were given antidepressant drugs, but also consistent increased immobility behaviour when animals were under the influence of pro-depressant factors (pre-natal stress,
chronic subordination stress, chronic corticosterone administration and amphetamine withdrawal) (Slattery, 2012).

Another limitation to consider is the use of cell cultures in the immunohistochemistry experiment to visualize the disruption of the D1-D2 receptor complex, as well as the Co-IP on the whole brain of a mouse in the Co-IP experiment. Together, these experiments show that TAT-D1-D2-I Pep is capable of reaching the CNS and disrupting the D1-D2 receptor complex after IV administration. However, they do not show that TAT-D1-D2-I Pep is adequately reaching the PFC, which is the area of interest for this experiment. An experiment to consider in the future is to perform Co-IP on rats so that the brain is big enough to extract only the PFC and get a more useful result. Another experiment to consider is Fluorescence Resonance Energy Transfer (FRET), a sophisticated way to test the interaction between receptors.

4.5 Future Directions

Some future directions have been mentioned in other sections of the discussion, but they will be summed up in this section. First, the next step for the development of the TAT-D1-D2-I Pep is to test it in a DDS. Our lab is currently developing a liposome to encapsulate peptides and mediate their delivery to the CNS. The goal of the liposome-mediated delivery is to protect the peptide from being metabolized, and also to improve delivery to the BBB. Delivery to the BBB is improved by conjugating the liposome with different BBB-targeting ligands.

A second future step is to quantify the amount of peptide that reaches the CNS using immunohistochemistry. We have shown that the TAT-D1-D2-I Pep reaches the
PFC, but the results were not quantitative. Understanding the quantity of peptide that reaches the PFC and comparing the quantities between types of delivery (IN, IV, IV-liposome, etc.) would give us a better understanding of which route of administration is ideal.

Third, testing for the disruption of the D1-D2 receptor complex in the PFC of a rat after intravenous administration of TAT-D1-D2-I Pep is an important next step to determine that the peptide disrupts the D1-D2 receptor complex in the PFC.

Fourth, a small molecule drug with the same D1-D2 interfering effect as our peptide could be beneficial as a marketable drug. Therefore, screening for a small molecule drug while using our peptide as a positive control is another future step to consider.

Fifth, more routes of administration must be explored. For example, subcutaneous and transdermal administration are other common routes of administration for peptide drugs and are other routes that can be explored for TAT-D1-D2-I Pep.

### 4.6 Final conclusions

Our experiments clearly show that the peptide is able to reach the frontal cortex and disrupt the D1-D2 receptor complex. Less clear are the behavioural antidepressant-like effects in rats in the FST. Although statistical measures show that the effect is not significant when all data points are considered, outlier removal shows that there is a significant effect at doses of 1.5nmol/g and higher, and for at least 2 hours following injection. Therefore, we believe that TAT-D1-D2-I Pep has an antidepressant effect when delivered intravenously at doses of 1.5nmol/g and
higher, and has an acute effect in rats for up to 2 hours. This project is a stepping-stone to testing this peptide with a DDS, or more specifically liposome-encapsulated delivery. Liposome-encapsulated delivery will improve the pharmacokinetic profile of the drug, as well as allow for the possible functioning as a sustained release system. Intravenous administration is the most common route of administration for liposome-encapsulated delivery, so this peptide first had to be delivered intravenously on its own to understand the effects so it can be compared with the effects of the peptide following liposome-encapsulated delivery.
References


Free RB, Hazelwood LA, Cabrera DM, Spalding HN, Namkung Y, Rankin ML, Sibley DR. 2007. D1 and D2 dopamine receptor expression is regulated by direct


Garau L, Govoni S, Stefanini E, Trabucchi M, Spano PF. 1978. Dopamine receptors: Pharmacological and anatomical evidences indicate that two distinct dopamine receptor populations are present in rat striatum. Life Sciences 23(17-18):1745-1750.


Route of Administration [Internet]. Silver Spring, MD: U.S. Food and Drug Administration. Access date: 10/02/2016. Web address: 

Niederehe G, Thase ME, Lavori PW, Lebowitz BD et al. 2006. Acute and 
Longer-Term Outcomes in Depressed Outpatients Requiring One or Several 

Neurosci 14(9):609-25.

Sanacora G, Treccani G, Popoli M. 2012. Towards a glutamate hypothesis of 
depression: an emerging frontier of neuropsychopharmacology for mood 


Schubert D, Martens GJM, Kolk SM. 2015. Molecular underpinnings of prefrontal 
cortex development in rodents provide insights into the etiology of 


Seamans JK, Yang CR. 2004. The principal features and mechanisms of dopamine 

Seo JS, Wei J, Qin L, Kim Y, Yan Z, Greengard P. 2016. Cellular and molecular basis 
for stress-induced depression. Mol Psychiatry.


Sheynikhovich D, Otani S, Arleo A. 2013. Dopaminergic control of long-term 
depression/long-term potentiation threshold in prefrontal cortex. J Neurosci 

Slattery DA, Cryan JF. 2012. Using the rat forced swim test to assess antidepressant-

Slattery DA, Hudson AL, Nutt DJ. 2004. Invited review: the evolution of 


