EFFICACY OF SYNAPSIN II ANTISENSE SEQUENCES

SYNAPSIN II REDUCTIONS AND SCHIZOPHRENIA: THE EFFECTS OF ANTISENSE KNOCKDOWN AND OTHER CONFOUNDS ON DISEASE MANIFESTATION

By

PATRICIA A. HUI

H.B.Sc

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2015) McMaster University
(Science – Neuroscience, MiNDS) Hamilton, Ontario

TITLE: Synapsin II Reductions and Schizophrenia: Investigating the Effects of Synapsin II Antisense Knockdown Technology and Other Confounds in Neonatal and Adult Pathophysiology of Schizophrenia

AUTHOR: Patricia A. Hui, H.B.Sc. (McMaster University)

SUPERVISOR: Dr. Ram K. Mishra

NUMBER OF PAGES: xx, 137

ABSTRACT

The complex heterogeneity of schizophrenia has proved difficult to replicate in preclinical animal models. Of the many molecular targets implicated with schizophrenia, this thesis focuses on synapsin II - a pre-synaptic protein critical for neurotransmission and synaptogenesis; and parvalbumin - a calcium-binding protein found in interneurons of the dorsolateral prefrontal cortex (DLPFC) and the striatum (STR).

Patients with schizophrenia display reduced levels of synapsin II mRNA in the DLPFC, while decreased activation of parvalbumin neurons in the same region has resulted in schizophrenia-like cognitive deficits. Knockdown of synapsin II in the medial prefrontal cortex (mPFC) of neonate and adult rats has previously induced schizophrenia-like alterations. However, there are concerns that must be addressed before novel animal models of schizophrenia can be developed using reductions in synapsin II.

This thesis was designed to 1) eliminate maternal separation (MS) between post-natal days (PD) 14-23, which correlates with a neurodevelopmental synapsin II model, as a means of inducing schizophrenia-like behaviours; 2) reassess the use of fully and partially phosphorothioated first-generation antisense oligonucleotides to reduce synapsin II levels, and 3) evaluate parvalbumin expression in the STR following synapsin II knockdown.

Results from this study indicate 1) a 36 hour MS regimen during PD 14-23 did not cause behavioural changes bearing resemblance to schizophrenia; 2) oligonucleotide sequences stabilized completely with phosphorothioate bonds were insufficient in

reducing synapsin II levels and caused localized necrosis, while partially modified sequences induced a slight knockdown effect without cell death; and 3) levels of striatal parvalbumin expression were decreased in rats receiving the partially, but not fully, modified antisense sequences.

The findings strengthen the face validity and safety profile of the synapsin II knockdown model. Novel evidence has also been provided for the role of parvalbumin in the striatum and suggests its influence on cognitive dysfunction in schizophrenia.

ACKNOWLEDGEMENTS

This past year has been an incredible learning experience, and I will always value the immense guidance that I received along the way. This acknowledgement only begins to express my appreciation for the wonderful individuals who have supported me during this process.

To my supervisor, Dr. Mishra, I extend my sincerest gratitude for your support and mentorship over the years. It has been a privilege to learn from you, and I thank you for the many opportunities that you have given me. Under your guidance, I have become a more competent and confident scientist. Thank you for your continuous belief in my potential and for always helping me pursue my goals. I am forever grateful to have you as a mentor, and for everything you have done for me.

I would also like to thank my supervisory committee members, Dr. Laurie Doering, Dr. Bhagwati Gupta, and Dr. Shucui Jiang, for their support and scientific insights. I appreciate the guidance with my research project, and the words of encouragement towards my personal and scientific development.

Throughout my years in the Mishra lab, I am fortunate to have worked with some of the kindest, most intelligent, and highly supportive individuals. To Mattea, Sharon, Tiffany, Farhat, Jay, Ritesh, Heta, Luke, Asim, Dipa, Shreya, and Ashley: thank you for making my time in the Mishra lab such a wonderful and unforgettable experience! To Nancy Thomas, thank you for being there to guide me during my initial years as an undergraduate student in the lab. I would have been lost without you.

Thank you to the friends who have always been there for me, and have helped keep me grounded. Jason, I am grateful for your continuous support and care, and your ability to cheer me up even when you say nothing at all.

Finally, to my family, my biggest source of inspiration, thank you. For the continued interest in understanding my research, for helping me through the good times and the bad, and for raising me up to be the person I am today, I am so thankful to have you all in my life. I could not imagine completing this stage of my life, or the next, without such love and unconditional support.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

8-ARM	eight-arm radial maze
aCSF	artificial cerebral spinal fluid
ANOVA	analysis of variance
A/P	anterior-posterior
AS	antisense oligonucleotides
CAF	Central Animal Facility
CT	computerized tomography
D1R	dopamine D1 receptor
D2R	dopamine D2 receptor
DA	dopamine
DISC1	disrupted-in-schizophrenia 1
DLPFC	dorsolateral prefrontal cortex
ECL	enhanced chemiluminescence
^{18}F-DOPA	^{18}F-dihydroxyphenyl-*L*-alanine
[^{18}F]FDG	fluorine-18-fluorodeoxyglucose
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GP_e	globus pallidus external

GP_i	globus pallidus internal
IDT	Integrated DNA Technology
M/L	medial-lateral
mPFC	medial prefrontal cortex
MS	maternal separation
NMDA	*N*-methy-D-aspartate
NMDAR	*N*-methy-D-aspartate receptor
NORT	novel object recognition task
nVH	neonate ventral hippocampus
OD	optical density
PBS	phosphate buffered saline
PSB-T	phosphate buffered saline with Tween®20
PCP	phencyclidine
PD	post-natal day
PET	positron emission tomography
PFC	prefrontal cortex
PPI	prepulse inhibition
PS	phosphorothioate
PV	parvalbumin
PVBC	parvalbumin basket cells

PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide
SN	substantia nigra
SPECT	single photon emission computed tomography
STR	striatum
STN	subthalamic nucleus
TBS	tris buffered saline
TBS-T	tris buffered saline with Tween®20
VH	ventral hippocampus

1. BACKGROUND

1.1 Schizophrenia

Schizophrenia is a chronic, debilitating psychiatric disorder with a high prevalence rate (Jones, Watson, and Fone 2011; Laruelle, Kegeles, and Abi-Dargham 2003). Despite an abundance of research in the field within the last century, the underlying mechanisms of schizophrenia have yet to be fully elucidated, therefore impeding the development of novel therapeutic drugs due to a lack of molecular targets (Jones, Watson, and Fone 2011). Currently, the majority of antipsychotic drugs act in a preventative manner, temporarily alleviating symptoms rather than curing patients of the disorder (Jones, Watson, and Fone 2011).

Our understanding of the neurological basis of schizophrenia remains limited, in part, due to its complex heterogeneity, and the few animal models available to accurately and reliably mimic the disorder (Jones, Watson, and Fone 2011). Of the commonly used animal models, most display behavioural phenotypes that mimic only the positive symptoms of schizophrenia, with fewer models showing the negative and cognitive symptoms of the disorder (Jones, Watson, and Fone 2011). Furthermore, many antipsychotic drugs, including haloperidol and olanzapine, attenuate the positive and negative symptoms of the disorder, but fail to provide treatment for the cognitive symptoms (Jones, Watson, and Fone 2011).

Despite promising developments in pharmacological treatment over the years, the economic burden of schizophrenia remains high. In Canada alone, the estimated annual

cost of schizophrenia exceeds $7 billion, primarily due to loss of workplace productivity, and to a lesser degree, direct healthcare expenditures (Goeree et al. 2005). It is evident that more must be done to reduce the cost of this severe mental illness in Canada.

Thus, in order to further our understanding of the disease pathology and develop novel, efficacious drugs, it is imperative to find more comprehensive animal models of schizophrenia that can accurately encompass all three domains of symptoms.

1.1.1 Incidence & Prevalence

Approximately 1% of the general population, amounting to 51 million people worldwide, suffers from schizophrenia (Jones, Watson, and Fone 2011; Laruelle, Kegeles, and Abi-Dargham 2003; NIMH). The number of new diagnoses of schizophrenia continues to rise each year, by about one in 4000 people (NIMH). A number of additional epidemiological factors can affect the prevalence rate in various population subsets. Genetic predisposition plays a large role, with prevalence rates of schizophrenia ranging from 8-10% in people with schizophrenic siblings, to 50-80% in identical twins (Jones, Watson, and Fone 2011; Tamminga and Holcomb 2005; O'Donovan, Williams, and Owen 2003). Although genetics is, by far, the strongest risk factor for schizophrenia, it is not the sole determinant of disease development. Schizophrenia can occur in those without prior family history of the disorder (Tamminga and Holcomb 2005). Pre-natal and early life events, including pre-natal maternal illness in the second trimester and maternal separation, can also confer increased risk for schizophrenia (Tamminga and Holcomb 2005; Ellenbroek and Riva 2003).

Although the progression of schizophrenia is life-long, symptoms classically present post-adolescence, between 16-25 years of age (Tamminga and Holcomb 2005; Jones, Watson, and Fone 2011). In childhood-onset schizophrenia, symptoms can manifest before 15 years of age, although such cases of the disorder occur at an incidence rate of 50 times less than that of classical schizophrenia (Tamminga and Holcomb 2005). From a gender perspective, males have a higher incidence rate of developing symptoms of psychosis, while it is more common for females to have a bimodal onset later in life (40-60 years old). Most patients with schizophrenia display immense deterioration in psychosocial function within the first few years of diagnosis, followed by a low plateau of symptomology over their lifetime. Interestingly, symptoms of schizophrenia may improve after 50 years of age, although it is undetermined whether the normal aging process has any therapeutic effect (Tamminga and Holcomb 2005). This course of disease is unique from both traditional neurodegenerative disorders (e.g. Parkinson's disease) where symptoms continuously decline, and neurodevelopmental disorders (e.g. mental retardation), in which disease progression is low and steady throughout the lifetime (Tamminga and Holcomb 2005).

1.1.2 Symptomology

The clinical phenotype of schizophrenia is characterized by three domains of symptoms: 1) positive, 2) negative, and 3) cognitive. Positive symptoms represent an excess of normal functioning, and include auditory and visual hallucinations as well as delusions (Tamminga and Holcomb 2005; Jones, Watson, and Fone 2011). Negative

symptoms including social withdrawal, anhedonia, and emotional blunting, are characteristic of a reduction in normal functioning (Tamminga and Holcomb 2005; Jones, Watson, and Fone 2011). The last category of symptoms, cognitive dysfunction, typically presents as attentional deficits, impaired working memory, and executive functioning (Tamminga and Holcomb 2005; Jones, Watson, and Fone 2011). Cognitive symptoms are considered the core prodromal symptom of schizophrenia, as they often manifest before the onset of positive and negative symptoms (Lencz et al. 2006). The extent of cognitive deficits is often the most predictive indicator of long-term outcome in response to antipsychotic drugs, and social functioning (Kraguljac, Srivastava, and Lahti 2013). The different domains of symptoms are not exclusive of another, although oftentimes, one cluster may predominate over the others (Tamminga and Holcomb 2005).

1.1.3 Pathophysiology of Schizophrenia

Altered function of various neurotransmitter systems has been heavily implicated in the underlying pathophysiology of schizophrenia. Evidence has shown that neurotransmitters, dopamine (DA), glutamate, and γ-aminobutyric acid (GABA), are involved in and play an integral role in the complex etiology of schizophrenia. Although this thesis consists primarily of behavioural studies, we will briefly discuss dopamine and glutamate, with an emphasis on GABA.

1.1.3.1 Dopamine

The most prevalent theory of schizophrenia involves the neurotransmitter, dopamine. The initial DA hypothesis emerged in the 1950s with the discovery of antipsychotic drugs and their ability to increase the metabolism of dopamine in rodents (Laruelle, Kegeles, and Abi-Dargham 2003). Excess transmission of DA in the striatum was linked with the positive symptoms of schizophrenia (Laruelle, Kegeles, and Abi-Dargham 2003). *In vivo* Positron Emission Tomography (PET) imaging studies utilizing ^{18}F-dihydroxyphenyl-*L*-alanine (^{18}F-DOPA) tracers have demonstrated elevated uptake levels in the substantia nigra and striatum (STR) of schizophrenic patients (Molinaro et al. 2015; Howes et al. 2013). In addition, Single Photon Emission Computed Tomography (SPECT) and PET studies have shown that patients with schizophrenia display elevated synthesis and release of DA in the basal ganglia (Carlsson et al. 2001). Blockade of the DA-D2 receptor (D2R) and other DA receptors by use of antipsychotic drugs, ameliorated positive symptoms but not the negative and cognitive deficits associated with the disorder (Howes and Kapur 2009). These findings lent support to the idea that elevated DA contributes to manifestation of positive symptoms only, including hallucinations and delusions. More recent research, however, has shown that reduced density of the DA-D1 receptors and low DA activity in the prefrontal cortex (PFC) of patients with schizophrenia correlates with cognitive impairment and poor performance in tasks involving working memory (Thierry et al. 2000; Laruelle, Kegeles, and Abi-Dargham 2003; Tamminga and Holcomb 2005). Furthermore, treatment with

phencyclidine (PCP) has been shown to produce dopaminergic hypofunction in the PFC of monkeys (Jentsch and Roth 1999). Thus, the modern dopamine hypothesis postulates that the positive symptoms of schizophrenia are connected with DA hyperfunction in the STR and mesolimbic regions, while the negative and cognitive symptoms arise, in part, due to dopaminergic hypofunction in the mesocortical regions.

1.1.3.2 Glutamate

The glutamate hypothesis originates from findings that *N*-methy-D-aspartate (NMDA) receptor antagonists, including the drugs ketamine and PCP, induce symptomatic manifestations similar to that of schizophrenia, including social withdrawal, emotional blunting, and delusions (Javitt and Zukin 1991). NMDA hypofrontality is therefore implicated in the etiology of schizophrenia (Tamminga and Holcomb 2005; Goff and Coyle 2001). Increasing NMDA receptor activity with NMDA agonists like glycine and D-serine can alleviate symptoms in patients with schizophrenia (Laruelle, Kegeles, and Abi-Dargham 2003; Greengard et al. 1993).

Evidence for both hypoglutamatergic and hyperdopaminergic activity in schizophrenia is not exclusive, but has rather been shown to behave in a complementary manner. NMDA hypofunction has been found to induce a reduction in mesocortical dopamine transmission and subsequent increase in subcortical mesolimbic dopamine activity, trends which are consistent with the DA hypothesis of schizophrenia (Laruelle, Kegeles, and Abi-Dargham 2003; Molinaro et al. 2015). Synaptic alterations within the

PFC may cause sustained dysfunction of glutamate neurotransmission and cause the observed abnormalities in DA transmission (Laruelle, Kegeles, and Abi-Dargham 2003).

1.1.3.3 GABA

Both glutamatergic and dopamine projections converge on the dendritic spines of GABAergic medium spiny neurons in the striatum (Laruelle, Kegeles, and Abi-Dargham 2003). GABA is the principle inhibitory neurotransmitter of the mammalian nervous system, and its signalling is partly regulated by the enzymatic activity of two isoforms of glutamic acid decarboxylase (GAD). Dysregulation in GABAergic neurotransmission has been observed in the dorsolateral prefrontal cortex (DLPFC) of patients with schizophrenia (Lewis et al. 2013). In particular, levels of protein and mRNA expression of the 67 kDa isoform of GAD (GAD67) have been consistently found to be reduced in post-mortem DLPFC samples from patients with schizophrenia, relative to healthy controls. Expression of the other isoform, GAD65 (65 kDa), within the cortex is not altered in schizophrenia (Lewis et al. 2013). Similarly, levels of GAD67 mRNA are markedly reduced in DLPFC interneurons which express the calcium-binding protein, parvalbumin (PV) (Lewis et al. 2013; Glausier, Fish, and Lewis 2014). PV mRNA levels have also been shown to be reduced in schizophrenia, although the number of PV neurons in the DLPFC does not differ between subject groups (Lewis et al. 2013).

Activity of PV-containing interneurons is critical to top-down control of working memory and cognitive control by the DLPFC (Rotaru, Lewis, and Gonzalez-Burgos 2012). Based on the temporal binding hypothesis, neural synchrony in the millisecond

range is necessary for object representation, attention, and working memory maintenance (Rotaru, Lewis, and Gonzalez-Burgos 2012). Therefore, cognitive function is reliant on the formation of synchronized neuronal assemblies in the DLPFC.

Precise control of pyramidal cell firing within the DLPFC requires selective recruitment of GABA-mediated synaptic inhibition, resulting in rhythmic cortical activity at different oscillatory frequencies (Rotaru, Lewis, and Gonzalez-Burgos 2012). Of particular interest is the formation of gamma oscillations (30-80 Hz) within the DLPFC (Lewis et al. 2013; Glausier, Fish, and Lewis 2014). Gamma oscillations require strong inhibition of excitatory pyramidal cells via GABA-synthesizing PV basket cells (PVBC). Fast, synchronized neuronal activity in the gamma frequency is necessary for supporting working memory and cognitive control processes (Glausier, Fish, and Lewis 2014; Frankle et al. 2015). Indeed, patients with schizophrenia have demonstrated reduced activation of the DLPFC and lower power of prefrontal gamma oscillations when performing tasks requiring working memory (Glausier, Fish, and Lewis 2014; Rotaru, Lewis, and Gonzalez-Burgos 2012). Furthermore, decreasing excitation of PV neurons impairs gamma oscillations and working memory in mice, while increasing excitatory drive to these neurons induces oscillations (Rotaru, Lewis, and Gonzalez-Burgos 2012). Interestingly, longstanding evidence has also shown the STR to be involved with mediating complex cognitive functions including working memory and executive functioning (Simpson, Kellendonk, and Kandel 2010). Thus, if the STR helps to modulate

cognitive activity, reduced PV levels and GABA transmission may also be observed in this region.

Until recently, there has been no direct, *in vivo* evidence that altered GABA transmission within the cortex is linked with schizophrenia and the associated cognitive disturbances (Frankle et al. 2015). Using a novel PET paradigm, Frankle *et al.* (2015) were able to conduct *in vivo* measurements of GABA neurotransmission by measuring amount binding of [^{11}C]flumazenil, a radiotracer that binds to the benzodiazepine site of the $GABA_A$ receptor (Frankle et al. 2015). After acute treatment of tiagabine, a GABA membrane transporter (GAT1) blocker, GABA accumulates extracellularly. Elevated levels of extracellular GABA enhance $GABA_A$ receptor affinity for benzodiazepines, including [^{11}C]flumazenil (Frankle et al. 2015). Subsequent findings from the Frankle *et al.* (2015) study demonstrated that patients with schizophrenia displayed lower levels of [^{11}C]flumazenil binding throughout the brain, compared to healthy controls, as well as altered cognitive function, lending further support for reduced GABA neurotransmission in schizophrenia.

Together, these findings suggest that impaired gamma band synchrony and subsequent impairment of cognitive function in schizophrenia may result from reduced GABA synthesis and PV-induced calcium buffering within PVBCs. It is evident that hypofunctional GABA activity plays a role in the pathophysiology of schizophrenia.

1.1.3.4 GABA-Glutamate Interaction

Alterations of both GABA and glutamate levels have been shown to mimic the cognitive symptoms of schizophrenia. Synchronous, rhythmic neuronal firing between excitatory pyramidal cells and inhibitory, parvalbumin-containing interneurons in the cortex and hippocampus is a critical mechanistic property involved in maintaining normal cognitive function (Rotaru, Lewis, and Gonzalez-Burgos 2012).

Interestingly, systemic NMDA receptor (NMDAR) antagonism has been shown to disinhibit pyramidal cells, and therefore increase their firing within the PFC (Homayoun and Moghaddam 2007). Prolonged exposure to NMDAR antagonists decreases PV interneuron activity, and causes alterations to GABAergic markers, resulting in impairments that mimic those of schizophrenia (Rotaru, Lewis, and Gonzalez-Burgos 2012). From these findings, it was postulated that NMDAR antagonists act directly on PV interneurons to reduce their activity, indirectly causing disinhibition of pyramidal cell firing (Homayoun and Moghaddam 2007; Rotaru, Lewis, and Gonzalez-Burgos 2012).

1.2 Neurocircuitry

While neurotransmitters, including dopamine, glutamate, and GABA, have been individually implicated in the pathophysiology of schizophrenia, it is important to understand the interactions between these neurotransmitter systems and how their dysregulation contributes to the complex disorder.

In a normal, healthy brain, dopamine release from the substantia nigra (SN) into subcortical regions (caudate putamen/STR) results in GABAergic activity projecting from

the latter regions into the globus pallidus external (GPe). In what is known as the indirect (D_2; inhibitory) pathway, inhibition caused by GABA output to the GPe results in GABAergic output to the subthalamic nucleus (STN). Activity of GABA neurons in the STN modulates glutamate activation into the globus pallidus internal (GPi), which leads to GABA output from the GPi into the thalamus. Subsequent glutamatergic transmission from the thalamus to the cortex regulates glutamate release from the cortex back into the subcortical regions. The direct (D_1; stimulatory) pathway bypasses the GPe (**Figure 1A**) (Carlsson et al. 2001; Laruelle, Kegeles, and Abi-Dargham 2003; Carr and Sesack 2000).

In a schizophrenic brain, it is hypothesized that there is excess dopaminergic activity projecting from the SN into the STR, combined with increased subcortical dopamine D_2 receptor activity. DA hyperactivity in the subcortical regions results in elevated GABAergic output to the GPe and increased inhibition, therefore leading to a decrease in GABA output from the GPe to the STN. Subsequently, glutamatergic activity into the GPi is increased, causing greater GABA release into the thalamus, and decreased glutamatergic transmission back into the cortex (**Figure 1B**) (Carlsson et al. 2001; Laruelle, Kegeles, and Abi-Dargham 2003; Carr and Sesack 2000).

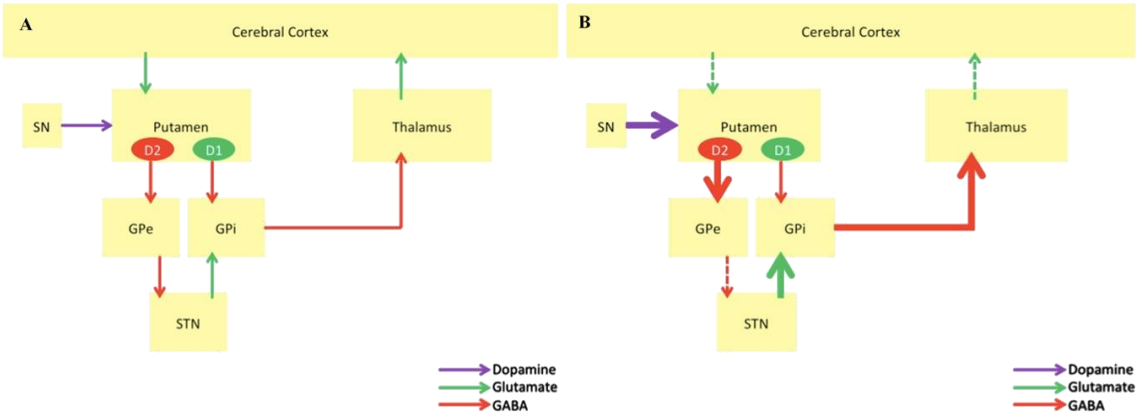

Figure 1: Oversimplified diagrams of the direct (D_1; stimulatory) and indirect (D_2; inhibitory) pathways. **A.** Diagram illustrates both these pathways in a normal brain. **B.** Diagram illustrates hypothesized neurocircuitry in a schizophrenic brain. Increases in dopaminergic activity (purple arrow) projecting into the putamen/striatum are observed, along with increased dopamine D2 receptor activity (bolded red arrow). This increase in subcortical dopaminergic activity results in increased GABAergic output to the GPe. Increased GABA-mediated inhibition to the GPe results in decreased GABA output to the STN and a subsequent increase in glutamatergic output from the STN into the GPi) Increased glutamate activation into the GPi results in increased GABA output from the GPi into the thalamus, and decreased glutamatergic transmission back into the cortex. In schizophrenia, overstimulation of the dopamine D2 receptor ties in closely with a decrease in glutamatergic transmission within the PFC. Figures modified from *Tan, 2014; Carlsson et al. (2001); Laruelle et al., 2003; Carr & Sesack, 2000).* GPe – globus pallidus external; GPi – globlus pallidus internal; SN – substantia nigra; STN – subthalamic nucleus.

1.3 Animal Models of Neurological Disorders

Our understanding of the mechanisms behind the complex, heterogeneous nature of neurological diseases, including schizophrenia, remains limited, thereby hindering the ability to develop novel drugs with improved therapeutic efficacy (Jones, Watson, and Fone 2011). When investigating these diseases, preclinical animal models offer a more rapid means of monitoring disease progression, and the ability to perform invasive monitoring of the neurological bases that underlie the cause of the disorder – both not possible in human patients (Jones, Watson, and Fone 2011). However, one of the main limitations of animal research stems from our attempts to quantify and assess uniquely human traits, including thoughts, hallucinations, and verbal learning (Powell and Miyakawa 2006).

In theory, all reliable and useful animal models should demonstrate a triad of face, predictive, and construct validities (Jones, Watson, and Fone 2011). Face validity refers to how phenotypically similar the animal model is when compared to the behavioural and physiological responses observed in human patients with the disorder. Predictive validity is an assessment of the expected pharmacological response, or lack thereof, to treatment by known therapeutics of the clinical disorder being modelled. Finally, construct validity, often the most difficult to establish in an animal model, refers to the replication of the theoretical neurobiological rationale and pathology (Jones, Watson, and Fone 2011). In an animal model of schizophrenia, it would ideally be expected to express positive, negative, and cognitive behavioural phenotypes of the disorder; attenuation of symptoms using

antipsychotic drugs such as olanzapine or haloperidol; and cortical glutamatergic hypofunction, and limbic dopamine dysregulation (**Figure 2**) (Jones, Watson, and Fone 2011).

The established animal models of schizophrenia can be classified into four different categories based on the induction method: 1) pharmacological; 2) lesion; 3) developmental; or 4) genetic manipulation (Jones, Watson, and Fone 2011). A majority of the models have been developed on the basis of the principle theory that dysregulation in dopamine signalling, and later glutamate dysfunction, was central to the pathophysiology of schizophrenia, resulting in models that replicate aspects of the positive symptoms of the disease. Fewer rodent models mimic the negative and cognitive phenotypes of schizophrenia (Jones, Watson, and Fone 2011).

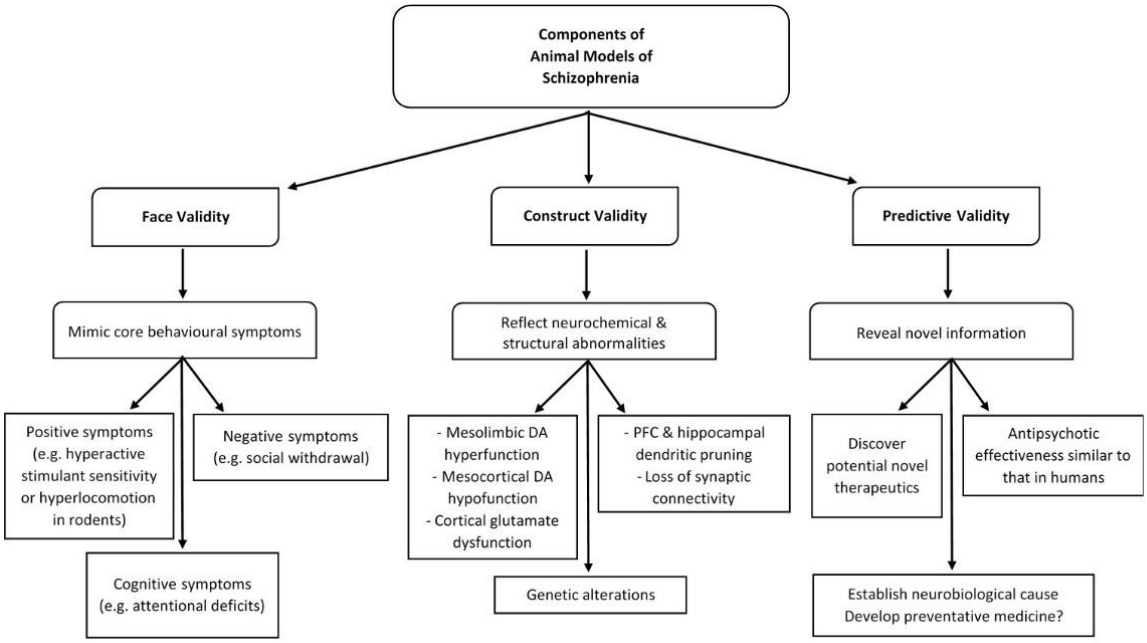

Figure 2: Components of Animal Models of Schizophrenia. Reliable animal models of schizophrenia should possess a triad of face, construct, and predictive validities. Face validity refers to how well the model mimics core behavioural symptoms of the disorder, including the positive, negative, and cognitive domains. Construct validity is in reference to neurochemical and structural abnormalities seen in the animal model, which reflect those abnormalities seen in human patients with schizophrenia. Such alterations can include dopaminergic hyperfunctionality, and glutamatergic dysfunction. The last component, predictive validity, often refers to the efficacy of antipsychotic use in attenuating symptoms presented in the animal model, to a similar extent of that in humans. Figure modified from Jones *et al.* (Jones, Watson, and Fone 2011).

1.3.1 Pharmacological Models of Schizophrenia

The two best characterized drug-induced models of schizophrenia are the amphetamine model and the PCP model. In the 1950s, amphetamine-induced psychosis was first noted, with clinical symptoms of auditory hallucinations and delusions reminiscent of the positive symptoms of schizophrenia (Jones, Watson, and Fone 2011). In rodents, acute and chronic amphetamine treatment resulted in the manifestation of a hyperactive state of motion, and long-term deficits in prepulse inhibition (PPI) (Featherstone, Kapur, and Fletcher 2007; Tenn, Fletcher, and Kapur 2005). However, amphetamine administration has not been shown to induce negative symptoms (social isolation) in both rodents and patients, while rodent cognitive impairment is region-specific following chronic injection (Fletcher et al. 2007; Featherstone, Kapur, and Fletcher 2007; Jones, Watson, and Fone 2011).

The PCP model of schizophrenia was developed based on evidence of dysregulation of glutamatergic signalling. A non-competitive NMDA receptor antagonist, PCP-treated individuals display several domains of symptoms akin to those seen in schizophrenia, including hallucinations and poverty of speech, representing the positive and negative symptoms, respectively (Javitt and Zukin 1991; Jones, Watson, and Fone 2011). Similarly, rodents receiving acute administration of PCP display hyperlocomotion, reduced startle response, and social withdrawal (Sams-Dodd 1998; Jones, Watson, and Fone 2011). Cognitive impairments, although transient, are also seen in PCP models, including attentional deficits as seen with use of the attentional set-shifting task (Rodefer

et al. 2008; Jones, Watson, and Fone 2011), impaired novel object recognition (McKibben et al. 2010; Jones, Watson, and Fone 2011), and delayed processing ability as measured using the five-choice serial reaction time task (Amitai and Markou 2009).

Both the amphetamine and PCP animal models of schizophrenia demonstrate face, construct, and predictive validities, and are considered to be reliable and valid to use in the preclinical study of the disorder. However, with increasing evidence to support additional causes of the underlying pathophysiology of schizophrenia, other models have been developed to represent these findings.

1.3.2 Lesion Models of Schizophrenia

Targeted lesion animal models have been developed with the attempt to better assess the neurodevelopmental and neurodegenerative hypotheses of schizophrenia (Marcotte, Pearson, and Srivastava 2001). Destruction of specific neuronal tissue is typically induced via excitotoxic agents, such as ibotenic acid, which can stimulate excitatory glutamate release, or act as direct glutamate receptor agonists (Marcotte, Pearson, and Srivastava 2001).

Adult rats with lesions to the PFC display increased locomotor activity, and reduced PPI response after apomorphine injections, among other phenotypes (Swerdlow et al. 1995; Marcotte, Pearson, and Srivastava 2001). Lesions induced in the adult hippocampus, specifically the ventral hippocampus (VH), result in increased DA-agonist-induced locomotor activity, and PPI deficits following apomorphine challenge; these behavioural changes are observed two weeks post-operation (Marcotte, Pearson, and

Srivastava 2001). No impairment to social interaction is observed in these models (Marcotte, Pearson, and Srivastava 2001). Although demonstrating face and predictive validities, the time point in which these lesions are induced limit their construct validity as a neurodevelopmental model of schizophrenia.

To further assess neurodevelopmental theories of schizophrenia, neonatal lesion models have been developed. Such animal models allow for the demonstration of delayed onset of symptoms, mimicking the clinical presentation of schizophrenia in humans (Marcotte, Pearson, and Srivastava 2001). Similar to the adult models, lesions in the PFC have resulted in post-pubertal increases in locomotor activity following amphetamine challenge. With far fewer neonate PFC lesion models developed, the full characterization of behavioural and biochemical changes has yet to be fully determined (Marcotte, Pearson, and Srivastava 2001). Instead, much of the neonatal lesion research focuses on the ventral hippocampus. The neonate ventral hippocampal (nVH) lesion model, developed in the early 1990s by the Lipska-Weinberger group, is induced by lesioning rat pups at post-natal day (PD) 7 using ibotenic acid (Lipska and Weinberger 1993; Jones, Watson, and Fone 2011). When tested at the pre-pubertal stage (PD 35), lesioned pups behaved similarly to control animals. However, delayed onset of hyperdopaminergic behaviours including increased locomotor activity, apomorphine-induced stereotypy, and impaired PPI response, was observed at the post-pubertal stage (PD 65) of development. Deficits in social behaviours appeared as early as PD 25 and persisted throughout development (Jones, Watson, and Fone 2011; Marcotte, Pearson, and Srivastava 2001;

Sams-Dodd 1998). Cognitive impairments have also been observed in this model, with lesions causing persistent impairments in spatial working memory tasks, including the eight-arm radial maze (8-ARM) paradigm (Jones, Watson, and Fone 2011). Behavioural changes, with the exception of social withdrawal and cognitive deficits, are attenuated after treatment with the antipsychotic, clozapine, suggesting that more research needs to be done to determine the predictive validity of the model (Jones, Watson, and Fone 2011; Marcotte, Pearson, and Srivastava 2001).

Despite showing moderate face, predictive, and construct validity, lesion models should be utilized cautiously with respect to novel drug development, as they reflect far greater neuronal damage than is seen in post-mortem brains of those diagnosed with schizophrenia.

1.3.3 Developmental Models of Schizophrenia

In general, developmental models of schizophrenia, including the neonatal lesion model, are difficult to produce due to maternal rejection of pups and premature pup death, resulting from extended periods of maternal separation (MS) during critical periods of development (Fatemi and Folsom 2009). Preclinical studies have suggested that early maternal separation - where animal pups are separated from their mother, but not littermates - may provide a model of early life stress that could result in the emergence of schizophrenia-like symptoms in adulthood (Rees et al. 2008; Anglin, Cohen, and Chen 2008; Ellenbroek, van den Kroonenberg, and Cools 1998; Bouet et al. 2011; Wang et al. 2015). Behavioural abnormalities included locomotor hyperactivity, decreased PPI, and

decreased spatial learning, all behaviours reminiscent of those resulting from various accepted pre-clinical animal models of schizophrenia (Anglin, Cohen, and Chen 2008; Ellenbroek, van den Kroonenberg, and Cools 1998; Wang et al. 2015). However, these behavioural phenotypes were not reproducible and/or observed between various MS models (Ellenbroek, van den Kroonenberg, and Cools 1998; Lehmann, Pryce, and Feldon 2000). It is of interest to note that despite minimal literature regarding clinical cases of MS and schizophrenia development, a study conducted by Hilgard and Newman (1963) found that certain populations of patients with schizophrenia experienced excess MS prior to reaching adulthood. Additional methods of inducing a preclinical neurodevelopmental model of schizophrenia involve maternal stress, malnutrition, infection or immune activation, or hypoxia (Jones, Watson, and Fone 2011).

Exposure to gestational and/or perinatal environmental insults has been closely associated with an increased risk of developing schizophrenia in humans (Jones, Watson, and Fone 2011). However, it is the timing, rather than the nature, of these early-life events that matters. The gestational MAM model, for example, is dependent on the time frame in which cortical neurogenesis occurs during gestation (Jones, Watson, and Fone 2011). When administered to pregnant dams, MAM, an anti-mitotic and anti-proliferative agent, selectively affects fetal brain structures undergoing the most rapid development, producing persisting anatomical and behavioural deficits in offspring. However, behavioural alterations seen in MAM offspring vary according to the gestational day in which MAM was administered to the dam (Jones, Watson, and Fone 2011).

The current working neurodevelopmental hypothesis postulates that a combination of exposure to adverse events early in life, and a genetic predisposition to schizophrenia, may lead to an altered pattern of neuronal development and neuro-connectivity that subsequently results in the presentation of a schizophrenic phenotype. Therefore, several of the described neonate models can also be combined with genetic interventions that could potentially expand the robustness and utility of the model.

1.3.4 Genetically Manipulated Models of Schizophrenia

Unequivocal evidence accumulated over the years has shown schizophrenia to be predominantly a genetic disorder (Jones, Watson, and Fone 2011). Twin studies demonstrate the heritability of schizophrenia to be approximately 80%, although there is no single genetic alteration responsible for its pathophysiology (Jones, Watson, and Fone 2011; Tamminga and Holcomb 2005). Through a number of genome-wide scans and meta-analyses, various candidate genes have been implicated in the etiology of schizophrenia. Most of the disrupted genes encode for proteins involved in neural plasticity, dopaminergic or glutamatergic function, and synaptogenesis (Jones, Watson, and Fone 2011). Numerous genetic models have been produced, with a majority of them developed on the basis of replicating observed changes in mRNA and protein expression in schizophrenia (Jones, Watson, and Fone 2011). Given the vast amount of models developed, for the purpose of this thesis, we will focus solely on the DISC1 gene and synapsin II.

The disrupted-in-schizophrenia 1 (DISC1) gene was one of the earliest genes implicated in the underlying etiology of schizophrenia, and is expressed early in development (Jones, Watson, and Fone 2011). Crucial to pre- and post-natal neuron development, DISC1 is involved with synaptogenesis, synaptic plasticity, and neuronal migration (Jaaro-Peled 2009; Jones, Watson, and Fone 2011). To investigate schizophrenia, different strains of transgenic mice have been produced containing inducible and/or DISC1 gene mutations, resulting in a partial loss of DISC1 function (Jaaro-Peled 2009). The behavioural and physiological effects of a DISC1 knockdown are disputed, however, among the models. Some, but not all, models display deficits in PPI attenuated by haloperidol and clozapine, locomotor hyperactivity, and slight deficits in working memory and sociability (Clapcote et al. 2007; Hikida et al. 2007; Pletnikov et al. 2008). Additionally, DISC1 knockdown mice have reductions in hippocampal dendritic complexity, and show decreased parvalbumin levels in the medial PFC (mPFC) and hippocampus – abnormalities consistent with cognitive impairment in schizophrenia (Jones, Watson, and Fone 2011). Genetic models involving synapsin II, another synaptic protein, will be discussed in the following section.

Based on the various models of schizophrenia that exist, it is suggested that genetic, environmental, and developmental factors may all act in a synergistic manner to determine the susceptibility of an individual in developing schizophrenia (Tseng et al. 2007).

1.4 Synapsins

Similar to the DISC1 gene, the synapsin group of proteins have been implicated in schizophrenia (Südhof 2004; Owen, O'Donovan, and Harrison 2005; Harrison and Owen 2003). The first pre-synaptic vesicle-associated family of proteins to be discovered, synapsins are evolutionarily conserved and the most abundant of neuron-specific phosphoproteins. Mammalian synapsins are encoded by 3 genes: synapsin I, synapsin II, and synapsin III, which are located on chromosome X, 3 and 22, respectively (Hilfiker et al. 1999). Synapsins are important in a variety of pre-synaptic regulatory functions, including synaptogenesis, synapse function, synapse maintenance, and synaptic plasticity (Südhof et al. 1989; Greengard et al. 1993; Cesca et al. 2010; Gitler 2004; Bogen et al. 2006). They can be found in both the central and peripheral nervous systems, and tend to be localized to synaptic vesicles. In particular, synapsins can be predominantly found tethered to pre-synaptic vesicles at the reserve, or distal, pool of vesicles, away from the plasma membrane. Synapsins can also be associated, to a lesser extent, with docked vesicles or the proximal pool (**Figure 3**) (Cesca et al. 2010).

Many nerve terminals within the central nervous system contain at least one synapsin isoform, but the various forms of synapsins exhibit different expression patterns (Cesca et al. 2010). Synapsins I and II are typically found in mature synapses, with expression coinciding with the time of progenitor cell commitment to differentiated neurons, and is particularly high during synaptogenesis. Synapsin III is often attributed to developing synapses during embryogenic development, with declining overall expression

throughout adulthood (Cesca et al. 2010; Südhof 2004; Greengard et al. 1993; Hilfiker et al. 1999). The different expression patterns suggest a different function of each synapsin protein. Synapsins I and II are both highly involved in neurite outgrowth, synapse formation, and synapsin maturation. Knockout models of synapsin I, II, or both, have demonstrated that synapsin I and II are critical for neurotransmission and maintaining the total number of pre-synaptic vesicles, especially vesicles within the reserve pool. However, a synapsin II knockout animal was phenotypically more severe than the synapsin I knockout animal, with a double knockout of I and II causing the most drastic changes including a 50% reduction in synaptic vesicle numbers (Molinaro et al. 2015; Südhof 2004). A synapsin III knockout model does not dramatically influence pre-synaptic morphology (Cesca et al. 2010; Greengard et al. 1993; Kao et al. 1999). Synapsin III plays a greater role in neuronal development, and is strongly involved with neuronal proliferation and survival, and axonal specification (Cesca et al. 2010; Ferreira et al. 2000).

Functional variances amongst synapsin proteins likely result from structural differences in the gene products. The short N-terminus (approximately 20 residues), as well as the central domains (A-C), are conserved between all three synapsin genes. Thus, any structural variation between isoforms is localized to the C-terminus (Molinaro et al. 2015). A conserved phosphorylation site for protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase I (CaM kinase I) is found in domain A, while domain B functions to link the N-terminus with domain C (Südhof 2004). There are also a

number of variable domains found in the synapsin family, although only seven have currently been identified (Molinaro et al. 2015; Südhof 2004; Hilfiker et al. 1999). Domain E is conserved amongst all "a" isoforms, and through interactions with cytoskeletal elements, is thought to play a role in the clustering of synaptic vesicles and maintenance of the reserve pool. Domain E also is involved in the formation of synaptic dimers, where "a" isoforms may dimerize to bring weaker targeting isoforms, such as Ib, to synaptic terminals (Molinaro et al. 2015; Fornasiero et al. 2010). Domains D, and F-J are poorly conserved between the isoforms, and are specific to each synapsin gene (**Figure 4**) (Molinaro et al. 2015).

Of the three mammalian synapsin genes, synapsin II has been most strongly associated with schizophrenia. The synapsin II gene is located on chromosome 3p2ss5, which was suggested to be a region of vulnerability for schizophrenia (Cesca et al. 2010; Dyck and Mishra 2011). Alternative splicing of the synapsin II gene produces two isoforms: synapsin IIa and synapsin IIb (**Figure 4**) (Cesca et al. 2010). Support for the association of synapsin II and schizophrenia has been found in genetic linkage studies, where positive associations have been found between schizophrenia development and single-nucleotide polymorphisms of the synapsin II gene in affected families of various genetic backgrounds, including a Han Chinese population, and Northern European ancestries (Chen et al. 2004a; Chen et al. 2004b; Saviouk et al. 2007; Lee et al. 2005). Several studies have provided additional data to support a relationship between synaptic dysfunction and the onset of schizophrenia. In particular, microarray analysis of post-

mortem tissue samples of patients suffering from schizophrenia have shown significant reductions in PFC synapsin II mRNA and synapsin IIa and IIb protein expression relative to normal control subjects (Mirnics et al. 2000; Vawter et al. 2002; Guest et al. 2010; Tan et al. 2014). Only mRNA of the synapsin IIa isoform, however, was up-regulated with lifelong use of antipsychotic agents (Tan et al. 2014).

Further evidence to substantiate the involvement of synapsin II dysfunction in the etiology of schizophrenia can be found in work utilizing preclinical animal models. Experiments conducted with gene knockdown technology (either by antisense oligonucleotides or SiRNA) in rodent subjects have revealed behavioural phenotypes similar to those resulting from various accepted preclinical animal models of schizophrenia (Dyck et al. 2011). Knockdown of synapsin II in the adult rat mPFC, but not in the hippocampus or cerebellum, resulted in similar behavioural phenotypes, including deficits in sensorimotor gating/PPI, locomotor hyperactivity, social withdrawal, and deficits in the 8-ARM paradigm, suggesting a specific association between the mPFC and synapsin II expression (Dyck et al. 2011). Furthermore, immunoblotting results demonstrated a reduction in glutamate and GABA signalling within the mPFC resulting from synapsin II knockdown. Specifically, reductions in the protein concentration of glutamate vesicular transporters (VGLUT-1, VGLUT-2), and vesicular GABA transporter (VGAT) were observed (Dyck et al. 2011). These findings suggest that synapsin II is involved in vesicular loading, and that deficits in synapsin II protein expression may

result in reduced neurotransmitter uptake and release. Thus, the study of synapsin II and its isoforms may be imperative to understanding the pathophysiology of schizophrenia.

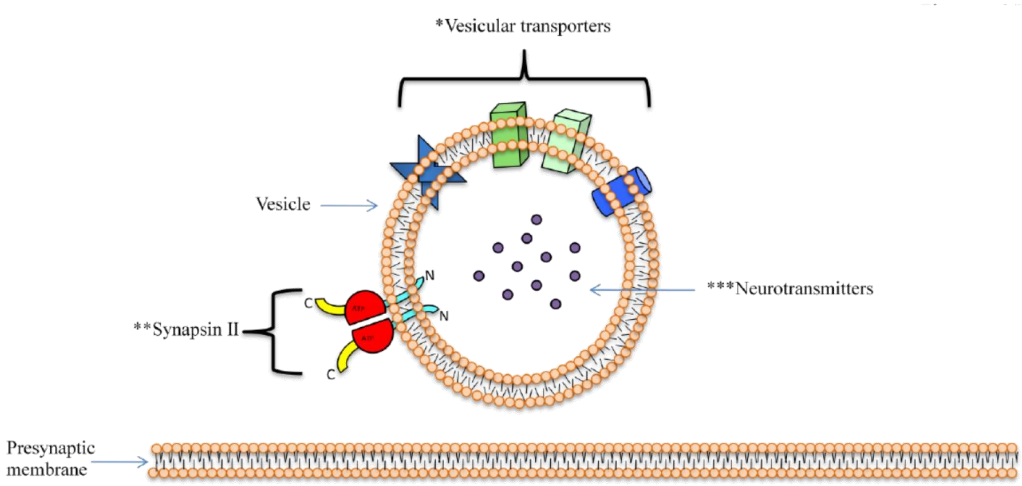

Figure 3: Oversimplified illustrated depiction of a synaptic vesicle and associated pre-synaptic membrane. At the pre-synaptic cleft, synapsins help tether synaptic vesicles to the reserve pool; shown here is the synapsin II protein. Specific vesicular transporters also associate with synaptic vesicles to help load their respective neurotransmitters into the vesicles at pre-synaptic terminals.

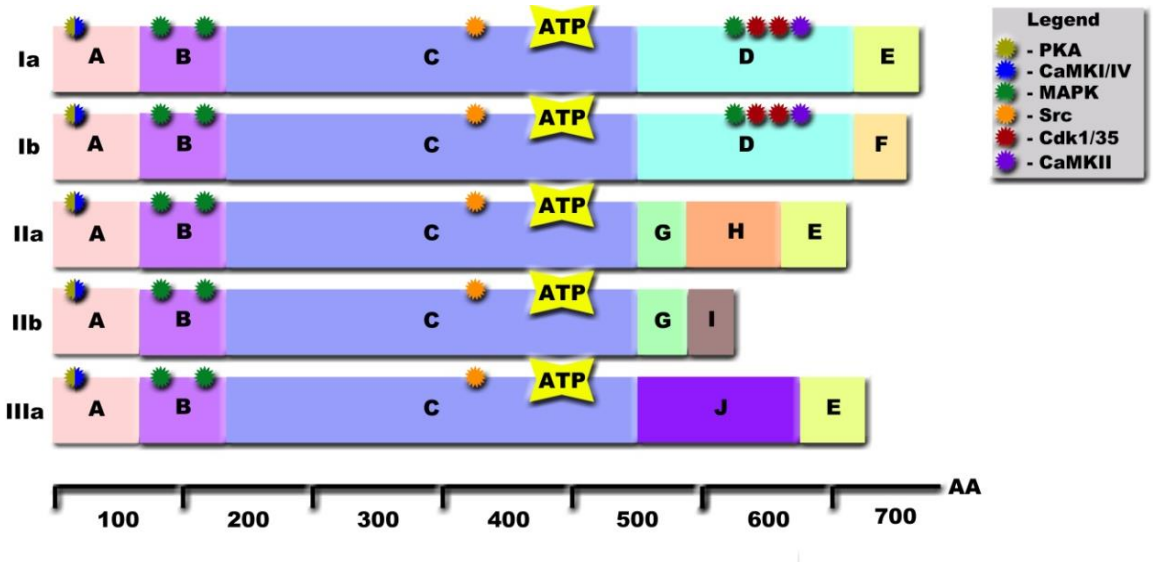

Figure 4: Illustration demonstrating the various mammalian synapsin gene products, in particular, synapsin IIa and IIb. Various domains are indicated, as well as known phosphorylation sites (●) and their respective kinases (colour coded). Domains A-C are conserved amongst the three synapsin genes. Domain E, which is shared between all "a" isoforms, is implicated in phospholipid vesicular clustering and dimerization. Domains D, and F-J are poorly conserved amongst synapsin and are specific to each gene product. Modified from: Hilfiker *et al.*, Fornasiero *et al.*, and Monaldi *et al.* (Hilfiker et al. 1999; Fornasiero et al. 2010; Monaldi et al. 2010).

1.5 Antisense Oligonucleotides

The use of antisense technologies to inhibit expression of a target gene was first discovered by Stephenson and Zamecnik (1978). Antisense (AS) oligonucleotides have since been developed for target validation and potential therapeutic purposes (Kurreck 2003). Oligonucleotides function in a sequence-specific manner, binding to their complementary target mRNA, preventing translation of mRNA into protein via two different mechanisms (Kurreck 2003). The first mechanism involves activating RNase H, which cleaves the RNA moiety of a DNA-RNA complex, therefore resulting in the degradation of the target gene mRNA. Most AS oligonucleotides are designed to activate RNase H. Oligonucleotides that do not induce RNase H cleavage can instead inhibit translation via steric blockade of the ribosome. By targeting AS oligonucleotides to the 5'-terminus of the mRNA strand, the binding and assembly of translational machinery is prevented (Kurreck 2003).

Oligonucleotides are typically 15-20 nucleotides long, and are designed to avoid significant homology with other mRNA sequences. To prove that observed effect(s) are due to AS-induced knockdown of the target mRNA, a number of negative control oligonucleotides are used. Mismatch oligonucleotides differ from AS sequences in only a few positions, while sense oligonucleotides are an inverted sequence of the target mRNA (Kurreck 2003). Of major concern when utilizing AS oligonucleotides is the stabilization of the sequence, as unmodified oligonucleotides are rapidly targeted and degraded by nucleases (Kurreck 2003). To circumvent this problem, several chemical modifications

have been used to stabilize AS sequences, resulting in different ‘generations’ of AS oligonucleotides.

First-generation AS oligonucleotides consist of phosphorothioated (PS) oligonucleotides, and are the most widely used modification of DNA analogs (Kurreck 2003). To prevent nuclease degradation, one non-bridging oxygen atom in the native phosphodiester bond is replaced with a sulphur atom. PS oligonucleotides have a half-life of 9-10 hours in human serum, compared to the 1 hour half-life of an unmodified oligonucleotide (Kurreck 2003). In addition to enhanced nuclease resistance, PS oligonucleotides are capable of activating RNase H and carry negative charges for cell delivery. However, there is the risk of cellular toxicity when using these first-generation sequences. PS oligonucleotides have been shown to bind to heparin-binding proteins, and inhibit the clotting cascade (Kurreck 2003). Several studies have demonstrated that chronic infusion of fully PS-modified oligonucleotides into rodents produces localized necrosis of brain tissue and severely affects animal health (Widnell et al. 1996; Guzowski et al. 2000). To circumvent these issues, oligonucleotides that were PS-modified only on the terminal base pairs, or on the last three base pairs at both the 5’- and 3’-ends, have been utilized.

Second- and third-generation AS nucleotides have been developed over the years to solve some of the problems associated with the first-generation, PS-modified oligonucleotides. Second-generation nucleotides contain alkyl modifications at the 2’ position of the ribose. Sequences comprised of these altered sugars are less toxic than PS-

modified DNA, although they cannot recruit RNase H, and therefore must induce their AS effect by steric hindrance of translational machinery (Kurreck 2003; Chan, Lim, and Wong 2006). Similarly, third-generation AS nucleotides also cannot induce RNase H cleavage of target mRNA, but rather, function generally by blocking ribosomal attachment and assembly. This group of AS agents is comprised of a vast number of differently modified nucleotides including peptide nucleic acids and morpholino oligonucleotides (Kurreck 2003; Chan, Lim, and Wong 2006). While several third-generation nucleotides have demonstrated high AS potency and low toxicity *in vivo*, the high costs of the DNA sequences make them difficult to utilize on a broad spectrum. For the purpose of this thesis, first-generation AS oligonucleotides were chosen due to their established knockdown effects and cost-efficient prices.

2. OBJECTIVES & RATIONALE

The overall objective of this project was to further investigate the efficacy of synapsin II antisense oligonucleotides, and other confounds, in the manifestation of schizophrenia-like abnormalities, and its effects on striatal parvalbumin expression. This objective was pursued via the combination of behavioural, imaging, and immunochemistry techniques.

2.1 Examining the effect of sub-chronic post-natal maternal separation on the development of schizophrenia

Previous experiments, conducted during my undergraduate studies, investigated a post-natal, selective synapsin II knockdown, and its effects on the developmental time course of schizophrenia manifestation. It was hypothesized in that experiment, that pups receiving synapsin II knockdown via AS technology would display schizophrenic-like behavioural abnormalities throughout development, relative to the negative controls: artificial cerebral spinal fluid (aCSF) and synapsin II sense oligonucleotide treated rats. Although promising results were observed from these studies, some behavioural discrepancies were also noted between the 3 treatment groups, notably that our negative controls also displayed schizophrenia-like tendencies in certain paradigms (**Figure 5**). It is unlikely that these unanticipated variances were a result of the surgical procedure itself, as this procedure has been perfected within our lab over several years. Thus, there may have been other confounding factors involved in this neonatal synapsin II model.

In the process of inducing the neonate synapsin II knockdown, our model required separating pups from their mothers for 4 hours per day, for a 9 day period (accounting for surgeries, recovery and infusions) during early development. Previous preclinical studies have suggested that early maternal separation may act as an early life stressor that could result in the emergence of schizophrenia-like symptoms in adulthood, although our particular MS regimen has never been studied before (Rees et al. 2008; Anglin, Cohen,

and Chen 2008; Ellenbroek, van den Kroonenberg, and Cools 1998; Bouet et al. 2011; Wang et al. 2015).

Therefore, the objective of this particular study was to examine the effects of repeated 4-hour long MS periods, during PD 14-23, on Sprague Dawley rat pups, to eliminate MS as a confound contributing to the manifestation of the observed behavioural variances in our neonate synapsin II model. Based on existing research, it was hypothesized that (1) sub-chronic maternal separation is not sufficient to induce changes in animal behaviour at pre-puberty and post-puberty; and (2) synapsin II protein levels will not vary amongst maternally separated and control rodents throughout development.

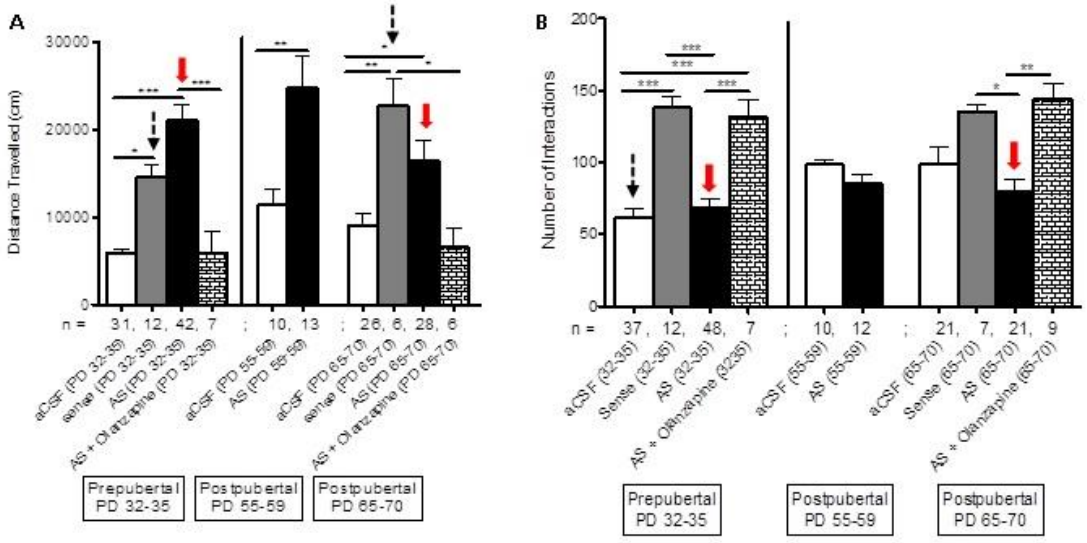

Figure 5: Behavioural and biochemical results obtained from preliminary neonate synapsin II knockdown studies conducted in our laboratory. Solid red arrows (⬇) indicate schizophrenia-like abnormalities observed in this preclinical model. Dashed arrows (⇣) indicate discrepancies from hypothesized results. **A**. Synapsin II AS animals demonstrated significantly increased locomotor activity over 180 minutes, relative to aCSF rats. However, sense rats also displayed significant hyperlocomotion relative to aCSF rats. The latter findings were unanticipated, as sense oligonucleotide treatment should ideally act as a negative control. **B**. Synapsin II AS rats displayed significant reductions in the total number of social interactions, as was also unexpectedly observed in aCSF rats at pre-pubertal testing. Graphs from Tan 2014.

2.2 Investigating cortical metabolic activity in an adult synapsin II knock-down rodent model, using fMRI/PET imaging

In schizophrenia, decreased metabolic activity in the PFC during cognitive activation has been a consistent finding (Floresco et al. 2006; Wolf et al. 2002). Several human imaging studies have employed PET imaging using fluorine-18 fluorodeoxyglucose ([^{18}F]FDG) to measure local cerebral glucose utilization in patients with schizophrenia. Glucose utilization is directly related to neuronal activity. Findings from these studies demonstrated absolute or relative metabolic hypofunction within certain brain regions, including the PFC, known to have altered neurochemical deficits in schizophrenia (Wolf et al. 2002; Birrell and Brown 2000).

In preclinical models of schizophrenia, similar findings have also been observed. Chronic PCP administration in rats resulted in schizophrenia-like behavioural abnormalities and also produced metabolic hypofunction (Rodefer et al. 2008). However, acute administration of MK-801, a non-competitive NMDAR antagonist, at 0.5 mg/kg, resulted in region specific metabolic hyperfunction (Daya et al. 2014). Therefore, the aim of these experiments was to determine whether mPFC synapsin II knockdown results in cortical hypo- or hyperfunction. Specifically, it was postulated that a site-specific knockdown of synapsin II would lead to 1) cortical hyspofrontality and 2) reduced PV expression in the striatum.

2.3 Examining biochemical and behavioural abnormalities induced by selective synapsin II knockdown in the adult rat, using partially phosphorothioated synapsin antisense sequences

The use of antisense oligonucleotide technology has been demonstrated in previous experiments from our lab to produce a specific, sustained decrease in synapsin II levels in the mPFC (Dyck et al. 2011). To prevent nuclease-mediated degradation of the oligonucleotides, fully phosphorothioated sequences were used. In our recent studies, two-week infusion of complete PS-modified oligonucleotides produced localized tissue damage, visible upon sacrifice and brain tissue extraction (unpublished). Similar toxic effects have been noted in other studies using fully PS-modified AS sequences to knockdown the gene of choice (Guzowski et al. 2000; Widnell et al. 1996). In those studies, researchers switched to chimeric phosphorothioate/phosphodiester oligonucleotide sequences, containing PS bonds on the three terminal bases of both the 5'- and 3'-terminals, with phosphodiester internal linkages (Guzowski et al. 2000; Widnell et al. 1996). Less toxic than completely phosphorothioated oligonucleotides, these partially modified sequences have been shown to still retain their biochemical specificity and efficacy (Widnell et al. 1996; Guzowski et al. 2000; Hooper, Chiasson, and Robertson 1994).

Based on the success of those results, the specific aims of this objective were to examine the effectiveness and toxicological profile of a partially phosphorothioated synapsin II antisense sequence, in a selective synapsin II knockdown model of

schizophrenia in the adult rat. In particular, it was hypothesized that: (1) selective knock-down of synapsin II using a chimeric antisense oligonucleotide sequence disrupts sensorimotor gating and induces locomotor hyperactivity, both one-week and two-weeks post-surgery; (2) protein expression levels of synapsin II decrease locally within the mPFC two-weeks post cannula and pump implantation; (3) levels of parvalbumin protein expression decreases in the striatum; and (4) no site-specific tissue damage or necrosis will be incurred.

2.4 Significance

Extensive research over the years has yielded effective treatments for schizophrenia in the form of antipsychotic drugs, yet these therapeutic options only serve to manage the symptoms of the disorder. Continued investigation into the underlying mechanisms of schizophrenia will aid our understanding of the disorder and help promote the development of novel and better targeted therapeutics. Disruptions in synapsin II gene expression and neurotransmitter systems within the mPFC have been linked with the pathophysiology of schizophrenia, as demonstrated from previous studies conducted in our laboratory (Dyck et al. 2011). Furthermore, the neurodevelopmental role of synapsin II in schizophrenia has also been investigated in our lab, with results lending more support to the involvement of impaired synapsin II in the etiology of the disorder. Despite these findings, more research is needed in order to establish the site-specific knockdown of synapsin II in the mPFC as a new animal model of schizophrenia.

Evidence from this thesis contributes to furthering our progress towards developing novel adult and neonate animal models of schizophrenia via knockdown of synapsin II. Our neurodevelopmental model requires a time-sensitive insult, but is also affected by confounding factors that may unwillingly induce the manifestation of schizophrenia. My research has demonstrated that, with respect to our particular model, early-life maternal separation does not result in schizophrenic-like behavioural and physiological abnormalities, providing additional support to the idea that synapsin II is critical to normal neural development. In addition, I have found that partial modification of our synapsin II antisense oligonucleotides produces a safer toxicological profile than fully modified sequences do in our adult model, while maintaining the ability to reduce synapsin II protein expression in the mPFC. For the first time, I observed decreased parvalbumin levels within the STR in synapsin II knockdown animals, further implicating striatal abnormalities in cognitive dysfunction, and lending support to the GABAergic hypofunctionality hypothesis.

Ultimately, this evidence helps clarify whether reductions of the neuronal phosphoprotein, synapsin II, are a causal element in schizophrenia. Improved understanding of the underlying pathophysiology of the disorder forms the basis for the development and use of novel, more specifically targeted and efficacious therapeutic interventions, such as gene therapy, in the treatment of schizophrenia, and thus having broad clinical implications for patients around the world.

3. METHODOLOGY

3.1 Animals

Three cohorts of animals were utilized during the course of this thesis. All animals were housed in the McMaster University Central Animal Facility (CAF) and cared for in compliance with the Canadian Council of Animal Care guidelines. Rodents were housed in standardized cages in a room maintained at 22^{o}C and kept on a 12 hour reverse dark/light cycle, beginning at 7am/7pm. All cohorts of animals were allowed a one-week acclimation period to their new surroundings and were handled by the experimenters during this time.

3.1.1 Objective 1: Maternal Separation Study

Three pregnant female Sprague Dawley rats at 13-15 days gestation were commercially obtained from Charles River, Quebec. The dams were housed individually and provided with *ad libitum* access to water and a high-fat diet. Each dam gave birth to a litter of pups after a gestational period of 21 days (n_{total} = 36 pups). Pups were left to nurse with their mother until being individually weaned on PD 24, at which point they were given *ad libitum* access to water and food.

3.1.2 Objective 2: Adult Imaging Study

In this study, 11 male Sprague Dawley rats were obtained from Charles River, Quebec at a weight range of 250-300 grams. Animals were housed individually upon arrival to the CAF and given *ad libitum* access to water. Beginning one week post-arrival, rodents were restricted to 15 grams of food pellets per day. The animals were fed

consistently around 4pm each day. Individual body weight was recorded every week to ensure that the animals did not lose more than 85% of their initial pre-restriction body weight.

3.1.3 Objective 3: New Oligonucleotide Study

Another 12 male Sprague Dawley rats, between 250-300 grams, were obtained from Charles River, Quebec for the last study. Animals were housed individually upon arrival and given *ad libitum* access to food and water for the duration of the study.

3.2 Maternal Separation (Objective 1)

To mimic the separation periods experienced by synapsin II knockdown pups (Tan 2014), a group of pups (n = 18) were removed from their respective mothers for 4 hours a day between PD 14-15, and PD 17-23 for a total of 36 hours of maternal separation. Separated pups were kept with their littermates in a cage placed away from any possible exposure to their mother. Pups experiencing maternal separation were returned to their respective cages at the end of the 4 hour period. Non-separated rats (n = 18) remained in their home cage with their respective mothers. This process was repeated for the remaining 8 days. All rodents were individually weaned on PD 24 and left to mature until behavioural and biochemical testing at pre-pubertal (PD 32-35) and post-pubertal (PD 65-69) stages.

3.3 Antisense Sequences and Preparation (Objectives 2 & 3)

Synapsin II knockdown within the adult rat mPFC was induced via antisense sequences designed to block cellular mRNA and inhibit translation of synapsin II **(Tables 1-2)**. Sequences were synthesized and purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA).

With respect to the second objective (otherwise known as the adult imaging study), every nucleotide within the sequences contained a PS cap to prevent nuclease degradation (**Table 1**) (Lebedeva and Stein 2001). For the second cohort of rodents (Objective 3), the first and last three nucleotides at the 5'- and 3'- ends of each sequence, respectively, were phosphorothioated **(Table 2)**. The remaining nucleotides were left uncapped and linked via phosphodiester bonds. The sense oligonucleotide sequence for the coding region of synapsin II served as a negative control in both cohorts, and did not bind to the target mRNA sequence (Dyck et al. 2011; Kurreck 2003).

All oligonucleotides were dissolved in nuclease-free water (IDT, Coralville, IA, USA) to a final concentration of 1 mM. The solution was then filtered through sterile Acrodisc® 25 mm Syringe Filters with 0.2 µm Supor® Membrane (Pall Corporation, Ann Arbor, MI, USA). The filtered solutions were stored at -20^{o}C until required.

OBJECTIVE 2: Adult Imaging Study	
	Nucleotide Sequence
Antisense Sequence 1	5’ - A*G*T*T*C*A*T*C*A*T*C*T*G*G*C*T*T*G*A*G*G*G*A - 3’
Antisense Sequence 2	5’ - C*G*A*C*C*A*A*A*G*G*G*T*G*G*T*C*C*G*C*G*T*C*T*C - 3’
Sense	5’ - T*C*C*C*T*C*A*A*G*C*C*A*G*A*T*G*A*T*G*A*A*C*T - 3’

Table 1: Nucleotide composition of fully modified synapsin II antisense and sense oligonucleotide sequences. Each nucleotide was capped with phosphorothioates to prevent *in vivo* nuclease destruction. The two synapsin II AS sequences overlap with one another to provide a complete knockdown of the synapsin II gene. The sense oligonucleotide is an inverted sequence of the target mRNA, designed to prevent binding with mRNA, and therefore serving as a negative control for the oligonucleotide technology.

OBJECTIVE 3: New Oligonucleotide Study	
	Nucleotide Sequence
Antisense Sequence 1	5’ - A*G*T*TCATCATCTGGCTTGAG*G*G*A - 3’
Antisense Sequence 2	5’ - C*G*A*CCAAAGGGTGGTCCGCGT*C*T*C - 3’
Sense	5’ - T*C*C*CTCAAGCCAGATGATGA*A*C*T - 3’

Table 2: Nucleotide composition of partially modified synapsin II antisense and sense oligonucleotide sequences. The first and last three nucleotides at the 5’- and 3’- end of each sequence were capped with phosphorothioates to prevent *in vivo* nuclease destruction. The two synapsin II AS sequences overlap with one another to provide a complete knockdown of the synapsin II gene. The sense oligonucleotide is an inverted sequence of the target mRNA, designed to prevent binding with mRNA and therefore serving as a negative control for the oligonucleotide technology.

3.4 Pump Preparation (Objectives 2 & 3)

In both adult cohorts, each rodent received a bilateral infusion of solution into the mPFC, requiring two mini-pumps per rat. Each pump had a reservoir of 200 µl and a flow rate of 0.5 µl/hour, with a duration of two weeks (ALZET® Osmotic Pumps, DURECT Corporation, Cupertino, CA, USA). Pumps were filled an hour before surgeries using sterile technique according to the guidelines provided by ALZET® Osmotic Pumps.

In the first cohort of surgically-treated rats, animals were either administered: 1) sense oligonucleotides (n = 5); or 2) a cocktail solution of AS sequences #1 and #2 (n = 6; Table 2). Regarding the second cohort of adult rats, animals were assigned to one of four groups: i) aCSF (n = 2); ii) sense oligonucleotide (n = 5); iii) AS sequence #1 (n = 2; Table 3); or iv) a cocktail solution of AS sequences #1 and #2 (n = 2; Table 3). When combined, the solution of both AS sequences should result in a complete knockdown of the synapsin II gene. Synapsin II AS sequence #1 was designed to knockdown the synapsin IIa isoform only.

3.5 Surgery (Objectives 2 & 3)

Animals were anaesthetized with gaseous anesthetic (Isoflurane, Pharmaceutical Partners of Canada Inc, Richmond Hill, ON) and mounted on a stereotactic apparatus (David Kopf Instruments, Tujunga, CA). Using a scalpel, a midline incision was made between the eyes extending to the nape of the neck, exposing the animal's skull. Each rat had two guide cannulae implanted (ALZET® Osmotic Pumps, DURECT Corporation, Cupertino, CA, USA) into the mPFC (coordinates relative to bregma: +2.7mm A/P,

±1.5mm M/L, and -3.0mm D/V) (Schneider and Koch 2005). The filled pumps were attached to the guide cannulae via plastic tubing prior to cannulae implantation. Using a scapula, two pockets were made under the animal's skin, extending past each shoulder blade, where the pumps were inserted into the pockets. The incision was closed using 9mm wound clips (ALZET® Osmotic Pumps, DURECT Corporation, Cupertino, CA, USA), and VetBond (tissue adhesive). Following immediate recovery from surgery, rats were returned to their home cage and monitored for five consecutive days. During this time, rats were given *ad libitum* access to food and water during recovery, and body weight was assessed daily. Staples were removed from each rat ten days post-surgery.

3.6 Histology

To visually confirm successful infusion into the mPFC, the following histological approach was used. Evans Blue dye (2%, Sigma Aldrich) was dissolved in 0.9% saline solution. Pumps were filled with 200 µL of dye as per the methodology described in Section 3.4. The dye was bilaterally infused into the mPFC at the aforementioned coordinates in three Long Evans discard rats obtained from the McMaster CAF. The animals were sacrificed one week following surgery via rapid decapitation and the area of dye diffusion in coronal sections was visually captured using a standard point-and-shoot camera.

3.7 Behavioural Testing

Each of the three cohorts of animals utilized in this thesis were subject to behavioural testing, as described below.

3.7.1 Maternal Separation (Objective 1)

Behavioural testing for all pups (n = 36) was performed at pre-pubertal (PD 32-36) and post-pubertal time-points (PD 65-70) to assess for the presence of schizophrenia-like behavioural abnormalities. Due to the nature of this study, baseline behavioural testing was not performed. These animals were tested on the following behavioural paradigms: prepulse inhibition, locomotor activity, social interaction, and the novel object recognition test (NORT).

3.7.2 Adult Synapsin II Knockdown - Fully Phosphorothioated Oligonucleotides (Objective 2)

This cohort of animals (n = 11) was tested on the eight-arm radial maze, a reliable measure of cognitive deficits in a synapsin II model of schizophrenia (Dyck et al. 2011). Habituation and training for the 8-ARM paradigm took place three weeks prior to surgeries. Rats also performed baseline testing on prepulse inhibition before surgeries to determine treatment groups. Following recovery from surgeries, each animal underwent eight testing sessions of the 8-ARM over the course of one week. A single post-surgery PPI test was also performed during this one week period.

3.7.3 Adult Synapsin II Knockdown - Partially Phosphorothioated Oligonucleotides (Objective 3)

Behavioural testing for these animals (n = 12) was performed both one-week and two-weeks post-surgery. Baseline behavioural testing was performed several days before surgery and results were used to determine treatment groups. The following behavioural paradigms were used to assess for development of schizophrenia-like abnormalities: prepulse inhibition and locomotor activity.

Details for all behavioural tests utilized are listed below:

3.7.4 Prepulse Inhibition

PPI is a measure of sensorimotor gating ability, referring to the ability to "gate out" excess stimuli from awareness in order to selectively focus attention to salient environmental aspects (Braff and Geyer 1990). Deficits in sensorimotor gating have been well established in patients diagnosed with schizophrenia, and are consistently observed in established animal models of the disorder (Dyck et al. 2011; Braff, Geyer, and Swerdlow 2001). The PPI test assesses reflex startle response to a brief acoustic stimulus, also known as a 'prepulse' (68 dB, 71dB, 77dB), presented prior to a 'startle' pulse (120 dB). The prepulse stimulus inhibits the startle response to the stronger pulse stimulus. Healthy, control animals demonstrate greater percent inhibition resulting in a reduced startle response, relative to animals with a schizophrenic phenotype.

Three days prior to testing, animals were given 30 minutes of habituation time to the testing room, and 20 minutes of habituation to the PPI chambers. On the day of testing, rats were allowed 30 minutes to acclimatize to the room. Rodents were placed in a clear Plexiglas cylinder which was then put into the PPI chamber. Prepulse and startle stimuli were presented to the rats via a microphone mounted within the chamber, directly above the cylinder. A piezoelectric accelerometer located directly below the cylinder captured startle responses, and subsequently, PPI responses were recorded using the SR-Lab Startle Response System (San Diego Instruments, San Diego, CA, USA). Methods were adapted from Braff *et al.* and Geyer *et al.* (Braff and Geyer 1990; Geyer et al. 1990).

3.7.5 Locomotor Activity

Hyperlocomotor activity is a well-established measure of the positive symptoms of schizophrenia among preclinical animal models of the disorder. Although not a valid measure of the hallucinations and delusions demonstrated in human patients with schizophrenia, excess locomotion is believed to reflect increased levels of dopamine in the subcortical regions of the brain, consistent with findings in the brains of human patients (Carlsson et al. 2001; Seeman 2011; Laruelle, Kegeles, and Abi-Dargham 2003; Kapur et al. 2000). As such, hyperactivity can be considered to have reliable, translational relevance (Jones, Watson, and Fone 2011). For three days prior to testing, rodents underwent 30 minutes of habituation to the procedural room and another 30 minutes of acclimation to the locomotor chambers. Rats were placed in computerized, clear, Plexiglas cages (Accuscan Instruments, Columbus, OH, USA) for 180 minutes, wherein

their movement (total distance travelled) was recorded in 10 minute intervals (Dyck et al. 2011). Movement was measured in centimeters (cm) via interruptions of infrared light beams. The first 30 minutes of activity were considered habituation, with the last 150 minutes deemed as actual activity.

3.7.6 Social Interaction

Deficits in social behaviour, often indicated by decreased time in social interaction, are one of the first indicators of schizophrenia, and are easily detectable in patients with the disorder and preclinical animal models (Dyck et al. 2011; Sams-Dodd 1998). To assess for social withdrawal in rodents, pairs of animals were randomly determined within treatment group, with one animal coloured with a non-toxic black marker for identification purposes. The rats were placed into an open, black Plexiglas arena (100 cm x 75 cm x 40 cm). Interactions were recorded via a ceiling-mounted video camera and scored by two experimenters who were blind to treatment groups. Each pair of rats was recorded for 10 minutes, although activity was only scored for 5 minutes. Total time spent interacting was recorded (in minutes), as well as the number of active interactions (sniffing, following, crawling, aggressive behaviour, grooming) and passive interactions (close proximity). In between each trial, arena was sprayed with 75% ethanol and cleaned to remove any trace odours. Methodology adapted from File and Sams-Dodd (File 1980; Sams-Dodd 1998).

3.7.7 Novel Object Recognition Task

The NORT is a measure of recognition memory in rodents, and is analogous, in part, to declarative memory in humans, a cognitive domain often disrupted in schizophrenia (Rajagopal et al. 2014). Declarative memory is defined as the conscious memory for facts and events, and can be acquired with relatively minimal exposure to the event and/or object (Squire and Zola 1996). For that reason, object recognition tests are often used in preclinical models to study mammalian declarative memory, with the spontaneous NORT being the paradigm of choice for use in rodents (Winters, Saksida, and Bussey 2008). This task is advantageous because it capitalizes on rodents' natural tendency to explore novel stimuli in preference to familiar stimuli.

With modifications from the original protocol supplied by Winters (2004), the NORT was run in an eight-arm radial maze apparatus that was adjusted to be a Y-shaped apparatus. Of the eight arms, six were blocked off to prohibit entry into those arms; two adjacent arms and the centre platform remained open for entry (see below for description of the eight-arm maze). Activity in the maze was recorded using a tripod-mounted video camera, positioned overtop the maze. Beginning one week prior to testing, each animal was habituated to the apparatus and its surroundings for 20 minutes per day, for three consecutive days. A single NORT trial consisted of an acquisition and retention phase, separated by a 1 minute retention delay period. At the start of every session, each rat was placed on the center platform and was allowed 3 minutes of acclimation. After the initial 3 minutes elapsed, the rodent was removed from the maze and returned to its home cage

for a 1 minute delay period, following which it was returned to the centre platform of the apparatus for the acquisition phase. During this first delay, two identical objects (e.g. small orange pylon) were placed into the apparatus, one in each open arm. The rat was allowed 3 minutes of interaction with the objects before being removed and returned to its home cage once again. Researchers ensured that the animal was visiting both objects equally, to eliminate any location bias. At the end of the second delay period (1 minute), the rat was reintroduced to the Y-maze for the retention phase, which now contained a replica of the original object and a novel object (e.g. white sphere) (**Figure 6**). The amount of time the animal spent interacting with each object, during the 3 minute retention phase, was recorded. Interactions were classified as sniffing the object, and facing the object at a maximum distance of 2 cm away. Standing on the object and urination were not considered as interactions. In between each rodent's session, the maze and all objects were cleaned with 75% ethanol to remove trace odours; no cleaning of the apparatus occurred during the delay periods.

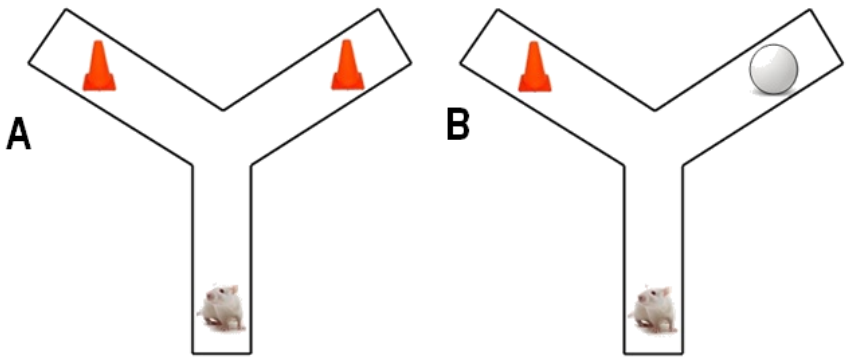

Figure 6: Modified radial arm maze for use of testing object recognition in rodents. **A.** In the spontaneous novel object recognition task, animals are exposed to two identical objects (e.g. orange pylons) in the acquisition phase. The animal has 3 minutes to explore both objects before being removed from the maze. **B.** Following a 1 minute retention delay, the rodent is returned to the apparatus for the start of the choice phase. Now, the animal is exposed to a replica of the original object and a novel retention object (e.g. white sphere). Researchers record the total amount of time the rat spends interacting with each object during the 3 minute period.

3.7.8 Eight-Arm Radial Maze

Performance in the 8-ARM has been shown to be an indicator of spatial, working, and reference memory (Bardgett et al. 2008). Working and reference memory performance was assessed using a delayed win-shift procedure (Olton 1987). The 8-ARM apparatus consisted of eight arms (70 cm long x 25.5 cm high x 12.5 cm wide), extending from an octagonal center platform (52 cm diameter), with guillotine doors placed proximal to the center platform to prohibit entry into a given arm. At the distal end of each arm was a small circular well where the food bait was placed. In this paradigm, animals were trained to find food rewards in blocked and open arms of the maze. Animals were food restricted to encourage motivation to complete the task.

For seven consecutive days, rats performed a habituation phase wherein they had access to all eight arms. A quarter of a Froot Loop (Kellogg's®, Mississauga, ON) was placed at the end of each arm. Each rat was allowed a maximum of 10 minutes to find and eat all treats. The session was terminated once all eight treats were consumed or the 10 minutes had elapsed. For the next 14 days, the rats underwent training in preparation for the win-shift format of testing. The win-shift format consists of one trial divided into a forced run and a free run, separated by a 1 minute or 15 minute delay. During the forced run, four arms of the maze were left open and baited with a quarter of a treat, while the other four arms were blocked. The sequence of open and blocked arms was randomly selected each day. Each rat was placed in the center platform and given 5 minutes to find and eat the four treats. Upon consuming all four treats or the expiration of time, the rat

was removed from the maze and placed in its home cage for a delay period of either 1 minute or 15 minutes. During this delay, the maze was cleaned with 75% ethanol, the four panels were removed leaving all eight arms accessible, and the previously blocked arms were baited. In the free run, the rat was returned to the center platform and given 5 minutes to eat the four treats. The following data was recorded during the free run: 1) the number of correct responses during the first four arm choices post-delay (correct response considered when animal enters correct arm and eats the treat), 2) the number of retroactive errors (i.e. visits to arms that were baited during the forced run), and 3) the number of revisits to arms that were baited during the free choice run. Retroactive errors and revisit errors have been shown to be indicative of reference memory and working memory impairment, respectively (Olton 1987; Spieker et al. 2012; Bardgett et al. 2008). Protocol modified from Bardgett *et al.* and Daya *et al.* (Bardgetts et al. 2008; Daya et al. 2014).

Each rat completed one trial per day for two weeks prior to surgeries. On any given day, half of the cohort completed a trial with the 1 minute delay while the other half completed the 15 minute delay trial. During training, rats must have achieved a criterion of one or fewer errors for three consecutive days, or achieve a stable state of performance before surgeries were completed. Testing on the 8-ARM paradigm was identical to the training sessions, and took place for eight consecutive days post-recovery from surgeries

3.8 Metabolic Function: [^{18}F]FDG Uptake

In collaboration with the McMaster Centre for Pre-clinical and Translational Imaging, PET and computerized tomography (CT) imaging was performed in the first cohort of adult rats (receiving fully phosphorothioated oligonucleotide sequences). Local cerebral glucose utilization values were obtained by measuring glucose uptake in the prefrontal cortex. The PFC was selected as the primary region of interest due to irregular metabolic function observed in the frontal cortex of schizophrenic patients (Floresco et al. 2006; Wolf et al. 2002).

Animals from the sense and AS groups (n = 4/group) were selected for imaging. The rodents were fasted for 12 hours prior to imaging and lightly anesthetized using 1.5% isoflurane (Pharmaceutical Partners of Canada Inc) for administration of 500 µCi of [^{18}F]FDG in 400 µL of saline via tail vein injection. Immediately following the injection and recovery from anesthetic, the rats were transferred to the 8-ARM to cerebrally stimulate the animal. All animals were previously habituated and trained to the 8-ARM paradigm for three weeks prior (Section 3.7.8). Following completion of the cognitive task, the rat was allowed to rest in its home cage until 30 minutes had elapsed since the [^{18}F]FDG injection. After the time had elapsed, the rat was anesthetized using 2% isoflurane and positioned in a bio-containment chamber designed for imaging. Each animal underwent a 15 minute static emission PET scan followed by a 5 minute high resolution CT acquisition. The animals remained anesthetized throughout the imaging process. Imaging was conducted in a Philips Mosaic dedicated Animal PET system

(Philips Medical Systems, Cleveland, OH, USA), and Gamma-Medica Ideas X-SPECT (Gamma-Medica Ideas, NorthRidge, California, USA).

3.9 Sacrifice and Tissue Dissection

All animals were anesthetized with 3.5% isoflurane (Pharmaceutical Partners of Canada Inc) before rapid decapitation. Brains were quickly removed and the following brain regions were dissected over ice: mPFC, PFC, STR, and cerebellum. The regions were stored at -80^{o}C until further use. Specific details about the sacrifice process for each objective are described below.

3.9.1 Objective 1: Maternal Separation Study

Approximately half of the animals (n = 9/ treatment group) were sacrificed at the pre-pubertal stage (PD 35), with the remaining animals being sacrificed following post-pubertal stage (PD 70) testing.

3.9.2 Objective 2: Imaging Study

All 11 animals were sacrificed 14 days post-surgery, upon completion of behavioural testing and PET/CT imaging.

3.9.3 Objective 3: New Oligonucleotide Study

To verify the infusion site and determine the efficacy of synapsin II knockdown, two animals (n = 1 from the sense and single AS sequence groups) were sacrificed one-week post-surgery. The remaining 10 rats were sacrificed two-weeks after surgery.

3.10 Protein Isolation and Quantification

Brain regions were homogenized by hand in Tris-EDTA buffer (50 nM Tris, 1 nM EDTA, pH 7.4) with a Mini-C Protease Inhibitor Tablet (Roche, Mississauga, ON) for approximately 10 seconds. The protein was further broken down by sonication for 5 seconds. To determine protein concentration, a Bradford Assay was run with Bio-Rad Protein Assay reagent (Bio-Rad, Mississauga, ON) and a CU-640 spectrophotometer (Beckman-Coulter, Mississauga, ON). Sample optical density (OD) was measured at 595 nm, in duplicates. Samples which fell outside the linear range of the purified bovine serum albumin protein standard of 1.2 – 10.0 µg/mL were further diluted with the homogenization buffer (Tris-EDTA + Mini-C tablet), and the OD was re-estimated. Samples were stored at -20^{o}C until further use.

3.11 Immunoblotting

Immunoblotting was performed to verify knockdown of the synapsin II protein in the medial prefrontal cortical tissue of all three cohorts of rats. Additional immunoblotting was performed in the prefrontal cortical and striatal tissue. Western blotting for the synapsin II antibody was performed as previously described by Dyck *et al*. (2011). After determining sample concentration, 15 µg of protein was separated by a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. The protein was then transferred onto a 0.2 µm polyvinylidene fluoride (PVDF) membrane. Membranes were blocked for 1 hour with 5% milk buffer in Tris Buffered Saline (TBS) with Tween® 20 (TBS-T). After blocking, blots were incubated with the synapsin II

primary antibody (overnight, 4ºC) and the respective secondary antibody (1.5-2 hours, room temperature) in TBS-T (**Table 3**). Concurrent probing for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), accounted for variations in sample loading and allowed for the normalization of the respective protein bands of interest.

Protocol for performing western blotting for PV, was modified from Drs. Luke Harris and Maria Gallo from the University of British Columbia and University of Alberta, respectively. Briefly, 15 µg of protein was separated by a 15% SDS-PAGE gel electrophoresis. The protein was then transferred onto a 0.2 µm PVDF membrane for 12 hours, at 4ºC. Membranes were blocked for 1 hour with 5% milk buffer in Phosphate Buffered Saline (PBS) with Tween® 20 (PSB-T). Following blocking, the membranes were incubated with parvalbumin primary antibody and its respective secondary antibody (1 hour each, room temperature) in 5% milk solution. Concurrent probing for GAPDH accounted for variations in sample loading and allowed for the normalization of the respective protein bands of interest.

Visualization of protein bands was achieved with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Pittsburgh, PA, USA). Synapsin II membranes were exposed to film for 1, 5, 10 seconds, 30 seconds, 1 minute, and 2 minutes, while PV membranes were exposed for a single 20 minute duration.

PRIMARY ANTIBODY		
	Antibody	**Concentration**
Synaptic Protein	Synapsin II	1:2,500
GABA	Parvalbumin	1:2,500
Housekeeping	GAPDH	1:20,000
SECONDARY ANTIBODY		
	Antibody	**Concentration**
	Anti-rabbit	1:5,000
	Anti-mouse	1:10,000

Table 3: Optimized primary and secondary antibody concentrations used for immunoblotting. Both synapsin II and PV antibodies interact with a polyclonal anti-rabbit secondary antibody, whereas GAPDH was raised in a mouse.

3.12 Data Analysis

All behavioural and biochemical data was graphed and analyzed using GraphPad Prism 4.0 software (San Diego, CA, USA). When analyzing data obtained from both the MS and imaging studies, the student's unpaired two-tailed t-test was used, as each study only had two treatment groups. In the new oligonucleotide study, data was analyzed by means of a one-way ANOVA with Tukey's post-hoc test. Statistical significance was defined as $p < 0.05$ (95% confidence levels). Outliers were identified using Grubb's test with a significance level of $\alpha \leq 0.05$.

3.12.1 Prepulse Inhibition

PPI analysis was performed according to the procedure previously described by Dyck *et al*. (2011). Percent inhibition (% PPI) was calculated using the following formula: $\%PPI = \frac{S-P}{S} \times 100$, where S is the average response to a startle-pulse only trial, and P is the average startle response to a prepulse-startle pulse trial. % PPI was calculated for each prepulse decibel level, and obtained between treatment groups (Dyck et al. 2011). Cohorts were either analyzed using the student's unpaired t-test or the one-way ANOVA, depending on the number of treatment groups.

3.12.2 Locomotor Activity

Total distance travelled (cm) was recorded and analyzed over a 180 minute period. Total distance travelled was also analyzed over 150 minutes, excluding the first 30

minutes of activity as habituation. Cohorts were either analyzed using a student's unpaired t-test or a one-way ANOVA, depending on the number of treatment groups.

3.12.3 Social Interaction

The following parameters were analyzed for each rat, within each treatment group: 1) total amount of time spent interacting (min), 2) total number of interactions, 3) total number of active interactions, and 4) total number of passive interactions. Of the recorded 10 minute interaction, the first 1-2 minutes were excluded as habituation. The number and length of interactions could then be analyzed over a 5 minute period from the remaining 8 minutes of the recording. Analysis began at a point when the two animals were situated on either side of the chamber, and not while they were in the middle of an interaction. Social interaction data was analyzed using the student's unpaired t-test to determine statistical significance.

3.12.4 Novel Object Recognition Task

During the retention phase, two times were recorded per animal: 1) the time spent interacting with the familiar object, and 2) the time spent interacting with the novel object. Times were recorded in seconds. To represent the animal's preference of two objects, a discrimination ratio was then calculated using the following formula: $discrimination\ ratio = \frac{time_{novel}}{time_{novel}+time_{familiar}} \times 100.$ The ratio was calculated following 1 minute and 3 minutes of recording. Dual assessment accounts for any

recognition bias that may have occurred after the first minute of interaction. Calculated ratios from both groups were compared by means of a student's unpaired t-test.

3.12.5 Eight-Arm Radial Maze

The three parameters recorded in this paradigm, 1) the number of correct responses during the first four arm choices, 2) the number of retroactive errors, and 3) the number of revisits to arms that were baited during the free choice run, were analyzed by means of a student's unpaired t-test. Group performances were analyzed by day, and also cumulatively at the end of the testing period.

3.12.6 ^{18}F[FDG] Uptake

PET data was reconstructed using a quantitative 3D iterative reconstruction algorithm and the CT data was reconstructed using a Feldkamp filtered backprojection cone beam reconstruction algorithm. Prior to analyzing [^{18}F]FDG uptake, our lab applied Amira 3D Software (Hillsboro, Oregon, USA) to process raw MRI images of a healthy brain, and convert them into 3D images which were then fused together to form 'labels' of various brain regions. Following creation of the labels, our PET and CT images were fused together and aligned with our existing MRI images using the software programs: FreeSurfer and FMRIB Software Library. Utilizing MatLab software (Natick, Massachusetts, USA), activity in the cortex was quantified by assessing [^{18}F]FDG uptake relative to the control labels. Values were obtained for each sample and region, and were then analyzed for significance using a student's unpaired t-test.

3.12.7 Immunoblotting

Intensity of the resulting protein bands were quantified using the NIH ImageJ program. Quantification was done individually for both synapsin IIa and II isoforms, PV, and GAPDH. Values of our proteins of interest were normalized to GAPDH before being graphed and compared between groups. Cohorts were either analyzed using a student's unpaired t-test or a one-way ANOVA followed by a Tukey's post-hoc test, depending on the number of treatment groups.

4. RESULTS

4.1 Effect of sub-chronic maternal separation between PD 14-23 on the development of schizophrenia-like behavioural abnormalities

4.1.1 Effect of early-life maternal separation on prepulse inhibition

A MS period of 4 hours per day for 9 days (total = 36 hours of MS), did not induce significant deficits ($p>0.05$) in prepulse inhibition at the 68dB, 71dB, and 77dB prepulses (**Figure 7**). No PPI deficits were observed at both pre-pubertal and post-pubertal stages between MS and non-MS rats.

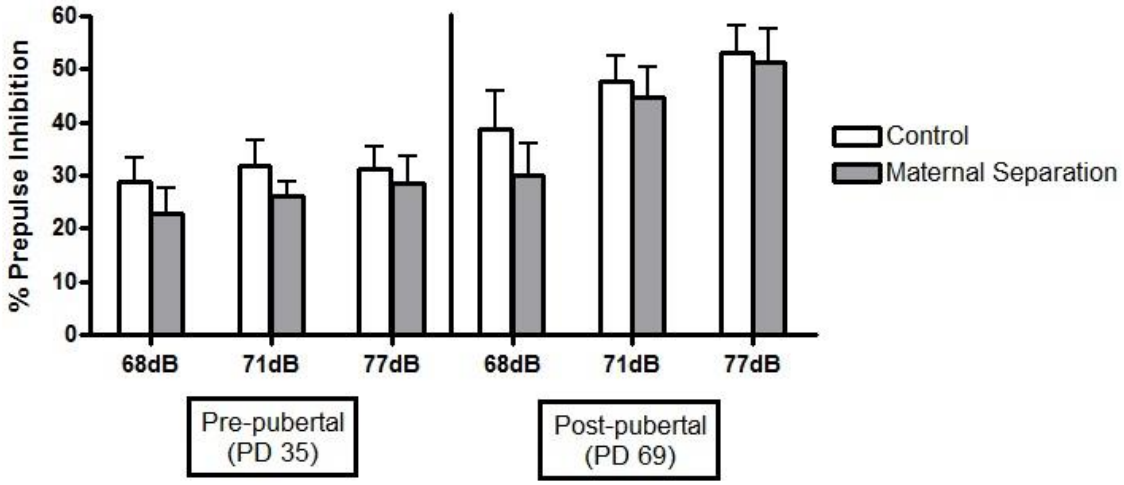

Figure 7: Percent prepulse inhibition measured at three different prepulses: 68dB, 71dB, and 77dB. Following a 9 day MS regimen (4hrs/day) between PD 14-23, no significant deficits in % PPI were observed at any of the measured prepulses ($p>0.05$), before and after puberty. Non-MS group is indicated by the white bars, and MS-group is indicated by the grey bar. Vertical line drawn between pre- and post-pubertal stages signifies puberty.

4.1.2 Effect of early-life maternal separation on locomotor activity

Sub-chronic MS did not induce differences in locomotor activity, between MS and non-MS rats at pre-pubertal (PD 32-35) and post-pubertal (PD 65-69) stages of development ($p>0.05$). Non-significance was observed in both 180 minutes (**Figure 8A**) and 150 minutes (**Figure 8B**) of locomotor activity recording.

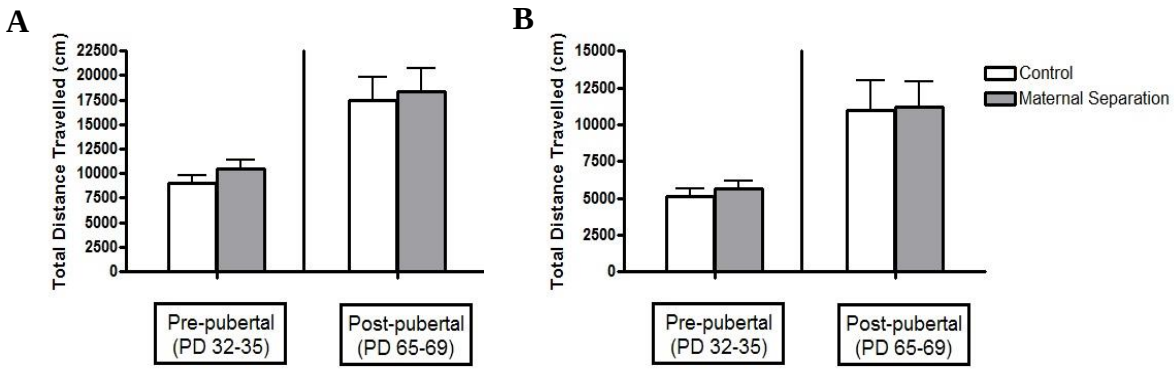

Figure 8: Locomotor activity measured as total distance travelled in centimeters (cm). **A**. Total distance travelled in 180 minutes. No significant difference ($p>0.05$) in locomotor activity was observed between non-MS and MS groups at both pre-pubertal and post-pubertal stages. **B**. Total distance travelled in 150 minutes, excluding the first 30 minutes as habituation. Repeated maternal separation during development did not result in significant changes ($p>0.05$) in locomotor activity relative to control animals, at pre-pubertal and post-pubertal stages. Non-MS group is indicated by the white bars, and MS-group is indicated by the grey bar. Vertical line is drawn between pre- and post-pubertal stages to signify puberty.

4.1.3 Social Interaction:

Following 36 hours of early-life MS over the course of 9 days, no changes in social interaction were observed at pre-pubertal (PD 32-35) and post-pubertal (PD 65-69) time points. Specifically, animals that were separated from their mothers did not display reduced numbers of active and passive interactions ($p>0.05$) (**Figure 9A-B**), nor did they differ from non-separated rodents in the total number of interactions (**Figure 9C**) or the total time spent interacting ($p>0.05$) (**Figure 9D**).

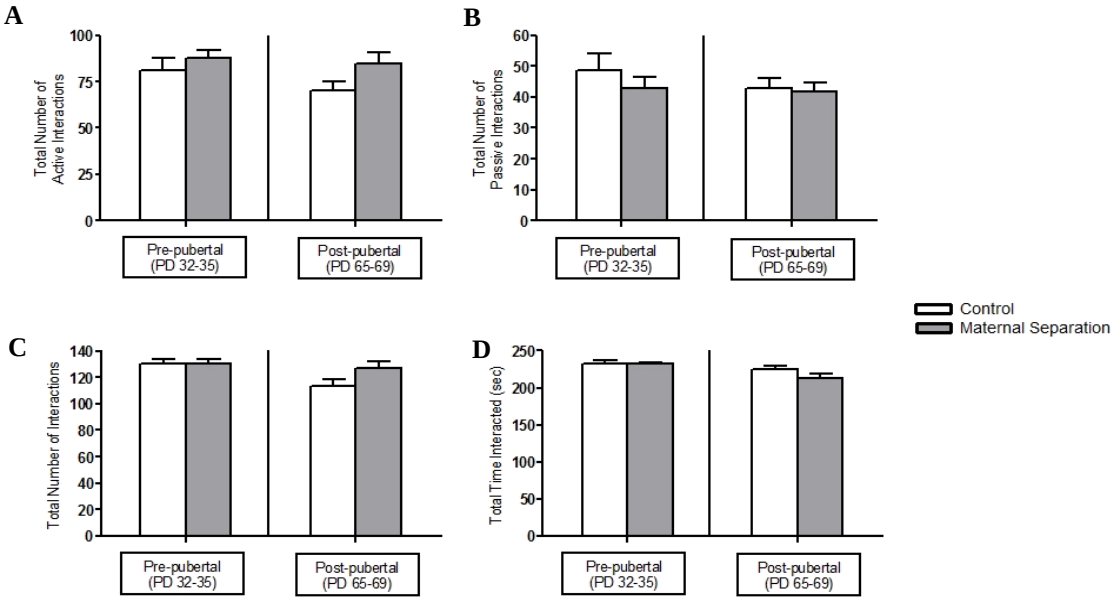

Figure 9: Social interaction paradigm. **A**. Total number of active interactions. No significant difference ($p>0.05$) in active interactions (e.g. fighting) was observed between non-MS and MS groups at both pre-pubertal and post-pubertal stages. **B**. Total number of passive interactions. Repeated maternal separation during development did not result in significant changes ($p>0.05$) in passive interactions (e.g. sniffing) relative to control animals, at pre-pubertal and post-pubertal stages. **C**. Total number of interactions, both active and passive. No significant changes in the total number of interactions ($p>0.05$) was observed in MS rats when compared to the control group. **D**. Total time interacted as measured in seconds. No significant difference ($p>0.05$) in the time spent interacting was observed between non-MS and MS groups. Control group is indicated by the white bars, and MS group is indicated by the grey bar. Vertical line is drawn between pre- and post-pubertal stages to signify puberty.

4.1.4 Effect of early-life maternal separation on novel object recognition

A sub-chronic MS regimen did not result in any significant changes between groups in NORT performance, when measured for 1 minute and 3 minutes (**Figure 10**). Non-significant differences were noted at both pre-pubertal (PD 32-35) and post-pubertal (PD 65-69) stages of development.

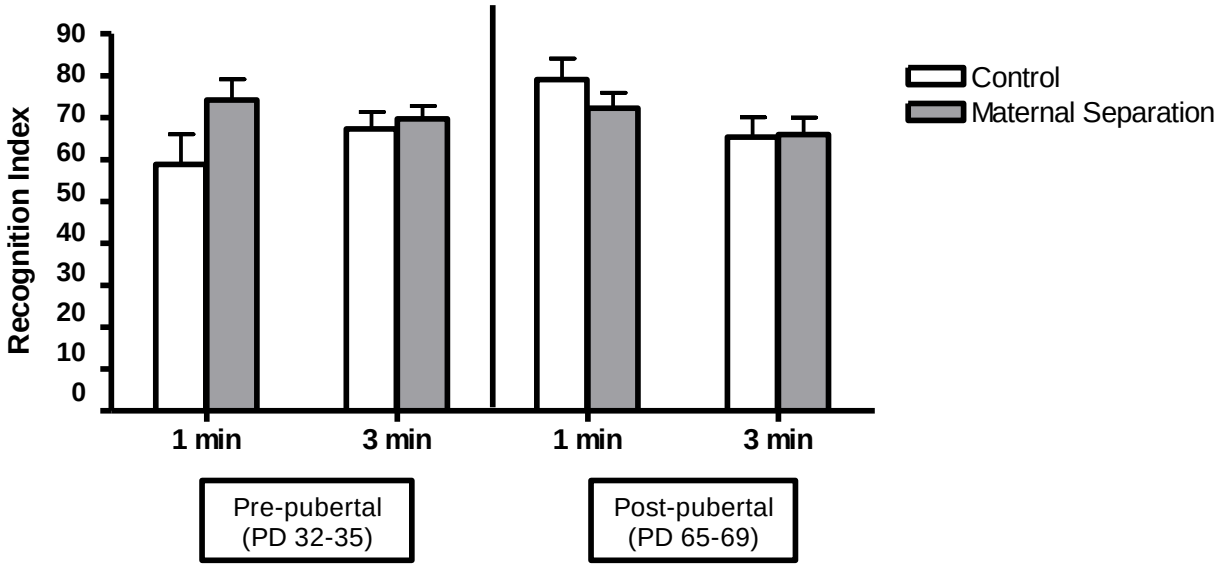

Figure 10: Novel object recognition test. Data obtained from the NORT was analyzed using the calculation: $Discrimination\ Index = \frac{time_{novel}}{time_{novel}+time_{familiar}} \times 100$. Sub-chronic maternal separation during development (PD 14-23) did not result in any significant performance deficits when measured for 1 minute and 3 minutes ($p>0.05$), at both pre-pubertal and post-pubertal time points. Non-MS group is indicated by the white bars, and MS-group is indicated by the grey bar. Vertical line drawn between pre- and post-pubertal stages signifies puberty.

4.2 Effect of sub-chronic maternal separation between PD 14-23 on the development of schizophrenia-like biochemical abnormalities

4.2.1 Effect of maternal separation on protein expression levels of synapsin II

Immunoblotting results indicate a non-significant difference ($p>0.05$) in synapsin IIa and IIb expression, in the mPFC of MS and non-MS animals (**Figure 11**). When assessed at the pre-pubertal stage, levels of synapsin IIa and IIb were non-significantly increased in MS rats, relative to the control group. At the post-pubertal stage, levels of synapsin IIa and IIb were virtually identical between treatment groups.

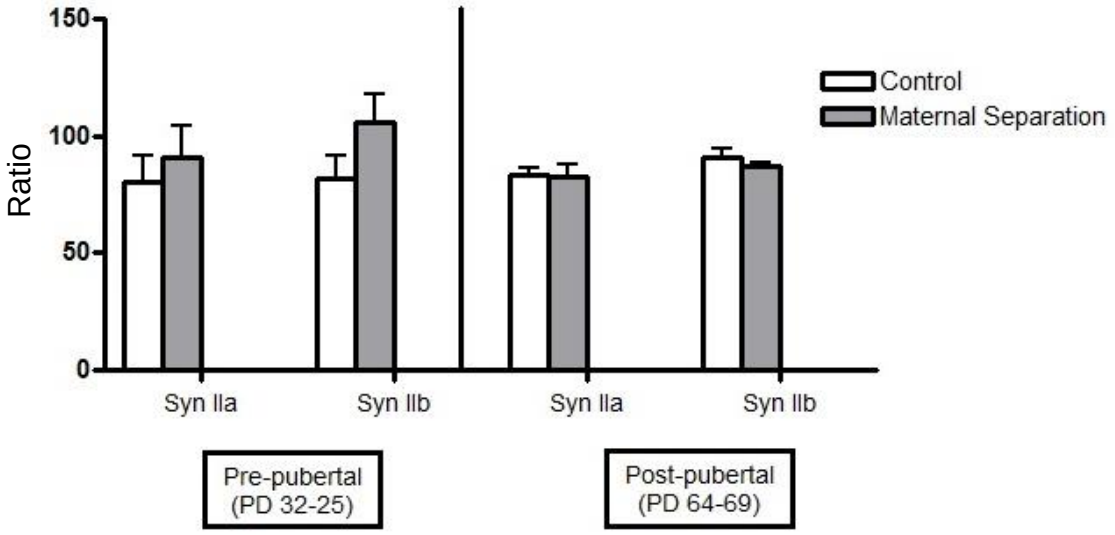

Figure 11: Synapsin II protein expression levels. Graph indicates normalized protein expression levels of synapsin isoforms IIa and IIb in the mPFC following pre-weaning maternal separation (PD 14-23). Immunoblotting assessed protein expression levels of synapsin IIa and IIb at both prepubertal (PD 32-35) and post-pubertal (PD 65-70) stages. There were no significant changes in synapsin IIa and IIb expression levels at both pre-pubertal and post-pubertal stages ($p>0.05$). Non-MS group is indicated by the white bars, and MS-group is indicated by the grey bar. Vertical line is drawn between pre- and post-pubertal stages to signify puberty.

4.3 Effect of 14-day bilateral infusion into the mPFC of fully phosphorothioated synapsin II antisense sequence on development of behavioural abnormalities

4.3.1 Effect of fully modified synapsin II AS sequence on prepulse inhibition

A two-week infusion period of synapsin II antisense oligonucleotides into the mPFC of adult rats resulted in a slight, but non-significant ($p>0.05$), deficit in prepulse inhibition at the 71 dB and 77 dB prepulses. However, when compared to sense-treated animals at the 68 dB prepulse, no decrease in PPI was observed in AS rats (**Figure 12**).

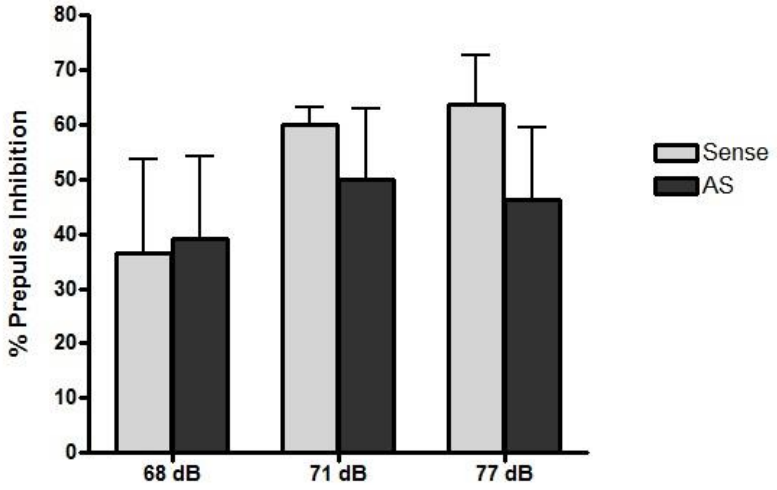

Figure 12: Percent prepulse inhibition measured at three different prepulses: 68dB, 71dB, and 77dB. Following a continuous 14-day bilateral infusion of fully modified synapsin II antisense oligonucleotides into the mPFC, no significant deficits in % PPI were observed at any of the measured prepulses ($p>0.05$), although there was a slight reduction seen at the higher decibel prepulses. The sense group is indicated by the light grey bars, and AS-treated rats are indicated by the dark grey bar.

4.3.2 Effect of fully modified synapsin II AS sequences on eight-arm radial maze performance

All animals were tested on the 8-ARM for ten days post-surgery. Analysis revealed a non-significant effect of synapsin II knockdown on the first four arm entries ($p>0.05$), retroactive errors ($p>0.05$), and revisit errors ($p>0.05$).

A one-minute delay period between the forced and free runs revealed a noticeable, yet non-significant ($p>0.05$), increase in the number of retroactive errors made by antisense-treated animals (**Figure 13B**). The same trend was observed between groups with respect to the number of revisits made in the free run (**Figure 13C**). Conversely, the number of correct responses made by AS animals did not differ from sense-treated animals (**Figure 13A**). Unlike the 1 minute delay, a 15 minute delay period did not result in any observable decrease in cognitive performance between sense and antisense treated groups (**Figure 13**).

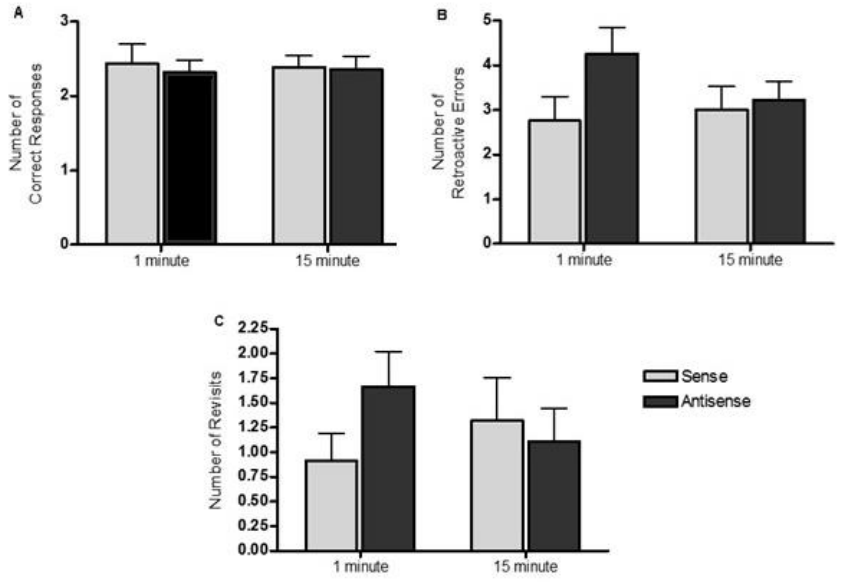

Figure 13: Eight-arm radial maze performance. **A**. The number of correct responses during the first four arm choices made. Infusion of fully modified synapsin II antisense oligonucleotides for 14 consecutive days did not result in any significant results between groups ($p>0.05$), following both a 1 minute and 15 minute delay period. **B**. Total number of retroactive errors. With a 1 minute delay, a noticeable, yet non-significant increase ($p>0.05$) in retroactive errors was made by AS-treated animals when compared to sense animals. No trend was observed following a 15 minute delay ($p>0.05$). **C**. Total number of revisits to arms that were baited during the free choice run. Similar to the number of retroactive errors made, AS-treated animals undergoing the 1 minute delay demonstrated an increased, but non-significant, number of revisits. No such trend was observed following a 15 minute delay. The sense group is indicated by the light grey bars, and AS-treated rats are indicated by the dark grey bar.

4.4 Effect of 14-day bilateral mPFC infusion of fully phosphorothioated synapsin II antisense sequences on cortical activity

The two-week infusion of first-generation synapsin II antisense oligonucleotides into the rodent mPFC did not result in significantly altered cortical metabolic activity ($p>0.05$) (**Figure 14**). Metabolism of [^{18}F]FDG in the cortex was assessed following cognitive stimulation with the eight-arm radial maze behavioural paradigm. Rats receiving the bilateral AS synapsin II knockdown solution displayed similar levels of [^{18}F]FDG mean standard uptake values when compared to rats that received bilateral sense synapsin II solution ($p>0.05$) (**Figure 15**). Due to the nature of the study, baseline metabolic activity with no prior cognitive stimulation was unable to be observed.

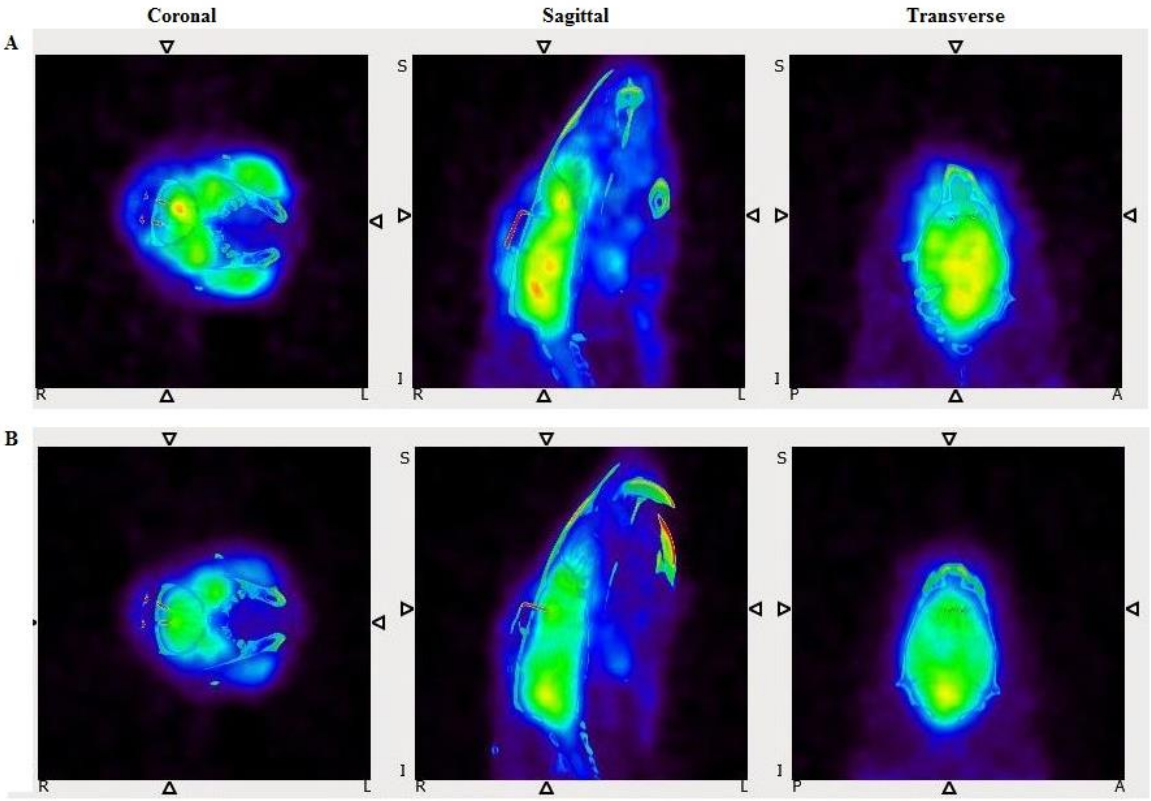

Figure 14: [^{18}F]FDG metabolism in the adult rat, assessed after cognitive stimulation with the eight-arm radial maze paradigm. Faint dots observable in the coronal images represent bilateral cannula implantations into the mPFC, in reference to bregma +2.7 mm A/P; ± 1.5 mm M/L; -3 mm D/V. In the sagittal plane, the 3mm, 28 gauge stainless steel tube and cannulae are visible. **A**. [^{18}F]FDG metabolism in a rat bilaterally infused with synapsin II negative control sense solution. **B**. [^{18}F]FDG metabolism in rats bilaterally treated in the mPFC with synapsin II AS solution. Slight hypofrontality was observed in the antisense treated rats, relative to sense rats, as visualized by the lack of yellow and red 'hotspots' in the cortex ($p>0.05$).

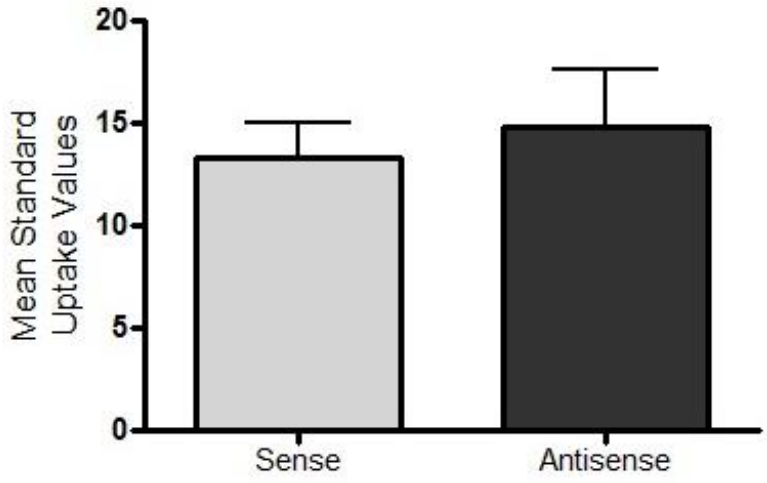

Figure 15: [^{18}F]FDG mean standard uptake values in the mPFC of rats bilaterally infused with sense or antisense solutions (n = 4/group). Uptake values can indicate metabolic activity and therefore, neuronal activity, within the brain region of interest. Following cognitive stimulation with the eight-arm radial maze paradigm, rats infused with the synapsin II antisense knockdown solution displayed minimal increase in mean uptake values of [^{18}F]FDG ($p>0.05$) in comparison to animals infused with the synapsin II sense control solution. The mean uptake values do not, however, appear to correlate well with the PET/CT scans which showed slight prefrontal cortical hypofrontality in AS rats (Figure 14).

4.5 Effect of 14-day bilateral infusion of fully phosphorothioated synapsin II antisense sequences into the mPFC on development of biochemical abnormalities

4.5.1 Effect of fully modified synapsin II AS sequence on protein expression levels of synapsin II

Immunoblotting results indicate a non-significant difference ($p>0.05$) in synapsin IIa and IIb expression, in the mPFC of sense and antisense treated animals (**Figure 16**). Interestingly, following 14 consecutive days of bilateral infusion, sense treated animals displayed a general, but non-significant, reduction of synapsin IIa and IIb expression relative to the group of rodents that received antisense infusions. Furthermore, levels of synapsin IIb were slightly elevated in both groups compared to synapsin IIa levels ($p>0.05$).

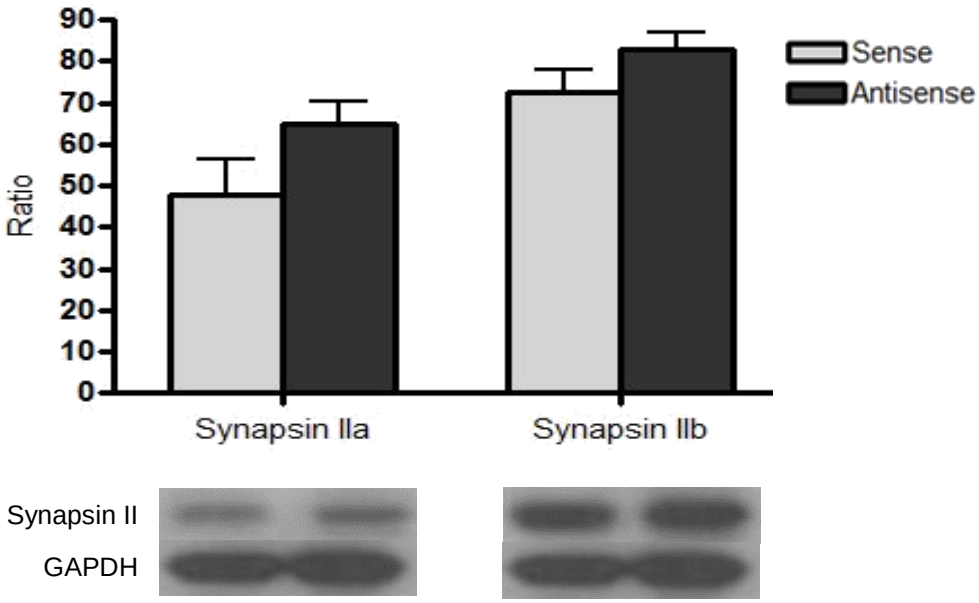

Figure 16: Normalized synapsin II protein expression levels in the mPFC following 14 consecutive days of bilateral infusion of fully phosphorothioated oligonucleotides. There were no significant changes in synapsin IIa and IIb expression levels between groups ($p>0.05$), although a slight reduction of protein expression was observed in sense treated animals relative to antisense treated animals. Synapsin bands were normalized to GAPDH (housekeeping gene). The sense group is indicated by the light grey bars, and AS-treated rats are indicated by the dark grey bar.

4.5.2 Effect of fully modified synapsin II AS sequences on striatal protein expression levels of parvalbumin

Immunoblotting results demonstrate a non-significant difference ($p>0.05$) in striatal parvalbumin levels following bilateral infusion of synapsin II antisense sequences into the mPFC (**Figure 17**).

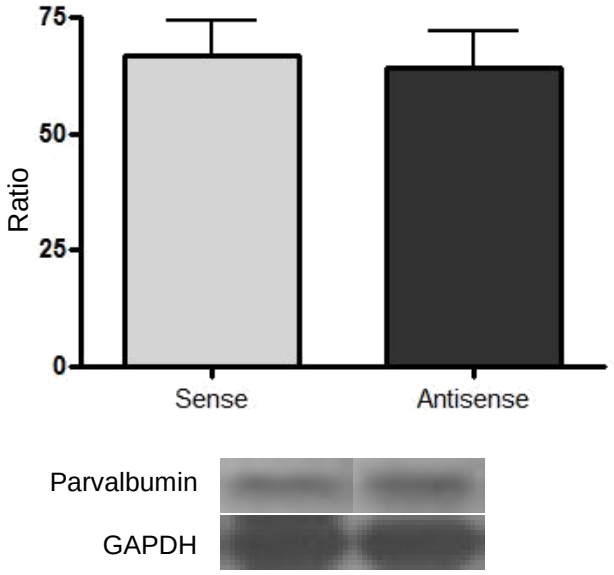

Figure 17: Normalized parvalbumin protein expression levels in the striatum. No significant difference ($p>0.05$) in parvalbumin levels was observed in the striatum after two continuous weeks of bilateral infusion of fully phosphorothioated synapsin II antisense sequences into the mPFC, PV bands were normalized to GAPDH (housekeeping gene). The sense group is indicated by the light grey bars, and AS-treated rats are indicated by the dark grey bar.

4.6 Bilateral infusion of partially phosphorothioated synapsin II antisense sequences into the mPFC and its effect on development of behavioural abnormalities

4.6.1 Effect of partially modified synapsin II AS sequence on prepulse inhibition

PPI was measured both one week and two weeks following cannula implantation into the mPFC, using three different prepulse intensities: 68dB, 71dB, and 77dB. When assessed after one week of infusions, a non-significant trend was observed between groups at the 71dB prepulse intensity. Animals receiving infusions of either a single AS sequence or the cocktail of AS sequences displayed reduced percent inhibition ($p>0.05$) relative to the aCSF infused rodents. Sense treated rats demonstrated a sensorimotor gating ability that was slightly reduced relative to aCSF rats, but was slightly elevated when compared to both AS groups (**Figure 18A**). When startle response was assessed the following week, at the end of the two week infusion period, the previously observed trend did not hold. Animals in the AS groups displayed greater percent inhibition in comparison to the aCSF and sense groups ($p>0.05$) (**Figure 18B**). Data collected at the 68dB and 77dB prepulse intensities revealed no significant difference and no observable trends between the groups (data not shown).

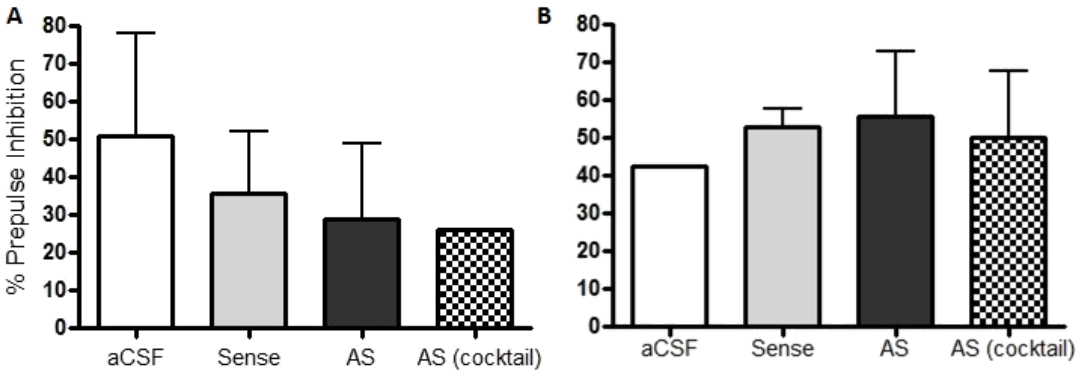

Figure 18: Percent prepulse inhibition measured at the 71dB prepulse intensity. **A.** One week after the start of infusions into the mPFC, animals treated with either a single AS sequence or a cocktail of AS sequences displayed reduced percent PPI relative to aCSF and sense infused animals ($p>0.05$). **B.** When assessed at the end of the continuous 14-day bilateral infusion, both AS groups displayed elevated percent PPI when compared to the control groups ($p>0.05$). No significant deficits or trends were observed at the other measured prepulses ($p>0.05$). The aCSF group is indicated by solid white bars, sense group is indicated by light grey bars, single antisense sequence group is indicated by the dark grey bars, and the cocktail of antisense sequences group is indicated by the checkered bars.

4.6.2 Effect of partially modified synapsin II AS sequence on locomotor activity

One-way ANOVA analysis revealed no significant difference in total distance travelled between all four treatment groups at the one-week time point ($p>0.05$), when measured over 150 minutes (**Figure 19A**). Conversely, there was a significant increase in locomotor activity after two-weeks of infusion of a single synapsin II antisense oligonucleotide, compared to the sense control group ($*p<0.05$). Treatment with the cocktail of antisense sequences did not result in any significant hyperlocomotion (**Figure 19B**).

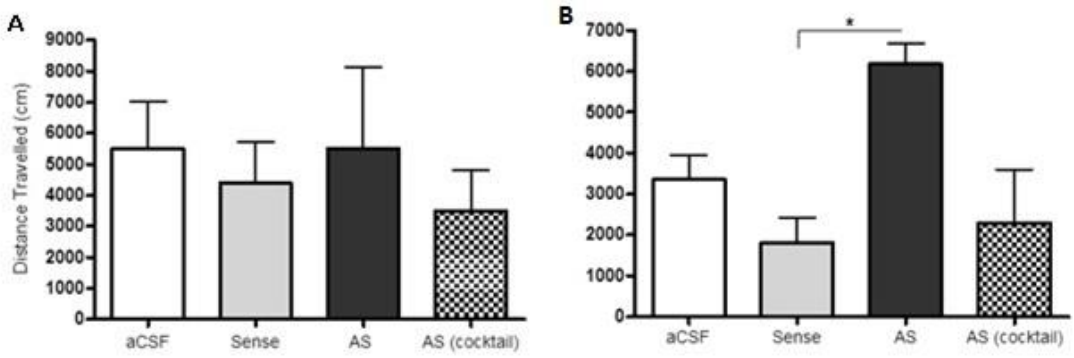

Figure 19: Locomotor activity measured as total distance travelled in centimeters (cm). Total distance travelled in 150 minutes, excluding the first 30 minutes as habituation. **A**. Locomotor activity measured one-week following cannula implantation. No significant difference in locomotor activity was observed between both synapsin II antisense groups and controls ($p>0.05$), as determined by one-way ANOVA with Tukey's post-hoc analysis. **B**. Locomotor activity measured two-weeks after cannula implantation. Animals infused with the single antisense sequence demonstrated significantly increased locomotor activity compared to the sense control group ($*p<0.05$). Treatment with the antisense cocktail solution did not result in hyperlocomotor activity ($p>0.05$). The aCSF group is indicated by solid white bars, sense group is indicated by light grey bars, single antisense sequence group is indicated by the dark grey bars, and the cocktail of antisense sequences group is indicated by the checkered bars.

4.7 Bilateral infusion of partial phosphorothioated synapsin II antisense sequences into the mPFC and its effect on development of biochemical abnormalities

4.7.1 Effect of partially PS modified synapsin II AS sequences on protein expression levels of synapsin II

Sub-chronic infusion of partially phosphorothioated AS oligonucleotides, either a single sequence or cocktail of two sequences, resulted in a slight but non-significant reduction of synapsin IIa expression when compared to animals infused with sense oligonucleotides ($p>0.05$). No differences in synapsin IIa protein expression were observed between the two AS groups ($p>0.05$). Control animals infused with aCSF displayed lower levels of the synapsin IIa isoform relative to the three experimental groups ($p>0.05$) (**Figure 20A**). With respect to the synapsin IIb isoform, levels of protein expression were similar between all groups, and no trends were observable, as determined by one-way ANOVA with Tukey's post-hoc test ($p>0.05$) (**Figure 20B**).

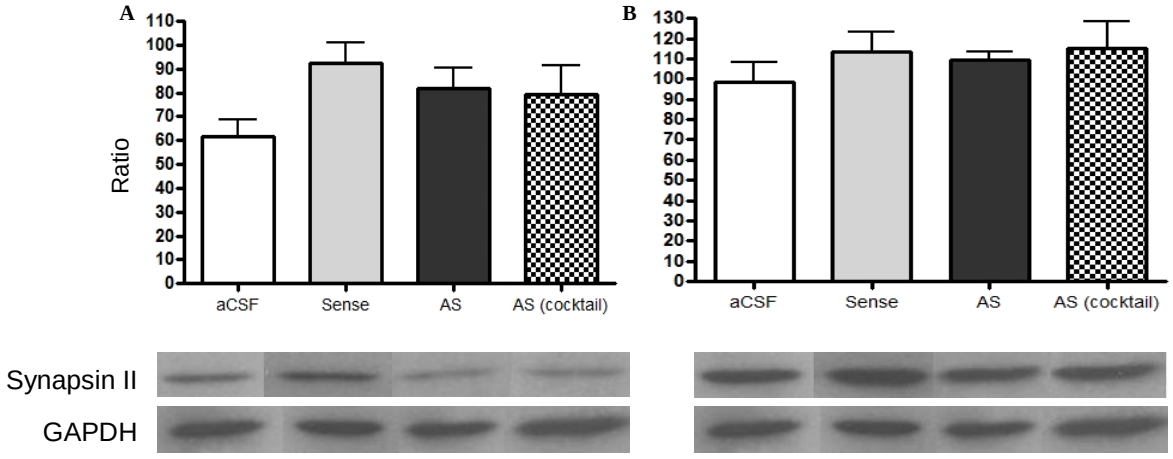

Figure 20: Synapsin II protein expression levels in the mPFC following 14 continuous days of bilateral infusion of partially phosphorothioated oligonucleotides. **A.** Normalized protein expression and western blot band visualization of the synapsin IIa isoform. A slight, but non-significant, decrease in protein expression was observed between the sense group and both AS treated groups ($p>0.05$). No difference in synapsin IIa expression was observed between animals infused with a single AS sequence or a cocktail of AS sequences ($p>0.05$). Control animals infused with aCSF displayed lower protein levels of the synapsin IIa isoform ($p>0.05$). **B.** Normalized protein expression and western blot band visualization of synapsin IIb isoform. There were no significant differences in synapsin IIb protein expression among all groups, as determined by one-way ANOVA with Tukey's post-hoc test ($p>0.05$). The aCSF group is indicated by solid white bars, sense group is indicated by light grey bars, single antisense sequence group is indicated by the dark grey bars, and the cocktail of antisense sequences group is indicated by the checkered bars.

4.7.2 Effect of partially PS modified synapsin II AS sequences on protein expression levels of parvalbumin

As determined by one-way ANOVA with Tukey's post-hoc test, rodents infused with a single partially phosphorothiaoted antisense sequence displayed reduced levels of parvalbumin protein expression within the striatum (*$p<0.05$), relative to both control groups as well as the other experimental group (**Figure 21**). Levels of protein expression were similar between the remaining three groups ($p>0.05$).

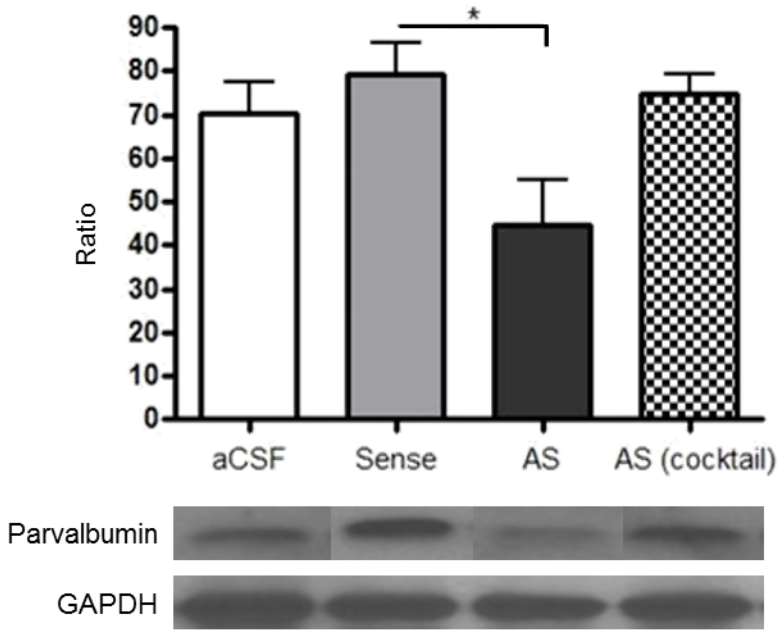

Figure 21: Normalized protein expression and western blot visualization of parvalbumin protein expression in the striatum. Following medial prefrontal cortical infusion of partially phosphorothioated nucleotide sequences, rodents treated with a single antisense sequence displayed reduced levels of parvalbumin ($p>0.05$) in comparison to the remaining three groups. No difference in parvalbumin expression was observed between rodents that received two antisense sequences, relative to the negative control aCSF and sense groups ($p>0.05$). The aCSF group is indicated by solid white bars, sense group is indicated by light grey bars, single antisense sequence group is indicated by the dark grey bars, and the cocktail of antisense sequences group is indicated by the checkered bars.

4.8 Visualization of successful infusion into the medial prefrontal cortex

Histological staining with Evans Blue dye visually confirmed the accuracy of surgical implantation of the cannula into the mPFC (**Figure 22**). Considering the area of dye diffusion, it is likely that AS infusion is highly localized within the mPFC and does not reach brain structures beyond the prefrontal cortex. Visualization of the dye occurred one week following implantation, and captured using a basic point-and-shoot camera.

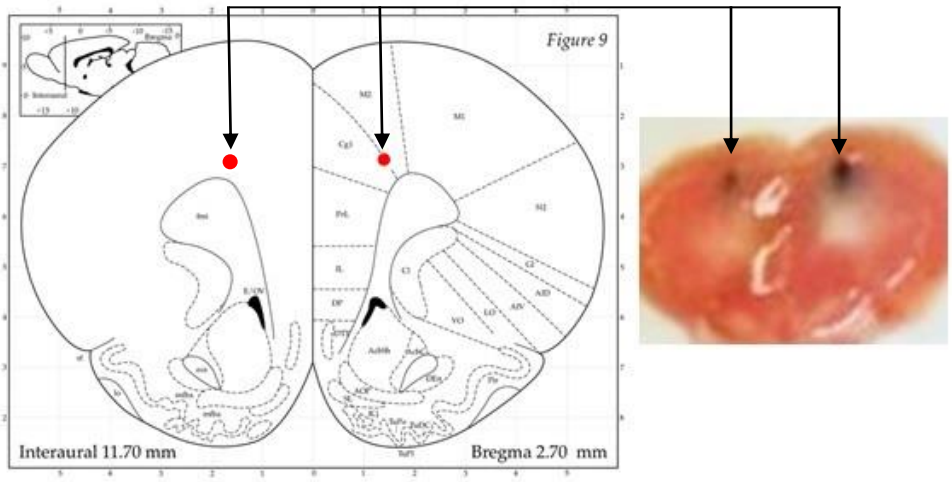

Figure 22: Visualization of infusion into the medial prefrontal cortex. Successful infusion into the target site as observed from a coronal slice obtained from an animal infused for one week with 2% Evans Blue dye into the medial prefrontal cortex. Corresponding illustration from the Rat Brain Atlas is displayed for anatomical reference (Paxinos and Watson 2006). The red dots on the illustration are a bilateral representation of the target site.

4.9 Assessment of local tissue following infusion of partially phosphorothioated synapsin II antisense oligonucleotides

As previously mentioned, based on information that infusion of first generation AS oligonucleotides with full phosphorothioate modifications has induced toxicity and localized necrosis *in vivo*, it was anticipated that use of partially phoshorothioated sequences would not result in toxicity and necrosis of the brain tissue surrounding the infusion site. Throughout the 14-day infusion period, animals were visually evaluated for signs of illness and were weighed every other day to verify healthy weight gain and/or maintenance. No animals in the study experienced life threatening antisense-induced toxicity, unlike in previous studies conducted in our lab over the past several years (data not shown). Furthermore, visual observation of the brains collected suggests that necrosis did not occur in animals infused with the chimeric antisense oligonucleotide sequences (**Figure 23**).

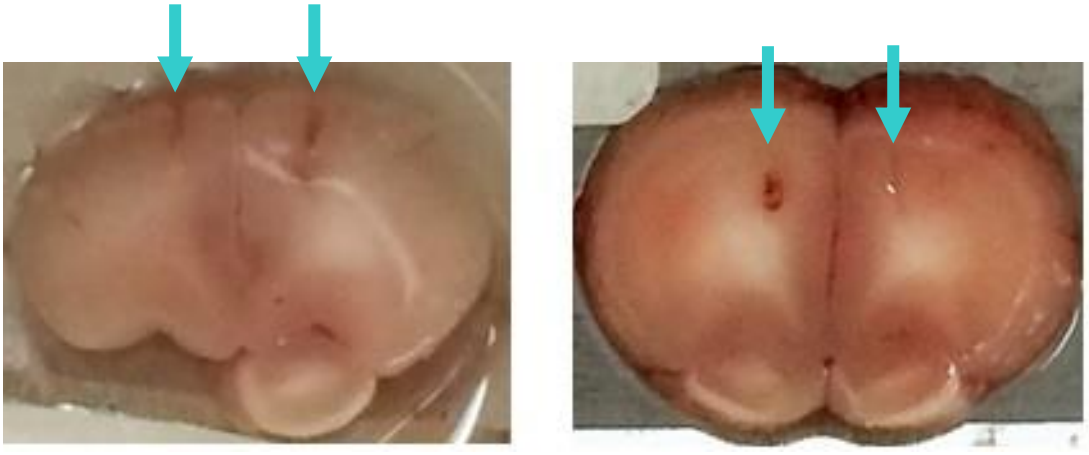

Figure 23: Visualization of local brain tissue. Coronal slices obtained from animals infused for two weeks with partially phosophorothioated synapsin II antisense oligonucleotides reveal a lack of site-specific necrosis. Lesions indicating the location of bilateral cannula implantation can be clearly identified. In previous studies using full phosphorothioate modifications, the mPFC and PFC were occasionally uncollectable due to extreme tissue damage.

5. DISCUSSION

5.1 Maternal separation during PD 14-23 is insufficient to induce schizophrenia-like behavioural abnormalities throughout development

The development of the mammalian brain is a dynamic process that begins with periods of overproduction of synapses, receptors, and neural connections. Immediately before birth, aggressive pruning of excess synapses occurs and continues through the transitional stages into adulthood (Andersen 2003). Within this lengthy timeframe, the brain is vulnerable to interruptions that can alter normal brain development and increase the likelihood of production of psychiatric diseases. The type of insult and when it must be administered are key factors to the manifestation of mental illnesses. Maternal separation is considered an adverse early life event, and is used in rats as a model of such experiences (Wang et al. 2015). Furthermore, a previous study from our laboratory determined the critical period in which an early-life knockdown of synapsin II results in the development of schizophrenia-like behaviours (Tan 2014), supplementing earlier studies from our laboratory which established that reduced levels of synapsin II protein correlate with the manifestation of schizophrenia-like behavioural abnormalities in both animals and patients with the disorder (Dyck et al. 2011; Tan et al. 2014). In order to verify the role of synapsin II knockdown during development, we sought to determine the effects of maternal separation in this study.

With respect to the exact pre-weaning maternal separation timeline, pups were removed from their mothers during the period when a knockdown of the synapsin II was

shown to induce behavioural abnormalities (Tan 2014). A maternal separation period of 4 hours a day, for 9 days (PD 14-23) did not result in schizophrenia-like behavioural abnormalities when assessed for during pre-pubertal (PD 32-35) and post-pubertal (PD 65-70) stages (i.e. no deficit in prepulse inhibition, no hyperactivity, no lack of social interaction, and no working memory impairment).

Although the findings from the study demonstrate a lack of effect from our implemented maternal separation paradigm, other research groups have found that pre-weaning environmental disturbances can lead to the emergence of schizophrenia-like behaviours reminiscent of those resulting from various accepted pre-clinical animal models of the disorder (Anglin, Cohen, and Chen 2008; Ellenbroek, van den Kroonenberg, and Cools 1998; Bouet et al. 2011; Wang et al. 2015). However, different phenotypes were observed with varying separation protocols. Studies that investigated the effect of a one-time separation of 24 consecutive hours at PD 9 found conflicting results (Ellenbroek, van den Kroonenberg, and Cools 1998; Lehmann, Pryce, and Feldon 2000). Conversely, rat pups that were deprived of their mothers for repeated separations of 3-4 hours per day, for a minimum of 14 consecutive days between PD 1-21, consistently had schizophrenia-like abnormalities manifest in adulthood (Sanders and Anticevic 2007; Wang et al. 2015). Sub-chronic separation regimens including ours, occurring any time between PD 3-23, for a total of 36 hours were insufficient in inducing schizophrenia-like behavioural phenotypes, and thus, do not provide an accurate representation of an early-life stressor (Shalev and Kafkafi 2002). While maternal separation undoubtedly is an

important risk factor in schizophrenia development, overall, our results support the proposed hypothesis and further suggest that any observed impairments in our preliminary synapsin II neonate model were due to the nature and timing of the knockdown.

5.2 Maternal separation during PD 14-23 is insufficient to induce schizophrenia-like biochemical abnormalities throughout development

Sub-chronic maternal separation did not induce a reduction in synapsin II levels within the mPFC, nor did it result in a loss of function in dopaminergic, glutamatergic, or GABAergic prefrontal cortical signalling (data not included). Unlike the synapsin II neonate model which displayed decreased glutamate activity in the PFC, this maternal separation model does not present any construct validity for schizophrenia. The results lend further support to the hypothesis that this particular MS regimen did not contribute to the pathological changes observed in the neonatal synapsin II knockdown model. However, further research was required to account for the abnormalities observed by Tan (2014) regarding the negative control rats in the synapsin II neurodevelopmental model of schizophrenia., thus leading to Objectives 2 and 3 (**Sections 2.2-2.3**).

5.3 Bilateral knockdown of synapsin II using fully phosphorothioated antisense sequences does lead to slight impairments in sensorimotor gating ability

Impaired sensorimotor gating ability is commonly observed in patients with schizophrenia, as measured using the prepulse inhibition test (Braff and Geyer 1990;

Braff, Geyer, and Swerdlow 2001). Reduced PPI is also seen in animal models of schizophrenia, and can be attenuated with antipsychotic drug treatment (Braff, Geyer, and Swerdlow 2001; Daya et al. 2014). PPI can be assessed using a number of sensory modalities, including visual, tactile and auditory (Braff, Geyer, and Swerdlow 2001). For the purpose of this thesis, acoustic startle response was measured using three different prepulse intensities: 68dB, 71dB, and 77dB, followed shortly after by a startle pulse stimulus of 120dB. PPI measures the ability of the prepulse to reduce the startle magnitude in response to the pulse stimulus. Stronger prepulse stimuli tend to induce greater levels of inhibition or lower startle responses. When sensorimotor gating ability is impaired, as in schizophrenia, the preceding prepulse has little to no effect on the startle magnitude to the pulse stimulus.

Rodents that received two weeks of continuous infusion of synapsin II antisense sequences into the medial prefrontal cortex displayed reduced, but non-significant, PPI when compared to negative control animals infused with sense sequence. Therefore, site-specific knockdown of synapsin II within the mPFC, using fully phosphorothioated antisense sequences, does appear to affect the underlying circuitry responsible for PPI response. The lack of significance in results can likely be attributed to a small sample size (n=4/group), and a large variance in standard error of means within each group.

5.4 Bilateral knockdown of synapsin II using fully phosphorothioated antisense sequences disrupts working and reference memory

The eight-arm radial maze behavioural paradigm is most often used as an indicator of working and reference memory performance in murine animals (Bardgett et al. 2008). Patients with schizophrenia are unable to successfully complete a virtual radial arm maze, similar to that used in rodents, and also demonstrate impaired working and reference memory (Spieker et al. 2012). In this study, a delayed win-shift procedure of the 8-ARM was utilized to examine working and reference memory, as well as executive functioning, in rats that received synapsin II antisense sequences in the mPFC.

The healthy rat can successfully perform the 8-ARM paradigm, making few working and reference memory errors while correctly choosing to only enter those arms baited with treats. Rats treated with synapsin II AS solution performed poorly in the 8-ARM, in comparison to the negative control, sense treated rats. Although there was no difference between the two groups in terms of the number of correct choices, AS rats made a noticeable, yet non-significant, increase in the number of retroactive errors and revisits to arms previously baited. Results therefore suggest that the knockdown of synapsin II within the medial prefrontal cortex interferes with executive functioning, a mental process dependent on the mPFC. An increase in retroactive and revisit errors can infer impaired reference and working memory, respectively. Furthermore, these cognitive abnormalities were observed only following a 1 minute delay period, and not the 15 minute delay, between the forced and free runs, implying a disruption in short-term

memory retrieval. Additional studies are required to bolster the sample size (currently n=4/group), which may increase the likelihood of significance, and help strengthen the link between reduced synapsin II in the mPFC and expression of cognitive deficits in schizophrenia.

5.5 Bilateral knockdown of synapsin II using fully phosphorothioated antisense sequences does not affect cortical activity

Metabolic dysfunction has been commonly observed in patients with schizophrenia, while assessment of brain metabolic function using PET/CT fused imaging in a live rodent model of schizophrenia is less common (Daya et al. 2014). Many neuroimaging studies have implicated abnormalities in dorsolateral prefrontal cortical activation in schizophrenia, with cortical hypofrontality being the most commonly observed phenotype in patient populations although hyperfrontality has also been observed (Callicott et al. 2003; Daya et al. 2014). It was therefore of interest to examine the effect of reduced levels of synapsin II in the mPFC on cortical activity.

Following cortical activation using the eight-arm radial maze, PET/CT fused imaging demonstrated that AS infused rodents did not show hypofrontality or hyperfrontality in comparison to the negative control sense group. Several clinical studies have also found negative results, wherein patients show neither over- nor under-activation (Curtis et al. 1999; Volz et al. 1999). However, these findings are grossly outnumbered by hypofrontality and hyperfrontality studies, suggesting that our negative findings are likely due to extrinsic factors including performance differences between groups, and

experimental design (Callicott et al. 2003; Kraguljac, Srivastava, and Lahti 2013). Furthermore, given the finding that levels of both synapsin II isoforms were not reduced in antisense animals, and were actually higher than that of control sense rats, it stands to infer that altered brain activation and metabolic function would not be observed in this study.

PET and CT imaging is a valuable tool when studying live animals, as it combines the functional measurements of tissue metabolism from PET imaging with the anatomical reference obtained from CT imaging. When used in live animals, the fused imaging method ensures accurate localization of functionally aberrant tissue, and allows for increased translatability, live animal analysis, and ease of measurement (Daya et al. 2014). The advantages of PET and CT fused imaging render this technique more favourable to identify abnormalities in cortical function in the live animal. To our knowledge, if synapsin II reductions had occurred, this would have been only the second study to model cortical hypofrontality and/or hyperfrontality, using PET imaging, in the live rat. Further studies are required to better determine whether synapsin II knockdown in the mPFC results in hypo- or hyperfrontality.

5.6 Bilateral 14-day infusion of antisense sequences into the medial prefrontal cortex did not result in reduced expression of synapsin II

For almost three decades, antisense oligonucleotides have been used as an effective and cost efficient means to modify specific gene expression both *in vivo* and *in vitro* (Scanlon et al. 1995). Oligonucleotide sequences are prone to degradation *in vivo*,

and can be stabilized using modifications such as replacing the existing phosphodiester bonds with phosphorothioate bonds. With regards to synapsin II and schizophrenia, our lab has shown that use of fully phosphorothioated synapsin II antisense sequences in the mPFC does induce biochemical and behavioural abnormalities, similar to those seen in patients and other animal models of the disorder (Dyck et al. 2011). Due to recent discrepancies in our studies attempting to replicate previous findings, we sought to confirm the validity and reliability of this knockdown technique and the use of control sequences.

Bilateral infusion of synapsin II antisense solution into the mPFC for 14 consecutive days did not yield a synapsin II knockdown in the region of interest, nor the surrounding neural areas including the PFC and the striatum (data not included). Results imply that the lack of reduction in synapsin II protein expression levels can account for the absence of schizophrenia-like symptoms noted in these rodents. Interestingly, rats infused with the negative control, sense oligonucleotides, displayed a slight decrease in synapsin II levels relative to the antisense treated animals ($p>0.05$). Taken together, it can be concluded that the AS oligonucleotides were ineffective at inhibiting the translational process, although the precise reasoning behind the lack of effect remains unknown.

The most likely of reasons stems from the toxicity that has been associated with first generation, fully phosphorothioated oligonucleotide sequences. As noted earlier, an undesirable feature of such oligonucleotides is their ability to bind non-specifically to proteins such as heparin-binding proteins, resulting in cellular toxicity (Kurreck 2003).

The presence of phosphorothioated DNA oligonucleotides has also been shown to alter normal clotting cascade functioning (Kurreck 2003), while sequences comprised entirely of PS linkages have induced localized necrosis (Guzowski et al. 2000; Widnell et al. 1996). Similar observations were made when sacrificing and collecting brain tissue from our rodents. In several AS infused rats, blood clots were visible in the medial prefrontal cortical region, and the tissue was necrotized (images not shown). It is possible that the oligonucleotide induced cell death prevented synapsin II knockdown in the mPFC, and therefore, led to the lack of manifestation of schizophrenia-like abnormalities in the rodents. It is of interest to note that the negative control group, infused with synapsin II sense oligonucleotides, also displayed localized necrosis although to a much lesser extent that seen in antisense infused rodents (images not shown). This finding may explain the reduction, albeit non-significant, in synapsin II levels in the sense group.

Considering the possibility of cellular toxicity with the use of phosphorothioated oligonucleotide sequences, other research groups have chosen to utilize chimeric phosphorotioate/phosphodiester linked sequences, and have found few disadvantages. Regarding our current study, further research was required to better assess the use of a partially modified synapsin II antisense oligonucleotide and determine its efficacy, potency, and toxicity.

5.7 Bilateral 14-day infusion of antisense sequences into the medial prefrontal cortex did not result in reduced striatal expression of parvalbumin

The use of completely PS-modified synapsin II antisense oligonucleotides did not result in reductions of subcortical parvalbumin expression. Striatal tissue was easily collected in both sense and AS treated rats, allowing for more accurate immunoblotting unlike some of the necrotized mPFC tissue samples that were obtained. Thus, it was deduced that our lack of findings was not influenced by cell death within the striatum.

Given the negative results observed in Section 5.5 regarding synapsin II protein expression in the mPFC, it was expected that PV expression would also not be affected. Until recently, the majority of studies investigating the cognitive deficiencies of schizophrenia have focused on the PFC and the hippocampus (Simpson, Kellendonk, and Kandel 2010). Indeed, it has been established that PV-regulated gamma oscillatory waves in the DLPFC are required for working memory and cognitive control (Bartos, Vida, and Jonas 2007) Despite its role in motor control, longstanding evidence has implicated the striatum and its cortical connections as having a possible role in the development of the prodromal symptoms of schizophrenia (Simpson, Kellendonk, and Kandel 2010). Striatal activation has been observed in healthy human subjects while they perform the Wisconsin Card Sorting Test, a test that assesses attentional set shifting and flexibility in thought (Rogers et al. 2000). Furthermore, research from the 1960s first demonstrated that lesioning the striatum of monkeys and murine animals resulted in cognitive performances that bore resemblance to the alterations seen in animals with PFC lesions (Battig,

Rosvold, and Mishkin 1962; Chorover and Gross 2010). Additional clinical and pre-clinical studies conducted over the past two decades have continued to theorize the role of the STR in modulating motor control, learning, and formation of complex behaviours including executive functioning (Simpson, Kellendonk, and Kandel 2010). Therefore, striatal PV protein expression may help mediate the region's ability to control working memory and executive functioning performance.

5.8 Bilateral knockdown of synapsin II in the medial prefrontal cortex, using chimeric antisense sequences leads to transient impairments in prepulse inhibition

Prepulse inhibition is a commonly used, reliable, valid, and highly translatable tool in preclinical schizophrenia research. Due to the strong face validity of the paradigm, assessing for deficits in sensorimotor gating was critical when testing the efficacy of our newly designed chimeric PS/phosphodiester oligonucleotides.

Results demonstrate that, one week following infusion of partially modified oligonucleotide sequences, a reduction in PPI occurred with antisense treatment ($p>0.05$), in relation to both negative control aCSF and sense groups. While infusion with sense oligonucleotides did not yield similar results to our alternate control aCSF group ($p>0.05$), percent PPI was slightly elevated when compared to both antisense infused groups. Despite a lack of significance, the findings suggest that the chimeric antisense and sense oligonucleotides differ in their functionality. However, when PPI was assessed at the end of the 14-day infusion period, the trend was no longer observed. Levels of PPI

were comparable amongst all groups ($p>0.05$), suggesting that external factors may have affected the PPI paradigm, or the knockdown effect had worn off by this time point.

In assessing rodent PPI, there are several factors known to influence results: gender, age, and animal handling (Swerdlow et al. 1995). Sex-specific differences in PPI have been observed in patients with schizophrenia (Kumari 2011), although previous findings from our lab have demonstrated that gender does not influence sensorimotor gating ability in a synapsin II knockdown animal model of schizophrenia (Tan 2014). Furthermore, no difference in PPI in response to acoustic stimuli was noted between normal female and male Sprague Dawley rats (Shaikh and Schmid 2014). The age of the rodent, at time of testing, has also been shown to affect PPI results. Prepubescent rats tend to exhibit weaker and more variable PPI responses than adults (Braff and Geyer 1990). However, older rats should not be used as they may experience frequency-dependent hearing loss (Braff and Geyer 1990). Our cohort of adult, male Sprague Dawley rats weighed between 320-330 grams when PPI was tested, corresponding to 11 weeks in age, which can be associated with peak adulthood in these animals. Therefore, the lack of PPI seen following 14 days of continuous antisense infusions should not have resulted from any underlying age or gender influences. Improper animal handling can induce stress in the animals and significantly alter startle behaviour due to the sensitivity of PPI testing. It is believed, however, that minimal stress was induced in this cohort of animals, as precautions were taken to reduce anxiety caused by the presence of the researchers, the testing chambers, and the surgical process.

Instead, negative PPI results likely were due to the nature of the infused, partially modified oligonucleotides, where the internal portion of the sequence, only bonded via phosphodiester linkages, was vulnerable to nuclease degradation. Consequently, beyond the one week infusion point, it is possible that the infused oligonucleotides had degraded and therefore, were no longer effective at inducing RNAse H cleavage of the synapsin II mRNA. However, *in vivo* oligonucleotide stability is affected primarily by nucleolytic activity occurring as 5' and 3' exonuclease activity (Dagle et al. 1991). Thus, by including 3 phosphorothioate linkages at both the 5' and 3' oligonucleotide ends, there was a greater likelihood of inhibiting inter-nucleotide exonuclease degradation. Results from locomotor activity and immunochemistry testing will help supplement our understanding of the situation.

5.9 Bilateral knockdown of synapsin II in the medial prefrontal cortex, using chimeric antisense sequences leads to excess locomotor activity

Increases in total distance travelled over a set period of time, known as hyperlocomotion, have been observed in animal models of schizophrenia. Hyperlocomotion is often considered as an accepted model of the positive symptoms of schizophrenia, despite lacking face validity. However, excess locomotor activity is consistently observed across many established preclinical animal models of the disorder, and can therefore continue to be utilized for research purposes.

Locomotor activity was assessed in this cohort of animals both one- and two-weeks following infusion of oligonucleotide sequences. Results from the one-week

analysis demonstrated that rodents infused with a single AS sequence traversed a greater distance than both control groups and rats that received the cocktail solution of two AS sequences ($p>0.05$). By the end of the 14 day infusion period, rats that received the single AS sequence displayed significantly more locomotor activity ($*p<0.05$) than the remaining groups. Combined, the results suggest that use of partially modified synapsin II antisense oligonucleotides is sufficient to induce some schizophrenia-like behavioural abnormalities. However, it was apparent that the effect of protein knockdown on the development of positive schizophrenic phenotypes was affected by the number of antisense oligonucleotide sequences infused.

The cocktail solution, comprised of two overlapping synapsin II antisense sequences, was designed to knockdown both the synapsin IIa and IIb isoforms (Dyck et al. 2011). Theoretically, rodents infused with this solution should have presented with a more robust schizophrenic phenotype, given findings of reduced synapsin IIa and IIb isoforms in patients with the disorder (Chen et al. 2004a; Chen et al. 2004b; Mirnics et al. 2000; Dyck et al. 2011). However, it appears that the two oligonucleotide sequences may have interfered with one another, hindering the synapsin II knockdown, and thus resulting in the normal locomotor activity observed in these rodents.

5.10 Bilateral 14-day infusion of chimeric antisense sequences into the medial prefrontal cortex resulted in a slight reduction of synapsin IIa reduction, but not the IIb isoform

Our previously reported findings (Section 5.5) demonstrated that infusion of fully phosphorothioated antisense oligonucleotides was insufficient in reducing synapsin II levels within the medial prefrontal cortex. The lack of knockdown was attributed to localized necrosis due to toxic effects from oligonucleotides joined together entirely by phosphorothioate linkages. To minimize the harmful effects of first-generation antisense sequences, we designed a chimeric oligonucleotide, comprised of phosophodiester internal bonds and PS bonds between the first and last 3 nucleotides.

Continuous bilateral infusion of these partially modified sequences into the mPFC resulted in a slight decrease ($p>0.05$) of synapsin IIa protein expression in rats that were treated with either a single or double antisense sequence, when compared to rodents that received negative control sense oligonucleotides. Expression levels of the synapsin IIb isoform were unchanged between all treatment groups. In post-mortem studies, mRNA and protein levels of synapsin IIa and IIb have been shown to be decreased in patients with schizophrenia (Tan et al. 2014; Chen et al. 2004; Chen et al. 2004; Mirnics et al. 2000; Guest et al. 2010). Interestingly, our findings do not mimic earlier results reported by our lab, which demonstrated that infusion of first-generation antisense oligonucleotides led to a reduction of medial prefrontal cortical protein expression of synapsin IIa and IIb isoforms in Sprague Dawley rats (Dyck et al. 2011). It remains

unclear as to why this discrepancy in results was observed between studies conducted by the same research group.

Upon further investigation, we concluded that the difference in findings between our current research and that conducted by Dyck et al. (2011) may have resulted from a difference in coordinates used to locate the medial prefrontal cortex, relative to bregma. Using the initial coordinates obtained from Savelli *et al.* (1995), synapsin II antisense oligonucleotides were infused into the ventral portion of the mPFC for 14 consecutive days. The coordinates were as follows: +2.7 mm anterior; ± 1.5 mm lateral; and -4.0 mm ventral to the surface of the skull (Dyck et al. 2011). In this thesis, the reference coordinates differed only in the depth that the cannula was inserted: -3.0 mm ventral from the surface of the skull. The current set of coordinates was chosen as they better correspond to our region of interest. However, since the change was made, our lab has been unable to observe the development of schizophrenia-like abnormalities to the same extent as was seen by Dyck et al. (2011). It was noted that, at a depth of 4.0 mm, the cannula appeared to be probing the dorsal portion of the corpus callosum.

Of the many brain regions that have been implicated with schizophrenic disturbance, the corpus callosum is not a common one. However, as the largest connecting tract within the human brain, and with key roles including integrating information and inter-hemispheric transfer, it is highly probable that abnormalities in the corpus callosum will impinge on other cortical structures (David 1994; Arnone et al. 2008). A meta-analysis of magnetic resonance imaging studies confirmed that patients

with schizophrenia display reduced corpus callosal areas in comparison to healthy subjects. Callosal atrophy was largest in acute, antipsychotic-naive cases of schizophrenia, and lesser so in chronic cases of the disorder (Arnone et al. 2008). Although the full extent of its involvement in schizophrenia development remains unclear, impairments to the callosum, whether by synapsin II knockdown or cannula-mediated tissue damage, may play a critical role in disease manifestation.

Another possible reason for the observed discrepancies in synapsin II knockdown may be a change in nucleotide sequence synthesis. As stated earlier, the utilized synapsin II sense and antisense oligonucleotides are synthesized and acquired from IDT (Coralville, IA, USA). Oligonucleotides are received in a dehydrated form, to be reconstituted prior to use. In recent years, the company has stated that their method of synthesis has changed in a manner that significantly reduces the final yield of product. While a reduction in oligonucleotide yield should not cause toxicity, the altered method of synthesis may be more prone to contamination at certain procedural time points. However, all IDT synthesized oligonucleotides are analyzed via mass spectrometry to ensure the presence of purified product. It is therefore interesting that the observed discrepancies in our synapsin II antisense model seem to coincide with use of these differently synthesized oligonucleotides.

5.11 Bilateral 14-day infusion of chimeric antisense sequences into the medial prefrontal cortex resulted in reduced striatal parvalbumin expression

In our earlier objective, we were unable to observe differences in striatal PV protein expression following infusion of synapsin II AS oligonucleotides linked entirely by PS bonds **(Section 5.6)**. Negative results were attributed to a lack of synapsin II knockdown in the mPFC due to localized necrosis. It was anticipated, therefore, that after redesigning our oligonucleotide sequences to minimize toxicity, changes in PV levels would occur within the STR in association with any alterations in synapsin II expression in the mPFC.

Results demonstrate a significant reduction of PV expression in the STR of rodents that received two continuous weeks of single AS sequence infusion into the mPFC (n = 2). Interestingly, animals that were infused with two AS sequences displayed levels of PV similar to those observed in rats treated with aCSF or synapsin II sense oligonucleotides. To our knowledge, this is the first study that has found lowered PV in the STR of an animal model of schizophrenia. Future studies are required to increase the sample size and to confirm the presence of reduced striatal PV expression.

The striatum is anatomically and functionally linked to the mPFC via parallel associative loops which project directly between the two regions. In rodents, this loop connects the mPFC with the dorsomedial STR, while the homologous loop in humans bridges the DLPFC to the dorsal striatum. Consequently, spatial working memory and attentional processing deficits can be induced by lesions to either the PFC or the dorsal

STR (Simpson, Kellendonk, and Kandel 2010). Collectively, research suggests that human and rodent working memory and executive functioning is jointly mediated by the PFC and STR. Whether disturbances in the PFC drive striatal dysfunction, or vice-versa, remains unknown.

It was initially postulated that dopaminergic signalling from the striatum may alter cognitive information received from the cortex (Simpson, Kellendonk, and Kandel 2010). Increased expression of D2 and subsequent dopamine transmission in the STR may indirectly regulate cortical activity via the thalamus (**Figure 1B**). Hyperfunctioning of the STR could arise prematurely in schizophrenia manifestation, accounting for the observance of prodromal cognitive deficits, followed by a compensatory reduction of DA expression in the PFC during development (Simpson, Kellendonk, and Kandel 2010). However, cognitive deficits persist following blockade of D2R using antipsychotic drugs, suggesting that alternate neurotransmitters are involved in these processes (Howes and Kapur 2009; Simpson, Kellendonk, and Kandel 2010). In early synapsin II neonate and adult models of schizophrenia, only protein levels of glutamate and GABA transporters were lowered in the cortex. It is further proposed that reduced synapsin II expression in the mPFC results in disrupted glutamate transmission downstream to the caudate/STR. We postulate that synapsin II-induced glutamate dysfunction in the STR may play a causal role in the manifestation of the cognitive deficits of schizophrenia. The reduction of NMDA transmission can decrease striatal PV interneuron activity, therefore preventing PV-mediated inhibition of excitatory pyramidal cells and gamma oscillation formation,

leading to the development of impaired working memory (Section 1.1.3.4) (Rotaru, Lewis, and Gonzalez-Burgos 2012). Hypoglutamatergic activity combined with hyperdopaminergic activity in the STR can also result in reduced GABAergic function within the region, and subsequent decrease of glutamatergic signalling back to the cortex via the direct (D_1) and indirect (D_2) basal ganglia pathways.

Our proposed mechanism of action provides an alternate perspective to the pathophysiology of schizophrenia and the manifestation of prodromal cognitive symptoms. Future research is required to better elucidate the role of PV and how it may be affected by reduced synapsin II expression.

5.12 Confirmation of infusion location and assessment of toxicity of chimeric phosphorothioate/phosphodiester oligonucleotide sequences

Histological analysis demonstrated that the coordinates utilized during stereotactic surgery corresponded correctly with the medial prefrontal cortex. Evans Blue dye (2%) was infused into the mPFC via the implanted bilateral cannula, and visualized upon brain sectioning. The dye had not diffused into the surrounding regions. Furthermore, visual assessment of the surrounding tissue demonstrated that use of chimeric PS/phosphodiester oligonucleotides provided a safer toxicological profile than nucleotide sequences that were linked entirely with PS bonds. Collectively, our findings reaffirmed that these partially modified oligonucleotide sequences were infused directly into the brain region of interest and did not cause excess harm, therefore eliminating

improper localization and toxicity as reasons for the lack of synapsin II knockdown observed in the mPFC.

5.13 Future Directions

Although our findings lend some support to the concept of a causal role of synapsin II in the pathophysiology of schizophrenia, and provides a safer method of reducing synapsin II protein expression in murine animals, further research must be conducted prior to our development of a novel, pre-clinical animal model of schizophrenia. Of utmost importance, the stability of our newly designed, partially phosphorothioated oligonucleotide sequences must be assessed to determine if any *in vivo* degradation occurred. Sense and AS sequences previously removed from the mini-pumps will be run on an agarose gel to observe for the production of a single band or multiple bands, indicating that degradation did not or did occur, respectively. Fresh sequences that had never been infused *in vivo* will be utilized as a control.

Following confirmation of oligonucleotide stability, future studies will be performed using sequences with phosphorothioate caps on the first and last three nucleotides, and phosphodiester internal linkages. These studies will involve in the infusion of synapsin II antisense oligonucleotides into the ventral mPFC, using the coordinates listed by Dyck *et al.* (2011): +2.7 mm anterior; ± 1.5 mm lateral; and -4.0 mm ventral to the surface of the skull. By conducting experiments using our lab's previous coordinates, we will hopefully gain a better understanding of site-dependent synapsin II knockdown in the manifestation of schizophrenic-like abnormalities. Findings from such

a study will confirm whether previously observed schizophrenic behaviours were a result of infusion into the dorsal region of the corpus callosum, and the more ventral region of the mPFC (Dyck et al. 2011). Once the efficacy of the oligonucleotides and the knockdown effect has been confirmed, we will be able to reassess the effects of a synapsin II mPFC knockdown in neonates. Considering the safer toxicological profile of the partially phosphorothioated sequences, it is likely that we will see more accurate results that are truly indicative of the administered treatments.

In light of our findings of reduced striatal PV in synapsin II AS infused rats, further studies must be performed to better elucidate the relationship between synapsin II and subcortical GABAergic signalling, and its influence on the manifestation of the cognitive symptoms of schizophrenia.

6. CONCLUSIONS

1. We provide evidence that sub-chronic, pre-weaning maternal separation is insufficient to cause schizophrenia-like changes throughout development. Separating pups from their mother, but not their littermates, for 9 days, between PD 14-23, for 4 hours a day, does not lead to behavioural abnormalities reminiscent of those seen in other animal models of schizophrenia. These findings are crucial to future neurodevelopmental studies that further investigate the effects of early-life synapsin II reduction in the medial prefrontal cortex. The surgical procedure and infusion of antisense oligonucleotides coincides with the PD 14-23 timeframe, as synapsin II is maximally expressed in rodents by PD 14. Our

negative results eliminate maternal separation as a confounding factor in the novel developmental animal model of schizophrenia.

2. Knockdown efficacy when using first-generation oligonucleotides is dependent on the amount of phosphorothioate bonds used to stabilize the DNA sequence. We find that infusion of synapsin II antisense sequences containing PS linkages between every nucleotide, has harmful side-effects that hinder the knockdown effect via localized tissue death. No abnormal schizophrenic-like behaviours are observed, nor is there a reduction of synapsin II in the mPFC when using fully-phosphorothioated antisense sequences. Instead, we observe a safer and more efficacious profile with partial oligonucleotide phosphorothioation. Stabilizing the initial and terminal 3 nucleotides of the synapsin II sequence, while maintaining the native internal composition, results in slightly reduced levels of medial prefrontal cortical synapsin II protein expression and increased locomotor activity in antisense rats. Future studies can assess for face, construct, and predictive validity, in hopes of developing a novel and more comprehensive animal model of schizophrenia.

3. We present novel evidence of reduced parvalbumin expression within the striatum of an animal model of schizophrenia. Additional analysis of striatal PV levels is currently underway in tissue samples obtained from successful synapsin II knockdown rats (Dyck et al. 2011), to assess for further construct validity in this

disease model. Further research is required to strengthen any findings from the synapsin II knockdown model of schizophrenia. The striatum has long been considered a region of motor control, but has also been implicated in the regulation of complex cognitive function. Based on existing evidence that working memory and cognitive control in the DLPFC is modulated by PV-mediated gamma oscillations, it can be inferred that PV plays a similar role in the striatum. Reduced synapsin II may down-regulate glutamatergic and PV activity in both the PFC and STR, contributing to the development of impairments in complex cognitive ability. Future studies can investigate the function of striatal parvalbumin and its link to the manifestation of cognitive deficits in schizophrenia, in hopes of identifying novel therapeutic targets for treatment of the prodromal symptoms that currently go untreated with present-day antipsychotic drugs.

7. REFERENCE LIST

Amitai, Nurith, and Athina Markou. 2009. "Increased Impulsivity and Disrupted Attention Induced by Repeated Phencyclidine Are Not Attenuated by Chronic Quetiapine Treatment." *Pharmacology Biochemistry and Behavior* 93 (3): 248–57. doi:10.1037/a0030561.Striving.

Andersen, Susan L. 2003. "Trajectories of Brain Development: Point of Vulnerability or Window of Opportunity?" *Neuroscience & Biobehavioral Reviews* 27 (1-2): 3–18. doi:10.1016/S0149-7634(03)00005-8.

Anglin, Deidre M., Patricia R. Cohen, and Henian Chen. 2008. "Duration of Early Maternal Separation and Prediction of Schizotypal Symptoms from Early Adolescence to Midlife." *Schizophrenia Research* 103 (1-3): 143–50. doi:10.1016/j.schres.2008.02.016.

Arnone, D, A M McIntosh, G M Y Tan, and K P Ebmeier. 2008. "Meta-Analysis of Magnetic Resonance Imaging Studies of the Corpus Callosum in Schizophrenia." *Schizophrenia Research* 101 (1-3): 124–32. doi:10.1016/j.schres.2008.01.005.

Bardgett, Mark E., Megan Points, Christian Ramsey-Faulkner, Jeff Topmiller, John Roflow, Travis McDaniel, Timberly Lamontagne, and Molly S. Griffith. 2008. "The Effects of Clonidine on Discrete-Trial Delayed Spatial Alternation in Two Rat Models of Memory Loss" 33 (8): 1980–91. doi:10.1016/j.drugalcdep.2008.02.002.A.

Bartos, Marlene, Imre Vida, and Peter Jonas. 2007. “Synaptic Mechanisms of Synchronized Gamma Oscillations in Inhibitory Interneuron Networks.” *Nature Reviews. Neuroscience* 8 (1): 45–56. doi:10.1038/nrn2044.

Battig, K, H E Rosvold, and M Mishkin. 1962. “Comparison of the Effects of Frontal and Caudate Lesions on Discrimination Learning in Monkeys.” *J Comp Physiol Psychol* 55 (4): 458–63.

Birrell, J M, and V J Brown. 2000. “Medial Frontal Cortex Mediates Perceptual Attentional Set Shifting in the Rat.” *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 20 (11): 4320–24. doi:20/11/4320 [pii].

Bogen, Inger Lise, Jean-Luc Boulland, Espen Mariussen, Marianne S. Wright, Frode Fonnum, Hung-Teh Kao, and S. Ivar Walaas. 2006. “Absence of Synapsin I and II Is Accompanied by Decreases in Vesicular Transport of Specific Neurotransmitters.” *Journal of Neurochemistry* 96 (5): 1458–66. doi:10.1111/j.1471-4159.2005.03636.x.

Bouet, Valentine, Blandine Lecrux, Gwenaelle Tran, and Thomas Freret. 2011. “Effect of Pre- versus Post-Weaning Environmental Disturbances on Social Behaviour in Mice.” *Neuroscience Letters* 488 (2). Elsevier Ireland Ltd: 221–24. doi:10.1016/j.neulet.2010.11.033.

Braff, David L., and Mark A. Geyer. 1990. “Sensorimotor Gating and Schizophrenia.” *Archives of General Psychiatry* 47 (2): 181–88.

Braff, David L., Mark a. Geyer, and Neal R. Swerdlow. 2001. "Human Studies of Prepulse Inhibition of Startle: Normal Subjects, Patient Groups, and Pharmacological Studies." *Psychopharmacology* 156 (2-3): 234–58. doi:10.1007/s002130100810.

Callicott, Joseph H., Venkata S. Mattay, Beth a. Verchinski, Stefano Marenco, Michael F. Egan, and Daniel R. Weinberger. 2003. "Complexity of Prefrontal Cortical Dysfunction in Schizophrenia: More Than Up or Down." *American Journal of Psychiatry* 160 (12): 2209–15. doi:10.1176/appi.ajp.160.12.2209.

Carlsson, Arvid, Nicholas Waters, Susanna Holm-Waters, Joakim Tedroff, Marie Nilsson, and Maria L. Carlsson. 2001. "Interactions between Monoamines, Glutamate, and GABA in Schizophrenia: New Evidence." *Annual Review of Pharmacology and Toxicology*, no. 41: 237–60.

Carr, D B, and S R Sesack. 2000. "Projections from the Rat Prefrontal Cortex to the Ventral Tegmental Area: Target Specificity in the Synaptic Associations with Mesoaccumbens and Mesocortical Neurons." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 20 (10): 3864–73. doi:http://www.jneurosci.org/content/20/10/3864.

Cesca, F., P. Baldelli, F. Valtorta, and F. Benfenati. 2010. "The Synapsins: Key Actors of Synapse Function and Plasticity." *Progress in Neurobiology* 91 (4). Elsevier Ltd: 313–48. doi:10.1016/j.pneurobio.2010.04.006.

Chan, Jasmine H P, Shuhui Lim, and W S Fred Wong. 2006. "Frontiers in Research Review : Cutting-Edge Molecular Approaches to Therapeutics ANTISENSE OLIGONUCLEOTIDES : FROM DESIGN TO THERAPEUTIC APPLICATION." *Clinical and Experimental Pharmacology and Physiology* 33 (February): 533–40. doi:10.1111/j.1440-1681.2006.04403.x.

Chen, Qi, Guang He, Wei Qin, Qing-ying Chen, Xin-zhi Zhao, Shi-wei Duan, Xin-min Liu, et al. 2004. "Family-Based Association Study of Synapsin II and Schizophrenia." *American Journal of Human Genetics* 75 (5): 873–77. doi:10.1086/425588.

Chen, Qi, Guang He, Xiao Yan Wang, Qing Ying Chen, Xin Min Liu, Zhong Zhong Gu, Jie Liu, et al. 2004. "Positive Association between Synapsin II and Schizophrenia." *Biological Psychiatry* 56 (3): 177–81. doi:10.1016/j.biopsych.2004.05.010.

Chorover, Stephan L, and Charles G Gross. 2010. "Caudate Nucleus Lesions : Behavioral Effects in the Rat Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/1711748" 141 (3583): 826–27.

Clapcote, Steven J., Tatiana V. Lipina, J. Kirsty Millar, Shaun Mackie, Sheila Christie, Fumiaki Ogawa, Jason P. Lerch, et al. 2007. "Behavioral Phenotypes of Disc1 Missense Mutations in Mice." *Neuron* 54 (3): 387–402. doi:10.1016/j.neuron.2007.04.015.

Curtis, V A, E T Bullmore, R G Morris, M J Brammer, S C Williams, A Simmons, T Sharma, R M Murray, and P K McGuire. 1999. "Attenuated Frontal Activation in Schizophrenia May Be Task Dependent." *Schizophrenia Research* 37 (1): 35–44. http://www.ncbi.nlm.nih.gov/pubmed/10227106.

Dagle, J M, M E Andracki, R J DeVine, and J a Walder. 1991. "Physical Properties of Oligonucleotides Containing Phosphoramidate-Modified Internucleoside Linkages." *Nucleic Acids Research* 19 (8): 1805–10. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=328108&tool=pmcentrez&rendertype=abstract.

David, A S. 1994. "Schizophrenia and the Corpus Callosum: Developmental, Structural and Functional Relationships." *Behavioural Brain Research* 64 (1-2): 203–11. http://www.ncbi.nlm.nih.gov/pubmed/7840887.

Daya, R. P., J. K. Bhandari, P. a. Hui, Y. Tian, T. Farncombe, and R. K. Mishra. 2014. "Effects of MK-801 Treatment across Several Pre-Clinical Analyses Including a Novel Assessment of Brain Metabolic Function Utilizing PET and CT Fused

Imaging in Live Rats." *Neuropharmacology* 77. Elsevier Ltd: 325–33. doi:10.1016/j.neuropharm.2013.10.001.

Dyck, Bailey A., Michael G R Beyaert, Mark A. Ferro, and Ram K. Mishra. 2011. "Medial Prefrontal Cortical Synapsin II Knock-down Induces Behavioral Abnormalities in the Rat: Examining Synapsin II in the Pathophysiology of Schizophrenia." *Schizophrenia Research* 130 (1-3). Elsevier B.V.: 250–59. doi:10.1016/j.schres.2011.05.017.

Dyck, Bailey A., and Ram K. Mishra. 2011. "Regulation of Synapsin II by Dopaminergic Mechanisms." In *Dopamine: Function, Regulation and Health Effects*. New York: Nova Science Publishers.

Ellenbroek, Bart a., and Marco a. Riva. 2003. "Early Maternal Deprivation as an Animal Model for Schizophrenia." *Clinical Neuroscience Research* 3 (4-5): 297–302. doi:10.1016/S1566-2772(03)00090-2.

Ellenbroek, Bart a., Peter T.J.M van den Kroonenberg, and Alexander R. Cools. 1998. "The Effects of an Early Stressful Life Event on Sensorimotor Gating in Adult Rats." *Schizophrenia Research* 30: 251–60.

Fatemi, S. H., and T. D. Folsom. 2009. "The Neurodevelopmental Hypothesis of Schizophrenia, Revisited." *Schizophrenia Bulletin* 35 (3): 528–48. doi:10.1093/schbul/sbn187.

Featherstone, R.E., S. Kapur, and P.J. Fletcher. 2007. "The Amphetamine-Induced Sensitized State as a Model of Schizophrenia." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 31 (8): 1556–71. doi:10.1016/j.pnpbp.2007.08.025.

Ferreira, a, H T Kao, J Feng, M Rapoport, and P Greengard. 2000. "Synapsin III: Developmental Expression, Subcellular Localization, and Role in Axon Formation." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 20 (10): 3736–44. http://www.ncbi.nlm.nih.gov/pubmed/10804215.

File, S E. 1980. "Naloxone Reduces Social and Exploratory Activity in the Rat." *Psychopharmacology* 71: 41–44. doi:10.1007/BF00433250.

Fletcher, Paul J, Catherine C Tenn, Judy Sinyard, Zoë Rizos, and Shitij Kapur. 2007. "A Sensitizing Regimen of Amphetamine Impairs Visual Attention in the 5-Choice Serial Reaction Time Test: Reversal by a D1 Receptor Agonist Injected into the Medial Prefrontal Cortex." *Neuropsychopharmacology* 32 (5): 1122–32. doi:10.1038/sj.npp.1301221.

Floresco, Stan B, Orsolya Magyar, Sarvin Ghods-Sharifi, Claudia Vexelman, and Maric T L Tse. 2006. "Multiple Dopamine Receptor Subtypes in the Medial Prefrontal Cortex of the Rat Regulate Set-Shifting." *Neuropsychopharmacology* 31 (2): 297–309. doi:10.1038/sj.npp.1300825.

Fornasiero, Eugenio F., Dario Bonanomi, Fabio Benfenati, and Flavia Valtorta. 2010. "The Role of Synapsins in Neuronal Development." *Cellular and Molecular Life Sciences* 67 (9): 1383–96. doi:10.1007/s00018-009-0227-8.

Frankle, W. Gordon, Raymond Y. Cho, Konasale M. Prasad, N. Scott Mason, Jennifer Paris, Michael L. Himes, Christopher Walker, David a. Lewis, and Rajesh Narendran. 2015. "In Vivo Measurement of GABA Transmission in Healthy Subjects and Schizophrenia Patients." *American Journal of Psychiatry*, no. 24: appi.ajp.2015.1. doi:10.1176/appi.ajp.2015.14081031.

Geyer, M. a., N. R. Swerdlow, R. S. Mansbach, and D. L. Braff. 1990. "Startle Response Models of Sensorimotor Gating and Habituation Deficits in Schizophrenia." *Brain Research Bulletin* 25 (3): 485–98. doi:10.1016/0361-9230(90)90241-Q.

Gitler, D. 2004. "Molecular Determinants of Synapsin Targeting to Presynaptic Terminals." *Journal of Neuroscience* 24 (14): 3711–20. doi:10.1523/JNEUROSCI.5225-03.2004.

Glausier, J R, K N Fish, and D a Lewis. 2014. "Altered Parvalbumin Basket Cell Inputs in the Dorsolateral Prefrontal Cortex of Schizophrenia Subjects." *Molecular Psychiatry* 19 (1): 30–36. doi:10.1038/mp.2013.152.

Goeree, R., F. Farahati, N. Burke, G. Blackhouse, D. O'Reilly, J. Pyne, and J.-E. Tarride. 2005. "The Economic Burden of Schizophrenia in Canada in 2004." *Current Medical Research and Opinion* 21 (12): 2017–28. doi:10.1185/030079905X75087.

Goff, Dc C, and Joseph T Coyle. 2001. "The Emerging Role of Glutamate in the Pathophysiology and Treatment of Schizophrenia." *American Journal of Psychiatry* 158 (16): 1367–77. doi:10.1176/appi.ajp.158.9.1367.

Greengard, P, F Valtorta, a J Czernik, and F Benfenati. 1993. "Synaptic Vesicle Phosphoproteins and Regulation of Synaptic Function." *Science* 259: 780–85.

Guest, Kelly a., Bailee a. Dyck, Shazli Shethwala, and Ram K. Mishra. 2010. "Atypical Antipsychotic Drugs Upregulate Synapsin II in the Prefrontal Cortex of Post-Mortem Samples Obtained from Patients with Schizophrenia." *Schizophrenia Research* 120 (1-3): 229–31. doi:10.1016/j.schres.2010.03.029.

Guzowski, J F, G L Lyford, G D Stevenson, F P Houston, J L McGaugh, P F Worley, and C a Barnes. 2000. "Inhibition of Activity-Dependent Arc Protein Expression in the Rat Hippocampus Impairs the Maintenance of Long-Term Potentiation and the Consolidation of Long-Term Memory." *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 20 (11): 3993–4001. doi:20/11/3993 [pii].

Harrison, Paul., and Michael J Owen. 2003. "Genes for Schizophrenia? Recent Findings and Their Psychopathological Implications." *The Lancet* 361: 417.

Hikida, Takatoshi, Hanna Jaaro-Peled, Saurav Seshadri, Kenichi Oishi, Caroline Hookway, Stephanie Kong, Di Wu, et al. 2007. "Dominant-Negative DISC1 Transgenic Mice Display Schizophrenia-Associated Phenotypes Detected by Measures Translatable to Humans." *Proceedings of the National Academy of Sciences of the United States of America* 104 (36): 14501–6. doi:10.1073/pnas.0704774104.

Hilfiker, S, V a Pieribone, a J Czernik, H T Kao, G J Augustine, and P Greengard. 1999. "Synapsins as Regulators of Neurotransmitter Release." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 354 (1381): 269–79. doi:10.1098/rstb.1999.0378.

Hilgard, Josephine R., and Martha F. Newman. 1963. "Early Parental Deprivation as a Functional Factor in the Etiology of Schizophrenia and Alcoholism." *American Journal of Orthopsychiatry* 33 (3): 409–20.

Homayoun, H., and B. Moghaddam. 2007. "NMDA Receptor Hypofunction Produces Opposite Effects on Prefrontal Cortex Interneurons and Pyramidal Neurons." *Journal of Neuroscience* 27 (43): 11496–500. doi:10.1523/JNEUROSCI.2213-07.2007.

Hooper, M L, B J Chiasson, and H a Robertson. 1994. "Infusion into the Brain of an Antisense Oligonucleotide to the Immediate-Early Gene c-Fos Suppresses

Production of Fos and Produces a Behavioral Effect." *Neuroscience* 63 (4): 917–24. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7700517.

Howes, O. D., M. Williams, K. Ibrahim, G. Leung, a. Egerton, P. K. McGuire, and F. Turkheimer. 2013. "Midbrain Dopamine Function in Schizophrenia and Depression: A Post-Mortem and Positron Emission Tomographic Imaging Study." *Brain* 136 (11): 3242–51. doi:10.1093/brain/awt264.

Howes, Oliver D., and Shitij Kapur. 2009. "The Dopamine Hypothesis of Schizophrenia: Version III - The Final Common Pathway." *Schizophrenia Bulletin* 35 (3): 549–62. doi:10.1093/schbul/sbp006.

Jaaro-Peled, Hanna. 2009. "Gene Models of Schizophrenia: DISC1 Mouse Models," no. October: 75–86. doi:10.1016/S0079-6123(09)17909-8.

Javitt, Daniel C., and Stephen R. Zukin. 1991. "Recent Advances in the Phencyclidine Model of Schizophrenia." *American Journal of Psychiatry* 148 (10): 1301–8. doi:10.1176/ajp.148.10.1301.

Jentsch, J. David, and Robert H. Roth. 1999. "The Neuropsychopharmacology of Phencyclidine: From NMDA Receptor Hypofunction to the Dopamine Hypothesis of Schizophrenia." *Neuropsychopharmacology* 20 (3): 201–25. doi:10.1016/S0893-133X(98)00060-8.

Jones, C A, D J G Watson, and K C F Fone. 2011. “Animal Models of Schizophrenia.” *British Journal of Pharmacology* 164 (4): 1162–94. doi:10.1111/j.1476-5381.2011.01386.x.

Kao, H T, B Porton, S Hilfiker, G Stefani, V a Pieribone, R DeSalle, and P Greengard. 1999. “Molecular Evolution of the Synapsin Gene Family.” *The Journal of Experimental Zoology* 285 (4): 360–77. doi:10.1002/(SICI)1097-010X(19991215)285:4<360::AID-JEZ4>3.0.CO;2-3.

Kapur, Shitij, Robert Zipursky, Corey Jones, C.S Shammi, Gary Remington, and Philip Seeman. 2000. “A Positron Emission Tomography Study of Quetiapine in Schizophrenia.” *JAMA Psychiatry* 57 (6): 553–59.

Kraguljac, Nina V, Annusha Srivastava, and Adrienne C Lahti. 2013. “Memory Deficits in Schizophrenia: A Selective Review of Functional Magnetic Resonance Imaging (FMRI) Studies.” *Behavioral Sciences (Basel, Switzerland)* 3 (3). Multidisciplinary Digital Publishing Institute: 330–47. doi:10.3390/bs3030330.

Kumari, Veena. 2011. “Sex Differences and Hormonal Influences in Human Sensorimotor Gating: Implicaitons for Schizophrenia.” *Current Topics in Behavioural Neurosciences* 8: 141–54. doi:10.1007/7854.

Kurreck, Jens. 2003. "Antisense Technologies. Improvement through Novel Chemical Modifications." *European Journal of Biochemistry* 270 (8): 1628–44. doi:10.1046/j.1432-1033.2003.03555.x.

Laruelle, Marc, Lawrence S. Kegeles, and Anissa Abi-Dargham. 2003. "Glutamate, Dopamine, and Schizophrenia from Pathophysiology to Treatment." *Annals of the New York Academy of Sciences* 1003 (October 2015): 138–58. doi:10.1196/annals.1300.063.

Lebedeva, Irina, and C. A. Stein. 2001. "Antisense Oligonucleotides: Promise and Reality." *Annual Review of Pharmacology and Toxicology* 41: 403–19.

Lee, Hee Jae, Ji Young Song, Jong Woo Kim, Sheng-Yu Jin, Mi Suk Hong, Jin Kyoung Park, Joo-Ho Chung, Hiroki Shibata, and Yasuyuki Fukumaki. 2005. "Association Study of Polymorphisms in Synaptic Vesicle-Associated Genes, SYN2 and CPLX2, with Schizophrenia." *Behavioral and Brain Functions : BBF* 1: 15. doi:10.1186/1744-9081-1-15.

Lehmann, J, C R Pryce, and J Feldon. 2000. "Lack of Effect of an Early Stressful Life Event on Sensorimotor Gating in Adult Rats." *Schizophrenia Research* 41 (2): 365–71.

Lencz, Todd, Christopher W Smith, Danielle McLaughlin, Andrea Auther, Emilie Nakayama, Lauren Hovey, and Barbara a Cornblatt. 2006. "Generalized and Specific

Neurocognitive Deficits in Prodromal Schizophrenia." *Biological Psychiatry* 59 (9): 863–71. doi:10.1016/j.biopsych.2005.09.005.

Lewis, David a, Allison a Curley, Jill Glausier, and David W Volk. 2013. "NIH Public Access" 35 (1): 57–67. doi:10.1016/j.tins.2011.10.004.Cortical.

Lipska, B K, and D R Weinberger. 1993. "Delayed Effects of Neonatal Hippocampal Damage on Haloperidol-Induced Catalepsy and Apomorphine-Induced Stereotypic Behaviors in the Rat." *Brain Research. Developmental Brain Research* 75 (2): 213–22. doi:10.1016/0006-3223(94)00261-Z.

Marcotte, E R, D M Pearson, and L K Srivastava. 2001. "Animal Models of Schizophrenia: A Critical Review." *Journal of Psychiatry & Neuroscience : JPN* 26 (5): 395–410.

McKibben, Claire E., Trisha a. Jenkins, Hayley N. Adams, Michael K. Harte, and Gavin P. Reynolds. 2010. "Effect of Pretreatment with Risperidone on Phencyclidine-Induced Disruptions in Object Recognition Memory and Prefrontal Cortex Parvalbumin Immunoreactivity in the Rat." *Behavioural Brain Research* 208 (1). Elsevier B.V.: 132–36. doi:10.1016/j.bbr.2009.11.018.

Mirnics, Károly, Frank a. Middleton, Adriana Marquez, David a. Lewis, and Pat Levitt. 2000. "Molecular Characterization of Schizophrenia Viewed by Microarray Analysis

of Gene Expression in Prefrontal Cortex." *Neuron* 28 (1): 53–67. doi:10.1016/S0896-6273(00)00085-4.

Molinaro, Luke, Patricia Hui, Mattea Tan, Ram K Mishra, Luke Molinaro, Patricia Hui, Mattea Tan, and Ram K Mishra. 2015. "Schizophrenia" 5 (3): 260–72. doi:10.5498/wjp.v5.i3.260.

Monaldi, Ilaria, Massimo Vassalli, Angela Bachi, Silvia Giovedì, Enrico Millo, Flavia Valtorta, Roberto Raiteri, Fabio Benfenati, and Anna Fassio. 2010. "The Highly Conserved Synapsin Domain E Mediates Synapsin Dimerization and Phospholipid Vesicle Clustering." *The Biochemical Journal* 426: 55–64. doi:10.1042/BJ20090762.

O'Donovan, M. C., N. M. Williams, and M. J. Owen. 2003. "Recent Advances in the Genetics of Schizophrenia." *Human Molecular Genetics* 12 (suppl 2): R125–33. doi:10.1093/hmg/ddg302.

Olton, D S. 1987. "The Radial Arm Maze as a Tool in Behavioral Pharmacology." *Physiology & Behavior* 40 (6): 793–97. http://www.ncbi.nlm.nih.gov/pubmed/3313453.

Owen, Michael J, Michael C O'Donovan, and Paul J Harrison. 2005. "Schizophrenia: A Genetic Disorder of the Synapse?" *BMJ (Clinical Research Ed.)* 330 (January): 158–59. doi:10.1136/bmj.330.7484.158.

Paxinos, George, and Charles Watson. 2006. *The Rat Brain in Stereotaxic Coordinates, 6th Edition*. Hard Cover. Academic Press.

Pletnikov, M V, Y Ayhan, O Nikolskaia, Y Xu, M V Ovanesov, H Huang, S Mori, T H Moran, and C a Ross. 2008. "Inducible Expression of Mutant Human DISC1 in Mice Is Associated with Brain and Behavioral Abnormalities Reminiscent of Schizophrenia." *Molecular Psychiatry* 13 (2): 173–86. doi:10.1038/sj.mp.4002079.

Powell, Craig M, and Tsuyoshi Miyakawa. 2006. "Uniquely Human Disorder ?" *Biological Psychiatry* 59 (12): 1198–1207. doi:10.1016/j.biopsych.2006.05.008.Schizophrenia-Relevant.

Rajagopal, Lakshmi, Bill Massey, Mei Huang, Yoshihiro Oyamada, and Herbert Meltzer. 2014. "The Novel Object Recognition Test in Rodents in Relation to Cognitive Impairment in Schizophrenia." *Current Pharmaceutical Design* 20 (31): 5104–14. doi:10.2174/1381612819666131216114240.

Rees, Stephanie L., Emis Akbari, Meir Steiner, and Alison S. Fleming. 2008. "Effects of Early Deprivation and Maternal Separation on Pup-Directed Behavior and HPA Axis Measures in the Juvenile Female Rat." *Developmental Psychobiology* 50 (4): 315–21. doi:10.1002/dev.20292.

Rodefer, Joshua S, Tuyet N Nguyen, Jens-Jakob Karlsson, and Jørn Arnt. 2008. "Reversal of Subchronic PCP-Induced Deficits in Attentional Set Shifting in Rats by Sertindole

and a 5-HT6 Receptor Antagonist: Comparison Among Antipsychotics." *Neuropsychopharmacology* 33 (11): 2657–66. doi:10.1038/sj.npp.1301654.

Rogers, R D, T C Andrews, P M Grasby, D J Brooks, and T W Robbins. 2000. "Contrasting Cortical and Subcortical Activations Produced by Attentional-Set Shifting and Reversal Learning in Humans." *Journal of Cognitive Neuroscience* 12: 142–62. doi:10.1162/089892900561931.

Rotaru, Diana C., David a. Lewis, and Guillermo Gonzalez-Burgos. 2012. "The Role of Glutamatergic Inputs onto Parvalbumin-Positive Interneurons: Relevance for Schizophrenia." *Review Neuroscience* 23 (1): 97–109. doi:10.1515/revneuro-2011-0059.The.

Sams-Dodd, Frank. 1998. "Effects of Continuous D-Amphetamine and Phencyclidine Administration on Social Behaviour, Stereotyped Behaviour, and Locomotor Activity in Rats." *Neuropsychopharmacology* 19 (1): 18–25. doi:10.1016/S0893-133X(97)00200-5.

Sanders, Brian J, and Alan Anticevic. 2007. "Maternal Separation Enhances Neuronal Activation and Cardiovascular Responses to Acute Stress in Borderline Hypertensive Rats." *Behavioural Brain Research* 183 (1): 25–30. doi:10.1016/j.bbr.2007.05.020.

Savelli, J E, a Chugh, C Cheng, R K Mishra, and R L Johnson. 1995. "Modulation of N-Methyl-D-Aspartate (NMDA) Antagonist-Induced Darting Behaviour by the Peptidomimetic PAMTA." *Brain Research* 682 (1-2): 41–49. doi:10.1016/0006-8993(95)00316-I.

Saviouk, Viatcheslav, Michael P Moreau, Irina V Tereshchenko, and Linda M Brzustowicz. 2007. "Association of Synapsin 2 with Schizophrenia in Families of Northern European Ancestry." *Schizophrenia Research* 96 (1-3): 100–111. doi:10.1016/j.schres.2007.07.031.

Scanlon, K J, Y Ohta, H Ishida, H Kijima, T Ohkawa, A Kaminski, J Tsai, G Horng, and M Kashani-Sabet. 1995. "Oligonucleotide-Mediated Modulation of Mammalian Gene Expression." *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 9 (13): 1288–96. http://www.ncbi.nlm.nih.gov/pubmed/7557018.

Schneider, Miriam, and Michael Koch. 2005. "Behavioral and Morphological Alterations Following Neonatal Excitotoxic Lesions of the Medial Prefrontal Cortex in Rats." *Experimental Neurology* 195 (1): 185–98. doi:10.1016/j.expneurol.2005.04.014.

Seeman, Philip. 2011. "All Roads to Schizophrenia Lead to Dopamine Supersensitivity and Elevated Dopamine D2High Receptors." *CNS Neuroscience & Therapeutics* 17 (2): 118–32. doi:10.1111/j.1755-5949.2010.00162.x.

Shaikh, Komal, and Susanne Schmid. 2014. “There Are No Sex-Specific Differences in Habituation and Prepulse Inhibition of Acoustic Startle Response in Sprague Dawley Rats.” *Western Undergraduate Research Journal: Health & Natural Sciences* 4.

Shalev, Uri, and Neri Kafkafi. 2002. “Repeated Maternal Separation Does Not Alter Sucrose-Reinforced and Open-Field Behaviors.” *Pharmacology Biochemistry and Behavior* 73 (1): 115–22. doi:10.1016/S0091-3057(02)00756-6.

Simpson, Eleanor H., Christoph Kellendonk, and Eric Kandel. 2010. “A Possible Role for the Striatum in the Pathogenesis of the Cognitive Symptoms of Schizophrenia.” *Neuron* 65 (5). Elsevier Inc.: 585–96. doi:10.1016/j.neuron.2010.02.014.

Spieker, Elena A., Robert S. Astur, Jeffrey T. West, Jacqueline A. Griego, and Laura M. Rowland. 2012. “Spatial Memory Deficits in a Virtual Reality Eight-Arm Radial Maze in Schizophrenia.” *Schizophrenia Research* 135: 84–89. doi:10.1016/j.schres.2011.11.014.Spatial.

Squire, L R, and S M Zola. 1996. “Structure and Function of Declarative and Nondeclarative Memory Systems.” *Proceedings of the National Academy of Sciences of the United States of America* 93 (24): 13515–22. doi:10.1073/pnas.93.24.13515.

Stephenson, M L, and P C Zamecnik. 1978. “Inhibition of Rous Sarcoma Viral RNA Translation by a Specific Oligodeoxyribonucleotide.” *Proceedings of the National*

Academy of Sciences of the United States of America 75 (1): 285–88. doi:10.1073/pnas.75.1.285.

Südhof, T C, a J Czernik, H T Kao, K Takei, P a Johnston, a Horiuchi, S D Kanazir, M a Wagner, M S Perin, and P De Camilli. 1989. "Synapsins: Mosaics of Shared and Individual Domains in a Family of Synaptic Vesicle Phosphoproteins." *Science (New York, N.Y.)* 245 (4925): 1474–80. doi:10.1126/science.2506642.

Südhof, Thomas C. 2004. "The Synaptic Vesicle Cycle." *Annual Review of Neuroscience* 27 (1): 509–47. doi:10.1146/annurev.neuro.26.041002.131412.

Swerdlow, N. R., D. L. Braff, M. a. Geyer, B. K. Lipska, D. R. Weinberger, and G. E. Jaskiw. 1995. "Increased Sensitivity to the Sensorimotor Gating-Disruptive Effects of Apomorphine after Lesions of Medial Prefrontal Cortex or Ventral Hippocampus in Adult Rats." *Psychopharmacology* 122 (1): 27–34. doi:10.1007/BF02246438.

Tamminga, C a, and H H Holcomb. 2005. "Phenotype of Schizophrenia: A Review and Formulation." *Molecular Psychiatry* 10 (1): 27–39. doi:10.1038/sj.mp.4001563.

Tan, M L, B a Dyck, J Gabriele, R P Daya, N Thomas, C Sookram, D Basu, M a Ferro, V Z Chong, and R K Mishra. 2014. "Synapsin II Gene Expression in the Dorsolateral Prefrontal Cortex of Brain Specimens from Patients with Schizophrenia and Bipolar Disorder: Effect of Lifetime Intake of Antipsychotic Drugs." *The Pharmacogenomics Journal* 14 (1): 63–69. doi:10.1038/tpj.2013.6.

Tan, Mattea L. 2014. “Role of Synapsin II in Neurodevelopment.”

Tenn, Catherine C., Paul J. Fletcher, and Shitij Kapur. 2005. “A Putative Animal Model of the ‘prodromal’ State of Schizophrenia.” *Biological Psychiatry* 57 (6): 586–93. doi:10.1016/j.biopsych.2004.12.013.

Thierry, Anne Marie, Yves Gioanni, Eric Dégénétais, and Jacques Glowinski. 2000. “Hippocampo-Prefrontal Cortex Pathway: Anatomical and Electrophysiological Characteristics.” *Hippocampus* 10 (4): 411–19. doi:10.1002/1098-1063(2000)10:4<411::AID-HIPO7>3.0.CO;2-A.

Tseng, Kuei-Yuan, Barbara L Lewis, Barbara K Lipska, and Patricio O’Donnell. 2007. “Post-Pubertal Disruption of Medial Prefrontal Cortical Dopamine-Glutamate Interactions in a Developmental Animal Model of Schizophrenia.” *Biological Psychiatry* 62 (7): 730–38. doi:10.1016/j.biopsych.2006.10.012.

Vawter, M P, L Thatcher, N Usen, T M Hyde, J E Kleinman, and W J Freed. 2002. “Reduction of Synapsin in the Hippocampus of Patients with Bipolar Disorder and Schizophrenia.” *Molecular Psychiatry* 7 (6): 571–78. doi:10.1038/sj.mp.4001158.

Volz, H, C Gaser, F Häger, R Rzanny, J Pönisch, H Mentzel, W A Kaiser, and H Sauer. 1999. “Decreased Frontal Activation in Schizophrenics during Stimulation with the Continuous Performance Test--a Functional Magnetic Resonance Imaging Study.”

European Psychiatry : The Journal of the Association of European Psychiatrists 14 (1): 17–24. http://www.ncbi.nlm.nih.gov/pubmed/10572321.

Wang, Qiong, Man Li, Wei Du, Feng Shao, and Weiwen Wang. 2015. "The Different Effects of Maternal Separation on Spatial Learning and Reversal Learning in Rats." *Behavioural Brain Research* 280. Elsevier B.V.: 16–23. doi:10.1016/j.bbr.2014.11.040.

Widnell, K L, D W Self, S B Lane, D S Russell, V a Vaidya, M J Miserendino, C S Rubin, R S Duman, and E J Nestler. 1996. "Regulation of CREB Expression: In Vivo Evidence for a Functional Role in Morphine Action in the Nucleus Accumbens." *The Journal of Pharmacology and Experimental Therapeutics* 276 (1): 306–15. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8558448.

Winters, B. D. 2004. "Double Dissociation between the Effects of Peri-Postrhinal Cortex and Hippocampal Lesions on Tests of Object Recognition and Spatial Memory: Heterogeneity of Function within the Temporal Lobe." *Journal of Neuroscience* 24 (26): 5901–8. doi:10.1523/JNEUROSCI.1346-04.2004.

Winters, Boyer D., Lisa M. Saksida, and Timothy J. Bussey. 2008. "Object Recognition Memory: Neurobiological Mechanisms of Encoding, Consolidation and Retrieval."

Neuroscience and Biobehavioral Reviews 32 (5): 1055–70. doi:10.1016/j.neubiorev.2008.04.004.

Wolf, Lorraine E., Barbara a. Cornblatt, Simone a. Roberts, Barbara Maminski Shapiro, and L. Erlenmeyer-Kimling. 2002. "Wisconsin Card Sorting Deficits in the Offspring of Schizophrenics in the New York High-Risk Project." *Schizophrenia Research* 57: 173–82. doi:10.1016/S0920-9964(01)00301-2.