THE ROLE OF SYNAPSIN II IN NEUROLOGICAL DISORDERS

INVESTIGATING THE ROLE OF SYNAPSIN II IN NEUROLOGICAL DISORDERS INVOLVING DYSREGULATED DOPAMINERGIC TRANSMISSION

By

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Abstract

Schizophrenia (SCZ) is a debilitating mental illness that affects roughly 1 % of the world's population. Current theories about the etiology of this disease highlight disruptions in dopamine (DA) and glutamate. However, a more recent theory, the 'synaptic hypothesis' proposes that the fundamental pathology of this illness involves disruptions in synaptic transmission. The synapsins are a family of neuron specific phosphoproteins that play an important role in neurotransmitter release, synapse formation and maintaining a reserve pool of synaptic vesicles. Previous research has suggested that synapsin II has a role in the etiology of SCZ. For example, synapsin II mRNA is significantly reduced in the medial prefrontal cortex (MPFC) of patients, and synapsin II knockout rats display a variety of behavioural abnormalities which mimic human SCZ. Considering that SCZ may result from changes at the synapse, we wanted to further elucidate the role of synapsin II by measuring protein expression in post-mortem PFC samples. Overall, our results revealed that synapsin IIa and IIb are not significantly different between patients and controls, however, we hypothesize that synapsin II expression has been normalized in patients due to antipsychotic drug (APD) use. In fact, we discovered that treatment with atypical APDs significantly increases synapsin II in the prefrontal cortex (PFC) of patients, which may underlie the beneficial effects of these drugs. Another objective of our work was to investigate the expression of various presynaptic proteins in post-mortem samples from patients with Parkinson's disease (PD). Parkinson's disease, like SCZ, is an illness which involves dysregulated dopaminergic transmission and synaptic dysfunction. Therefore, we hypothesized that

synapsin II might also be disrupted in patients with PD. Our results demonstrated that synapsin IIa and IIb are significantly reduced in the substantia nigra (SN), but not the striatum (STR) or PFC of patients, when compared to controls. Further, no changes were observed in the other synapsins (I or III), or synaptophysin, which suggests that synapsin II dysregulation may be specific to disorders which involve disruptions in dopamine (DA).

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Table of Contents

List of tables

List of Figures

List of abbreviations

5-HT	5-hydroxytryptamine (serotonin)
6-OHDA	6-hydroxydopamine
APD	Antipsychotic drug
Con MO	Control Morpholino
CTX	Cortex
DA	Dopamine
DA D1	Dopamine D1 receptor
DA D2	Dopamine D2 receptor
DA D3	Dopamine D3 receptor
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine transporter
DBS	Deep brain stimulation
DISC1	Disrupted in schizophrenia 1
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EPS	Extra pyramidal side effects
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GABA	Gamma aminobutyric acid
GAPDH	Glyceraldehdye 3-phosphate dehydrogenase
HBSS	Hanks balanced salt solution
HS	Horse serum

LB	Lewy bodies
LN	Lewy neurites
MPFC	Medial prefrontal cortex
MO	Morpholino oligonucleotide
mRNA	Messenger ribonucleic acid
NMDA	N-methyl-D-aspartate
NRG1	Neuregulin 1
NVH	Neonatal ventral hippocampus
PBS	Phosphate buffered saline
PC 12	Pheochromocytoma cells
PCP	Phencyclidine
PD	Parkinson's disease
PFC	Prefrontal cortex
PINK1	PTEN induced kinase 1
PPI	Prepulse inhibition
SCZ	Schizophrenia
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Short interfering ribonucleic acid
SN	Substantia nigra
STR	Striatum
Syn II	Synapsin II
Syn II MO	Synapsin II MO
TBS-T	Tris buffered saline-tween

VGAT	Vesicular GABA transporter
VGLUT1	Vesicular glutamate transporter 1
VGLUT2	Vesicular glutamate transporter 2
VTA	Ventral tegmental area

Organization of thesis

This thesis is divided into three main experimental objectives. The first set of experiments was designed to investigate synaptic vesicle proteins in two neurological disorders which are characterized by dysregulated dopaminergic neurotransmission. The first objective was to determine if there were reductions in synapsin II in the PFC of patients with SCZ, as previous studies have reported alternations in synapsin II at the mRNA level. Our second objective was to determine if changes in synapsin II were specific to SCZ, or if there were also disruptions in this protein in patients with PD. Therefore, the expression of various synaptic vesicle proteins, including the synapsins, were investigated in the SN, STR and PFC of patients with PD. Finally, our third objective was to attempt to develop a more robust animal model of SCZ by targeting the synapsin II gene using MOs. This set of experiments involved testing the antisense sequence in two cell lines; rat primary cell cultures and PC12 cells.

Objective one: Determining if there are alterations in the synapsin II protein in the PFC of patients with SCZ.

1.0 Introduction

1.1 Dopamine

Dopamine functions as a hormone and neurotransmitter, and is found in both the central and peripheral nervous system. In the brain, DA is found in four distinct pathways; the mesocortical, mesolimbic, nigrostriatal and tuberoinfundibular pathways. It has an important role in regulating endocrine function, food intake, cognition, and

locomotor activity in the central nervous system and in modulating hormone secretion, gastrointestinal motility and catecholamine release in the peripheral nervous system (Missale et al, 1998). As a precursor to epinephrine and norepinephrine, DA also has a role in reward behaviour and reinforcement. To date, five DA receptor subtypes have been identified (D1-D5), which are classified as 'D1' like (D1 and D5) and 'D2' like (D2,D3 and D4) (Missale et al, 1998). As a central neurotransmitter, DA has been implicated in various neurological disorders, such as SCZ and PD.

1.2 Schizophrenia

Schizophrenia is a psychiatric illness that affects roughly 1% of the population, or an estimated 300,000 Canadians. The combined healthcare and non-healthcare costs of SCZ is estimated to be 6.85 billion Canadian dollars a year, with most of the loss caused by high unemployment rates, productivity losses and mortality caused by the illness (Goeree et al, 2005). Aside from the economic costs of SCZ, people who suffer from this mental illness experience significant social, emotional and occupational dysfunction, which often leads to poor prognosis and a reduced quality of life. The illness affects men and women equally, and across all races and cultures. However, men tend to present with the illness earlier (late teens to early 20's), while women show symptoms later (late 20's, early 30's) (Shultz et al, 2007).

1.2.1 Symptoms

SCZ is characterized by an array of symptoms which are divided into three broad categories; the positive symptoms, the negative symptoms and the cognitive symptoms.

The positive symptoms include delusions, hallucinations, and disorganized thought/behaviour (Shultz et al, 2007), while the negative symptoms include social withdrawal, anhedonia (lack of pleasure) and blunted affect (Kirkpatrick et al, 2006). The cognitive symptoms of this disease include problems with working memory and attention, and difficulty with reasoning and problem solving (Bozikas et al, 2006). No single symptom of SCZ is definitive and there is profound variability between individuals, making diagnosis and treatment difficult.

1.2.2 Treatment

While no cure exists to date, most patients with SCZ benefit from a combination of APD treatment and/or other therapies, including social skills training and cognitive behavioural therapy. There are two general classes of APDs used to treat SCZ; the first generation or 'typical' APDs (such as haloperidol) and the second generation or 'atypical' APDs (such as olanzapine). Both classes of APDs differ in their pharmacological profiles and mechanism of action and thus, differentially treat the symptoms of SCZ. For example, typical APDs primarily bind to and block DA D2 receptors while atypical APDs primarily act on DA D2 and 5-HT (serotonin) receptors (Nasrallah, 2008). In addition, typical and atypical APDs differ in their binding affinity for the DA D2 receptor. Specifically, atypical APDs transiently occupy DA D2 receptors and dissociate quickly whereas typical APDs bind tightly and take longer to dissociate (Seeman, 2002). The typical APDs have proven to be effective at treating the positive symptoms of SCZ, but have limited efficacy in treating the cognitive and negative

symptoms (Blin, 1999). They are also accompanied by undesirable 'extrapyramidal' side effects (EPS), such as tardive dyskinesia and dystonia (Meltzer et al, 2004). Typical APDs also cause an increase in serum prolactin levels, which can lead to sexual dysfunction and infertility in individuals who are taking these drugs (Petty, 1999). Still, in clinical 'emergencies' , where patients present with severe mania or agitated/violent behaviour, typical APDs (such as haloperidol) are still widely prescribed for the short term management of such behaviours (Huf and Adams, 2007).

On the other hand, the newer class of APDs appear to have fewer side effects (Meltzer, 2004). This class was generated based on the prototype drug clozapine, which has been found to reduce the severity and frequency of delusions and hallucinations in patients who previously did not respond to neuroleptic drugs (Meltzer et al, 2004). The atypical APDs are superior to the first generation drugs because, in addition to treating the positive symptoms of this illness, they have been shown to improve the cognitive and negative symptoms. For example, treatment with risperidone has been shown to improve both alertness and selective attention in patients with SCZ (Stip and Lussier, 1996). Considering that the negative and cognitive symptoms of SCZ are better predictors of functional outcome then the positive symptoms are (Harvey et al, 2006), then the newer 'atypical' class of APDs are fundamental to managing the symptoms of SCZ, and may allow patients to experience an improved quality of life. However, the atypical APDs are not completely free from side effects. For example, some are accompanied by metabolic and cardiovascular problems, including diabetes, hypoglycaemia and weight gain (e.g. Meltzer et al, 2002).

1.2.3 Etiology

Although the exact cause of SCZ remains unknown, several theories about the underlying etiology of this illness have been proposed. One of the most prevalent theories about the etiology of SCZ is the DA hypothesis, which proposes that excess DAergic transmission in the mesolimbic system gives rise to the positive symptoms, and decreased DA in the mesocortical system underlies the negative symptoms (Davis et al, 1991). This theory arose following the observation that DA agonists (i.e. amphetamine) induce psychosis –like symptoms and exacerbate symptoms in patients with SCZ (e.g. Liberman et al, 1987, Angrist et al, 1970). Further support for the DA hypothesis comes from the observation that DA antagonists, including APDs, act on DA receptors to improve some of the positive symptoms of SCZ (Stone et al, 2007). In fact, the clinical effectiveness of APDs was found to be directly related to their affinity for DA receptors (Seeman et al, 1975).

However, a number of patients with SCZ do not respond to antipsychotics, suggesting that an alternate neurochemical system, other than dopamine, is involved in the pathophysiology of this illness (Davis et al, 1991). In fact, research has also found a role for reduced glutamatergic neurotransmission in SCZ, involving hypofunctionality of the N-methyl-D-aspartate (NMDA) receptor (Kim et al, 1980). This theory emerged following the observation that administration of non-competitive NMDA receptor antagonists, such as phencyclidine 'PCP' and ketamine , led to the development of symptoms which mimicked SCZ, including hallucinations, delusions, and cognitive

deficits (Krystal et al, 1994). Furthermore, the density of ionotropic glutamate receptors (AMPA, kainate and NMDA) have all been found to be altered in patients with SCZ (Zavitsanou et al, 2002).

In addition to the DA and glutamate hypotheses of SCZ, several studies have suggested that genetic factors may underlie this illness. Several studies that have implicated genes in the etiology of SCZ have found that most of the susceptibly genes affect proper functioning of the synapse; for example, NRG1 (Neuregulin 1) is important in synaptogenesis and axon guidance, and has been found to be altered in some individuals with SCZ (Harrison & Weinberger, 2005). However, SCZ appears to be caused by a combination of factors; both genetic and environmental. In fact, studies have discovered that certain environmental factors can increase an individual's risk of developing this psychiatric illness. These include maternal complications during pregnancy (i.e. infection, obstetric problems), developmental problems during childhood and living in an urban setting (Cannon and Clarke, 2005).

Previous studies have failed to find a common 'diagnostic' brain lesion for SCZ, but they have reported consistent alterations in neurotransmission and connectivity (Frankle et al, 2003). Thus, a more recent theory regarding the pathophysiology of SCZ is the synaptic hypothesis, which proposes that the fundamental pathology of this illness involves dysregulated synaptic transmission. Although overly simplistic, this hypothesis is important because it aims to bring together past ideas into a cohesive theory about the etiology of SCZ. In fact, microarray studies of post-mortem samples from patients have

revealed changes in proteins that are involved in presynaptic secretory release (Frankle et al, 2003). In particular, the synapsin proteins have been suggested to be involved in the etiology of this disease.

1.2.4 Characterization of synapsins

The synapsins are a family of neuron specific phosphoproteins that bind synaptic vesicles to the cytoskeleton in their dephosphorylated state (Hilfiker et al, 1999). When synapsins are phosphorylated, they dissociate from vesicles and mobilize, allowing for eventual exocytosis (Hosaka et al, 1999). They represent one of the most abundant families of synaptic proteins, comprising approximately 1% of total protein in the brain (Goelz et al, 1981). They are encoded by one of three genes, synapsin I-III, which gives rise to at least nine different isoforms through alternative splicing (Kao et al, 1999). Synapsins are present in most nerve cells, however, the distribution of synapsin isoforms differs between neuronal cell types, (Sudhof et al, 1989), with synapsin I and II being the major isoform present in neurons (Ferreira and Rapoport, 2002). Structurally, synapsins are comprised of ten domains, referred to as A through J. The primary structural difference of synapsins exists at the COOH- terminal, since the NH2-terminal is highly conserved across all isoforms. Domains A, B, and C are shared by all synapsins, and the E domain is specific to the 'a' type isoforms (Hilfiker et al 1999). (Figure 1)

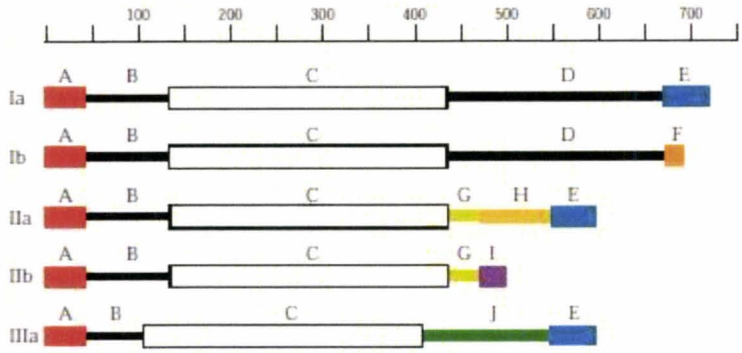


Figure 1: Diagram depicting the three synapsin genes. Domains are represented by letters A-J (the number of amino acid residues is displayed above) (Kao et al, 1999)

The synapsin isoforms have been shown to possess different combinations of the phosphorylation site that regulates their binding to synaptic vesicles and cytoskeletal elements (e.g. Greengard et al., 1993, Hilfiker et al, 1999). They have also been implicated in a variety of cellular processes, including neurotransmitter release, synaptogenesis and synapse stabilization (e.g. Kao et al, 2002, Ferreira and Rapoport, 2002, Hilfiker et al, 1999). Further, these phosphoproteins are thought to regulate synaptic transmission by controlling the storage and mobilization of the reserve pool of synaptic vesicles (Hilfiker et al, 1999), as well as the number of synaptic vesicles in excitatory and inhibitory nerve terminals (Gitler et al, 2004).

With regards to the specific role of each phosphoprotein, synapsin I has been found to have a role in axon elongation, and in regulating the kinetics of synaptic vesicle fusion (Coleman and Bykhovskaia, 2009). Both isoforms of synapsin I (Ia and Ib) also

have important roles in synapse formation (Ferreira et al, 1998) and have been found to minimize neurotransmitter loss, specifically at inhibitory synapses (Terada et al, 1999). Vesicular clustering and organization has also been shown to be impaired in synapsin I knockout mice, further suggesting that this phosphoprotein has a role in the proper functioning of the nerve terminal (Li et al, 1995).

While synapsin II has been found to have a role in similar cellular processes that synapsin I is involved in, studies have also revealed distinct roles for synapsin II. For instance, knockdown of synapsin II in hippocampal neurons results in deficient axon elongation (Ferreira et al, 1994) and disrupted synapse formation (Ferreira et al, 1995), evidence that this protein plays a role in synaptogenesis. In addition, synapsin II has been shown to be involved in vesicle docking, an important part of the synaptic vesicle cycle (Coleman et al, 2008). Furthermore, distinct roles for synapsin IIa and IIb, the two isoforms of synapsin II, have also been discovered. While synapsin IIa has been found to regulate the vesicular reserve pool at glutamatergic synapses (Gitler et al, 2008), synapsin IIb has been shown to have a critical role in the formation of presynaptic terminals (Han et al, 1991).

On the other hand, the third and most recently identified member of the synapsin gene family has been shown to be developmentally controlled and is highly expressed during early stages of nerve cell development (Ferreira et al, 2000). Synapsin III has been found to play a role in regulating neurogenesis in the mouse hippocampus (Kao et al, 2008) and has been shown to regulate neurotransmitter release in a manner different from

that of synapsin I and II. For example, knockout of synapsin III causes a decrease in synaptic depression and an increase in the size of the synaptic vesicle pools, the latter of which is the opposite to what is observed in synapsin I and II knockout animals. Similarly, mice lacking synapsin III show abnormal neurotransmitter release at inhibitory synapses, but not excitatory synapses (Feng et al, 2002). Unlike synapsin I and II which are found mainly at presynaptic nerve terminals, synapsin III is also found at extra synaptic sites and in growth cones of hippocampal neurons (Ferriera et al, 2000).

Regarding the relationship between the synapsins and neurotransmitter release, it appears that the role of these presynaptic proteins is distinct for vesicles containing different neurotransmitters. For example, in synapsin I and II double knockout animals, the expression of transporters for both glutamate (VGLUT1 and VGLUT2) and GABA (VGAT) are decreased, while the monoamine transporter (VMAT2) and VGLUT3 remained unchanged. However, it was also found that VGLUT3 and VMAT2 did not colocalize with either synapsin I or II (Bogen et al, 2006). Furthermore, loss of all three synapsins leads to an increase in catecholamine release, which can only be rescued by synapsin IIa, suggesting that synapsin II negatively regulates catecholamine release (Villanueva et al, 2006).

Evidence for the role of the synapsins in the aforementioned cellular processes mainly comes from studies which knockdown either one, two or all three synapsin genes, and measures the cellular consequences. For example Li et al (1995), reported that knockdown of synapsin I caused a significant reduction in vesicle clustering at

presynaptic sites, while Gitler et al (2004) found that knocking out all three synapsin genes caused differential effects at GABAergic and glutamatergic synapses, and an overall reduction in synaptic vesicle density. On the other hand, over-expression of the synapsins can also give us valuable information about their role both *in vitro* and *in vivo*. For example, over-expression of synapsin IIb in neuroblastoma cells causes the development of synapse-like cell-cell contacts and increases the number of synaptic vesicles, further support for their role in synapse formation and maintenance (Han et al, 1991). Similarly, over-expression of synapsin in *Aplysia* impairs basal synaptic strength and decreases the functional size of presynaptic vesicles (Fioravante et al, 2007). Thus, much of what we know about the role of synapsins in biological and cellular processes has come from these over-expression and knockout studies.

1.2.5 Synapsin II and SCZ

Of particular interest to the field of SCZ research is the role of synapsin II . This synaptic vesicle protein, which has two splice variants (synapsin IIa and IIb) has been suggested to have a causal role in the underlying pathophysiology of this illness. For example, Mirnics et al (2000) reported a significant reduction in synapsin II mRNA in the PFC of patients with SCZ, while Vawter et al (2002) found a reduction in synapsin II protein in the hippocampus. Distinct allelic variations have also been found between controls and patients, drawing a strong association between the synapsin II gene and SCZ (Chen et al, 2004). Further, mice lacking the synapsin II gene show an array of behavioural deficits which closely mimics human SCZ, including deficits in prepulse

inhibition (PPI), decreased social interaction and locomotor hyperactivity (Dyck et al, 2007, Dyck et al, 2009). A recent study by Dyck et al (submitted June 2010 to *Biological Psychiatry*) used antisense technology to knockdown expression of synapsin II in the MPFC of rats and discovered that they displayed a variety of schizophrenic-like behavioural abnormalities, including deficits in cognitive functioning. In addition, this synapsin II knockdown in the MPFC led to a significant decrease in prefrontal cortical glutamate (VGLUT1 & VGLUT2) and GABA (VGAT), but had no significant effect on DA(DAT), suggesting that synapsin II influences expression of both inhibitory and excitatory neurotransmitters. Preliminary results from our laboratory also show that synapsin IIa and IIb mRNA is reduced in the PFC of patients. This reduction in synapsin II was found to be specific to patients with SCZ, as bipolar subjects displayed synapsin IIa and IIb levels similar to control subjects (private communication from Dr Mishra, 2009). Taken together, these studies further implicate synapsin II in the pathophysiology of SCZ.

Synapsin II has also been shown to be regulated by DA D1 and DA D2 receptors. Treatment with the typical APD haloperidol, a DA D2 antagonist, significantly increases synapsin IIa and IIb mRNA and protein in the rat STR and synapsin II protein in the MPFC (Chong et al, 2002, Chong et al, 2004). On the other hand, treatment with the DA D1 receptor antagonist, *R*-[+]-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH23390) decreases synapsin II in mouse primary midbrain cultures. In addition, atypical APDs have been shown to significantly increase prefrontal cortical synapsin IIa and IIb in the PFC of post-mortem samples obtained from patients

with SCZ, while typical APDs increased synapsin II to levels that are comparable to normal controls (Guest et al, 2010).

The synapsin II gene has been shown to be under the control of three possible transcription factors; protein 2 alpha (AP-2α), early growth response factor 1 (EGR-1), or polyoma enhancer activator-3 (PEA-3). To delineate the possible mechanisms involved in DA receptor-mediated synapsin II regulation, Skoblenick et al (2010) performed separate in vitro knockdowns of the three transcription factors. They discovered that knockdown of AP-2α, but not EGR-1 or PEA-3, was accompanied by selective decreases in synapsin II in mouse primary midbrain neurons. Thus, a proposed model of synapsin II regulation by dopaminergic agents involves a cyclic AMP (cAMP)/ PKA-dependent mechanism. Blockage of the DA D2 receptor is proposed to increase cAMP levels and promote transcription of the synapsin II gene, explaining the increase in synapsin II following treatment with APDs (Skoblenick et al, 2010).

Rationale: The purpose of the present study is to determine if differences in synapsin II are present at the protein level in post-mortem PFC samples from patients with schizophrenia. Although previous studies have found reductions at the mRNA level, expression at the protein level has yet to be determined. In addition, although a relationship between APDs and synapsin II has been established in the rat, it has not been investigated in human patients with SCZ. Therefore, our goal is to determine the influence of APD usage on expression of this phosphoprotein. This will give us further insight into the mechanism of action of APDs at the synaptic level.

Objective two: Investigating the expression of synaptic vesicle proteins in the SN, STR and PFC of patients with PD.

1.3 Parkinson's Disease

1.3.1 Symptoms, Etiology and Treatment

Parkinson's disease is a progressive, neurodegenerative disorder which affects approximately 2% of individuals over the age of 60 and 4.4% of those over 85 (de Rijk et al, 2000). It is characterized by a variety of motor symptoms, including tremors, rigidity, bradykinesia (slowness of movement) and gait/balance disturbances (reviewed by Lew, 2007). Patients with PD also experience 'non-motor' symptoms, including cognitive impairments and neuropsychiatric disturbances. Some of the most common symptoms include hallucinations, anxiety, fatigue, and depression (Aarsland et al, 2009).

Parkinson's disease is characterized by the degeneration of DA-producing neurons in the substantia nigra pars compacta. In addition, inclusions known as Lewy bodies (LBs) and Lewy neurites (LNs) accumulate in several areas of the brain (Lucking & Brice, 2000). These highly insoluble protein aggregates are a diagnostic hallmark of PD, and have been shown to be composed largely of α-synuclein (α-syn) (Spillantini et al, 1998), a protein which is normally found in presynaptic terminals (Clayton & George, 1999). Unfortunately, diagnosis of PD usually doesn't occur until about 60-70% of DA neurons are abolished, and the subsequent motor symptoms appear (Schapira, 2006).

While the etiology of PD remains largely unknown, studies have identified that both environmental and genetic factors can contribute to the development of this illness. For instance, previous research has found that duplications, triplications, or mutations in the α-syn gene (PARK 1) cause some forms of familial PD (Singleton et al, 2003, Polymeropoulos et al, 1997). Other 'single gene' mutations have also been found in other familial cases of PD. For example, mutations of the PINK1 (PTEN induced kinase 1) gene has been found to be associated with a recessive form of early-onset PD. Most of the genes that have been identified as risk factors for PD involve proteins that are involved in mitochondrial function, oxidative stress and protein handling (Schapira, 2006). In addition to the genetic risk factors, research has also highlighted the role of environmental factors which may contribute to the development of this neurodegenerative disease. These include occupancy in rural settings, exposure to environmental toxins (such as pesticide/herbicides) and exposure to manganese, a component of herbicides/pesticides and gasoline (Schapira, 2006).

Similar to SCZ, there is no cure or no way to slow the progression of PD, there are only ways to manage the symptoms. Levodopa (L-DOPA), which is the molecular precursor of DA, remains the most commonly prescribed and most effective drug for the management of PD (Pezzoli & Zini, 2010). In order to avoid a series of negative side effects, it is most often prescribed in conjunction with a peripheral decarboxylase inhibitor, which prevents its conversion to DA in the peripheral nervous system. The clinical efficacy of L-DOPA is variable between patients, and most often targets only the key motor symptoms of this illness, namely, rigidity and bradykinesia (Pezzoli & Zini,

2010). The other motor and non-motor symptoms, such as cognitive impairments and postural/gait disturbances, do not usually respond well to targeted DA therapy, and thus still remain a problem for most patients with PD. Treatment with L-DOPA is also complicated by the appearance of drug-induced motor side effects (i.e. dyskinesia) and the relatively short half-life of the drug itself. As such, L-DOPA is typically prescribed to older patients who experience more severe symptoms, while other DA agonists are prescribed to younger patients with milder symptoms (Rao et al, 2006). For example, Bromocriptine (Parlodel), a DA agonist, has been shown to be effective in treating both early and advanced PD, and causes fewer motor side effects then L-DOPA. In addition, there is also evidence to suggest that some patients benefit from treatment with anticholinergics or monoamine oxidase-B (MAO-B)-inhibitors (Roa et al, 2006).

Besides the pharmacological interventions, there are also surgical procedures that may improve some of the symptoms of PD. For example, some patients may benefit from pallidotomy, a surgical procedure which involves lesioning a small area of the globus pallidus, or deep brain stimulation, a procedure which provides stimulation through an electrode implanted in the brain. In addition, a relatively new surgical procedure, cell transplantation, involves grafting fetal ventral mesencephalic tissue into the brain of patients. All three procedures have been found to improve the key motor symptoms of PD (e.g. Kishore et al, 1997, Walter & Vitek, 2004, Perlow et al, 1979, Freed et al, 2001, Olanow et al, 2003)

1.3.2 Animal models of PD

As with any complex neurodegenerative disorder, it is difficult to generate animal models of PD that closely mimic both the symptoms and pathological hallmarks of this disease. However, there are several established pre-clinical models of PD that have allowed researchers to discover critical information about the pathophysiology and treatment of this illness. The most widely used pre-clinical model of PD involves intracerebral infusion of 6-hydroxy dopamine (6-OHDA), a neurotoxin which causes widespread cell death of dopaminergic neurons in the nigrostriatal pathway. These animals display a variety of motor deficits, including rotational behaviour (indicative of a DA imbalance), slowness of movement as well as cognitive deficits (Simola et al, 2007). Neurochemical and morphological abnormalities, such as a reduction in the density of dendritic spines, as well as altered expression of DA and glutamate receptors, are also characteristic of this pre-clinical model (Simola et al, 2007).

Since the discovery that α-syn was a key component of LBs and LNs, the over-expression of this protein has also been a focus of several animal models of PD. For instance, Kirik et al (2002) used recombinant adeno-associated viral vectors to over-express wild-type and mutant human α- syn in the nigrostriatal system of the rat brain. They found several pathological and motor abnormalities, including deficits in rotational behaviour and α-syn positive inclusions. They also reported a 30-80% loss of dopaminergic neurons in the SN, and a 40-50% loss of DA in the STR.

1.3.3 Synaptic vesicle proteins in PD

Alpha-synuclein is a presynaptic protein that normally has a role in modulating synaptic plasticity (i.e. Clayton & George, 1999) and DA biosynthesis (i.e. Perez et al,

2002). However, the abnormal accumulation of this protein in LBs and LNs is a key diagnostic hallmark of PD. Aside from the role of α-syn in PD, very few studies have examined other presynaptic proteins and their potential role in this illness. Synapsin II has previously been shown to be disrupted in SCZ (Mirnics et al, 2000), a psychiatric illness which also involves dysregulated DA. This synaptic vesicle protein has also been shown to be regulated by DA D1 and DA D2 receptors (Chong et al, 2006), thus, it is possible that differences in this protein may underlie some of the pathological abnormalities that are found in PD, another illness which is characterized by disruptions in DAergic neurotransmission.

Rationale: Given the role that the synapsins play in several cellular processes, including synaptogenesis and vesicle exocytosis, the role of these phosphoproteins in PD warrants further research. Thus, the purpose of the present study is to determine if the synapsins display aberrant expression patterns in patients with PD. This may help us to understand the etiology of PD at the synaptic level. In addition, it will also tell us if the changes in synapsin II that are observed in SCZ are disease specific, or if it is common to another illness which is characterized by disruptions in DA signalling.

Objective three: To use cellular models to try and establish a novel technique to knockdown the synapsin II gene using MOs.

1.4 Animal Models of SCZ

While it is difficult to model a complex human disorder in a rodent or other mammal, there are several pre-clinical animal models of SCZ which are currently being used to help delineate the etiology of this illness. While some animal models mimic the symptoms of SCZ via genetic knockout of a particular gene, others display symptoms induced by treatment with pharmacological agents, such as amphetamine, PCP and MK-801. For example, the disrupted in schizophrenia 1 (DISC1) knockout mouse shows an array of behavioural and anatomical deficits characteristic of SCZ, including enlarged ventricles, increased locomotor activity and disruptions in sensorimotor gating (Hikida et al, 2007). In addition, both typical and atypical APDs have been found to reverse disrupted PPI and latent inhibition in mice with a DISC1 missense mutation (Clapcote et al, 2007). In a similar manner, rats that are challenged with MK-801 have been shown to display a variety of symptoms reflective of SCZ, including disrupted PPI, locomotor hyperactivity and social withdrawal. They also show disruptions in cognitive functioning, including deficits in rule acquisition and attentional set-shifting. Unlike amphetamine, treatment with MK-801 is capable of inducing all three symptoms types characteristic of SCZ in pre-clinical animal models (Rung et al, 2005, Stefani and Moghaddam, 2005).

There are also several 'developmental' animal models of SCZ, including prenatal immune challenges and isolation rearing. For example, Fone and Porkess (2008) found that rat pups who were reared in social isolation displayed several behavioural and neurochemical deficits, including impaired sensorimotor gating, cognitive deficits and mesocortical DA hypofunctionality. On the other hand, Shi et al (2003) showed that maternal infection with the human influenza virus lead to several behavioural

abnormalities in mouse pups, including deficits in PPI, disruptions in exploratory behaviour and decreases in social interaction. Further, these mice respond significantly to both APDs and psychomimetic drugs, which suggests that both the dopaminergic and glutamatergic systems are disturbed in this pre-clinical animal model.

Finally, there are some animal models of SCZ that are created by lesioning a particular area of the brain, for example, the neonatal ventral hippocampal lesion model. This pre-clinical model of SCZ displays several behavioural changes, including working memory deficits and social impairments, as well as various cellular changes, including reductions in DA transporter mRNA (Lipska, 2004).

However, despite the fact that there are several animal models to study SCZ, at best, these models 'mimic' the behavioural phenotype of this psychiatric illness, and must be used and interpreted with caution.

1.5 Genetic knockdown techniques

Genetic knockdown experiments are widely used in health research today to determine the impact of reducing expression of a target gene. Although there are several different classes of gene knockdown agents, three of the most commonly used include RNase H-dependent oligos (ex. phosphorothioate-linked DNA, 'S-DNA'), steric blocking oligos (ex. Morpholinos, 'MOs') and RNA interference oligos (ex. siRNA). The mechanism of action of RNase H-dependent oligos involves cleaving its target RNA sequence by exploiting cellular RNase H, the enzyme which cleaves RNA in an RNA/DNA duplex. S-DNA's are the most common type of antisense in this class, and

have a relatively long half-life in biological systems . They are structurally similar to DNA, with the exception of an oxygen atom being replaced by a sulphur in the intersubunit linkages (Summerton, 1999). Due to the sulphur atom on its backbone, S-DNA's have the advantage of being degraded slower, however, this sulphur also causes them to bind to several intracellular and extracellular proteins, which causes a variety of undesirable effects. For example, S-DNAs have been shown to activate the 'compliment cascade', which leads to convulsions and death when administered to animals (Wallace et al, 1996).

Another class of gene knockdown reagents, RNA interference oligos, use short interfering RNA (siRNA) to block translation of a complementary RNA sequence (Dorsett & Tuschl, 2004). This is accomplished when one strand of the siRNA combines with a cellular protein to form an RNA-induced silencing (RISC) complex. Small interfering RNA is capable of blocking translation of partially-complementary RNA sequences and degrading highly-complementary RNA sequences (Dorsett & Tuschl, 2004). Since they have a more 'natural' RNA sequence, they avoid most of the off target effects that are characteristic of S-DNAs, however, they are not free from all side effects. For example, siRNAs have been found to cause the induction of inflammatory cytokines (Sioud, 2005). Although they are still used today, both S-DNAs and siRNA have been shown to have poor sequence specificity and low targeting success rates. While S-DNAs are only effective at knocking down their intended target in 10 to 20% of cases, siRNAs have a targeting success rate of about 50% (Summerton, 2007).

The other class of antisense oligonucleotides, the steric blocking oligos, reduce gene expression by either blocking the splicing of the initial RNA, or by blocking translation of the mRNA by preventing the initiation complex from forming (Summerton, 2007). This class of antisense reagents include the recently developed MOs.

1.5.3 Morpholino oligonucleotides (MOs)

Morpholino oligonucleotides are different from other genetic knockdown tools in both their design and mechanism of action. They are comprised of a 6-membered morpholine ring with non-ionic phosphorodiamidate linkages (Figure 2). These sequences do not interact significantly with other proteins and are easy to deliver into cells, likely due to the lack of charge on the MO backbone (Summerton, 2007). In addition, they have been shown to be devoid of almost all off-target effects and show no symptoms of toxicity, even at very high doses (ex. Wu et al, 2010). MOs also appear to be very stable in biological systems and have a high specificity for their complimentary RNA sequence. Unlike other knockdown agents, MOs require a minimum of 14 bases to match a complementary sequence of a target gene, which contributes to their high targeting success rate (Summerton, 2007). Further, approximately 70-75% of MO sequences are effective at targeting a specific gene and they confer between 70 and 98% knockdown.

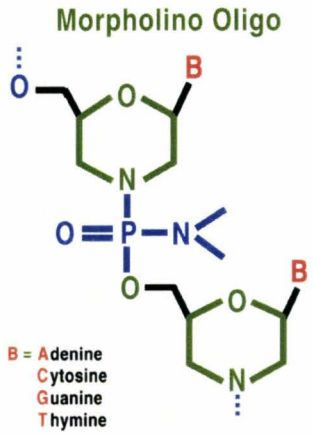


Figure 2: Structure of a morpholino oligonucleotide
Each subunit consists of a nucleic acid base, a morpholine ring and a non-ionic phosphorodiamidate intersubunit linkage (Summerton, 2007)

Delivery of MOs

Morpholino's come in two forms; 'bare' or 'vivo'. The former is for use *in vitro*, while the latter is specialized for use *in vivo*. In order for 'bare' MOs to enter cells, a delivery reagent is required. The safest and most effective method of delivering MOs is by using Endo-porter. Endo-porter is a weak base amphiphilic peptide that is capable of delivering substances into the cytosol of cells via an endocytosis-mediated process (Summerton, 2005). Once it is added to the cell mixture, endo-porter is rapidly adsorbed to the surface of the cell and then endocytosed, along with any other substances that are present in the medium. When the endosome is acidified, the endo-porter permeabilizes the membrane and releases its contents into the cytosol. Thus, endo-porter can effectively shuttle MOs, and other non-ionic substances into the cytosol or nuclear compartment of

cells without causing damage to the plasma membrane and without losing important cellular contents. As well, endo-porter does not cause the high degree of cell toxicity that is often reported with other reagents (Summerton, 2005).

Design of MOs

Morpholino oligonucleotides are designed by choosing a splicing site in the primary RNA transcript or in the mature mRNA, and targeting a region from the 5'cap through to the first 25-bases of the coding region (Summerton, 2007). This allows MOs to interfere with the initiation complex and prevent it from reaching the start codon, thereby preventing translation of the gene. MOs are typically 25-base pair sequences, and are designed with a Guanidine + Cytosine content of 35-65%. In addition, to ensure higher specificity and targeting ability, 4 or more consecutive guanines and 5 contiguous bases are generally avoided when designing a MO sequence (Summerton, 2007).

Advantages

Several of the advantages of MOs have been highlighted previously, including their high stability in biological systems, their lack of significant off target effects, and their high targeting success rate. In addition, MOs have been shown to have good solubility in aqueous solutions and are relatively resistant to degradation by nucleases (Summerton & Weller, 1997). With regards to the efficacy of MOs, there have been promising results reported using MOs both *in vitro* and *in vivo.* For example, while Tyson-Capper et al (2006) found a decrease in COX-2 mRNA and protein expression in myometrial cells using MOs, Arora et al (2002), reported an inhibition in cytochrome

P450 3A2 activity in rat skin and liver following topical application of antisense oligos. In addition, there are encouraging results using MOs in animal models of Duchenne muscular dystrophy. In fact, exon-skipping MOs are now being used in human clinical trials to restore the open reading frame of the mutated dystrophin gene in patients with this disease (Kinali et al, 2009).

Disadvantages

Morpholino's claim to be superior to other existing knockdown techniques, however, these antisense sequences are not completely free from side effects and other complications. For example, one of the issues surrounding the use of antisense oligonucleotides is selecting a suitable sequence to target. Knockdown reagents which contain DNA, such as S-DNAs, are able to form RNA-DNA hybrids, which RNase H can bind to and degrade its target. Thus, any sequence within the coding region of the target gene can be used as a potential antisense sequence (Corey & Abrams, 2001). On the other hand, MOs form RNA-MO hybrids which do not exploit RNase H to degrade its target RNA. Instead, MOs are targeted to the 5`UTR or the start codon and prevent the initiation complex from binding, thereby inhibiting protein translation. Therefore, if a MO is designed to target most of the coding region of the gene, it can be displaced by the ribosome as it translocates along the mRNA and will be ineffective in reducing its target gene (Corey & Abrams, 2001). Thus, there is a strict set of guidelines to design a MO that will be highly specific for its intended target.

In addition, although they claim to be virtually devoid of all off-target effects, some studies have reported significant issues with the use of MOs. For example, in sea urchins, the use of MOs to target the *S. purpuratus Runt gene* led to an early developmental arrest. Researchers discovered that this phenotype was caused by their MO targeting two histones, in addition to its intended gene (Coffman et al, 2004). Another study which reported non-specific effects due to MOs was conducted by Wright et al (2004). Results from this study revealed that a MO targeted to interfere with splicing of the gene *Magi1* unintentionally caused a narrowing of the midbrain and hindbrain in zebra fish embryo Collectively, these results suggest that MOs may cause unintentional side effects, and may not be as highly specific as they claim to be.

Rationale: There are several pre-clinical models of SCZ, including synapsin II knockdown in the rat PFC with antisense oligo's (Dyck et al, 2007). However, this technique has been shown to be accompanied by unwanted side effects (i.e. toxicity). Since MOs are accompanied by fewer side effects than other knockdown techniques, these unwanted effects should not be observed. In addition, MOs claim to knockdown 70-98% of its intended target, thus, it is possible that a more significant reduction in synapsin II will lead to more behavioural deficits that are characteristic of SCZ. Therefore, the purpose of the present study is to attempt to create a novel technique for knocking down synapsin II in the rat. Before attempting to knockdown the synapsin II gene in vivo, however, we tested the efficacy of the MO sequence in two cell lines; rat primary cell cultures and rat pheochromocytoma cells (PC 12). These studies would give

us an indication of how specific the sequence was, what dose would be effective, and what percent knockdown the sequence could confer.

1.5.1 Primary cell cultures

Primary cortical and striatal cells were chosen as a model for this experiment because they closely mimic the *in vivo* state and accurately represent the 'parent' tissue. *In vitro* models are advantageous because they allow researchers to carefully control for environmental influences (i.e. temperature and light exposure), and eliminate any variations that may arise when using *in vivo* models (i.e. individual variations in hormones, nutrition, illness etc) (Polikov et al, 2008). In addition, cell culture experiments can usually be replicated with ease, and offer easier experimental procedures and designs than most *in vivo* work. Further, *in vitro* cell studies offer the advantage of shorter experimental timelines and a decrease in the amount of materials used (i.e. drug treatments), when compared to whole animal studies (Polikov et al, 2008). Rat primary cortical cells were chosen as the dominant cell type to investigate MOs since a previous study found that knockdown of synapsin II in the mouse PFC led to a variety of behavioural abnormalities which mimic human SCZ (Dyck et al, 2007). As well, primary striatal cells were chosen as an added 'control' to determine if our MO sequence was capable of reducing expression of synapsin II in the STR. However, the future application of MOs *in vivo* would involve infusing the sequence into the rat PFC, since this is an area of the brain that is largely afflicted in SCZ, and has previously been shown to have dysregulated synapsin II expression (Mirnics et al, 2000).

1.5.4 PC 12 cells

PC12 cells are derived from a neuroendocrine tumour of the rat adrenal medulla. This particular cell line has been used widely in research to study cellular processes such as proliferation, survival and differentiation (Mingorance-Le Meur et al, 2009). The PC12 cell line was chosen to investigate the effects of our MO sequence after it was discovered that these cells express the synapsin IIb isoform (Leoni et al, 1999). In addition, these cells are easy to work with since they have a short doubling time, are easy to culture, and can be passaged an infinite number of times (Mingorance-Le Meur et al, 2009). Furthermore, they offer many of the advantages that were previously described with primary cells, including a reduction in the amount of materials needed and a shorter experimental time period.

2 Specific aims of thesis

Specific Aim 1: To determine if the expression of synapsin II is altered in the PFC of patients with SCZ, and to determine the influence of APD use on this phosphoprotein.

Specific Aim 2: To determine the pattern of expression of various presynaptic proteins, including the synapsins, in the SN, STR and PFC of patients with PD.

Specific Aim 3: To attempt to create a more robust pre-clinical animal model of SCZ using MOs by targeting the synapsin II gene in primary cell cultures and in PC12 cells.

3 Methods

3.1 Post-mortem studies of patients with SCZ and PD

Post-mortem samples

Post-mortem samples were obtained from the Human Brain and Spinal Fluid Resource Center (Los Angeles, California). The number of samples received, and other important information pertaining to the samples is outlined in Table 1. Briefly, from PD patients, we obtained the PFC (n=10), the SN (n=4) and the STR (n=4) and from SCZ patients we received the PFC (n=24), as well as normal control samples for both diseases. All samples were homogenized in a Tris-EDTA buffer with a protease inhibitor cocktail (Mini complete tablet EDTA-free, Roche Applied Science, Laval QC). The samples were first homogenized in a glass hand homogenizer, and then polytroned three times each at medium speed for 8 seconds, with a 20 second rest on ice in between. They were then aliquoted, and stored at -80°C for long term usage. Protein concentrations were determined using the Bradford protein assay (Bio-rad, Hercules, CA).

Table 1: Information pertaining to the human post-mortem samples obtained from the Human Brain and Spinal Fluid Resource Center

Sample Group	Number of samples	Brain region	Average age of patient (± SD)	Proportion of males: females	Average PMI
SCZ	24	PFC	56.9±20.5 yrs	15m/9f	26±22 hours
Controls	16	PFC	70.5±18.0 yrs	8m/8f	16±4 hours
PD	**18 (total)** • 4 • 4 • 10	 SN STR PFC	77.1±4.1 yrs 74.7±3.3 yrs 74.7±3.3 yrs 81.9±6.8 yrs	 2m/2f 2m/2f 5m/5f	14.3±4.0 hours 12±4.6 hours 12±4.6 hours 19.4±6.8 hours
Controls	**18 (total)** • 4 • 4 • 10	 SN STR PFC	69.2±5.6 yrs 66.0±9.9 yrs 66.0±9.9 yrs 75.8 ±14.4 yrs	 4m/0f 4m/0f 7m/3f	15.7±1.6 hours 14.8±2.4 hours 14.8±2.4 hours 17.7±3.0 hours

Note: PD= Parkinson's disease, PFC=prefrontal cortex, SN=substantia nigra, STR=striatu, PMI=post mortem interval, SD=standard deviation

Western Immunoblots

Ten micrograms of protein was loaded into each lane of a 10 % polyacrylamide gel. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane using a Bio-Rad Wet Blot transfer unit. Following transfer, blots were visualized with Ponceau-S

stain (ICN Biomedical, Aurora OH) to ensure efficient loading of protein. Blots were then incubated in 5% skim milk (2.5g fat free skim milk powder in TBS-T; 50mM Tris, 150mM NaCl, 0.2% Tween-20, pH 8.5) for 1 hour at room temperature. Following blocking, blots were briefly rinsed in TBS-T, and exposed to primary antibody diluted in TBS-T at 4°C overnight (Table 2). Following primary antibody incubation, blots were washed in TBS-T (2x 5mins, 1 x 15mins) and probed with secondary antibody (Table 2) for 1.5-2 hours at room temperature. Membranes were then washed once again in TBS-T (2x5mins, 1x10mins) and developed using electrochemiluminescence reagents (Amersham Pharmacia Biotech, Baie d'Urfe, QC) and exposed to Kodak BioMax Light Film (Kodak Industrie, Cahlon-sur-Saone, Cedex France). After probing for our protein of interest, blots were stripped with Re-Blot plus antibody stripping solution (Chemicon International, Temecula CA) and incubated with a housekeeping gene (Table 2). Films from western blots were analyzed using ImageJ software (NIH, Bethesda, MD) and data were analyzed by student's t test in GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). The size of the experimental effect was calculated using Cohen's D (d). Prior to data analysis, outliers were removed using the GraphPad Outlier calculator. Results are considered statistically significant if $p<0.05$.

Table 2: List of primary and secondary antibodies used in western blotting

Protein	Primary antibody dilution	Secondary antibody dilution
Synapsin I	1:300,000	Anti-rabbit, 1:5,000
Synapsin II	1:10,000	Anti-rabbit, 1:5.000
Synapsin III	1:5,000	Anti-rabbit, 1:5,000

Synaptophysin	1:200,000	Anti-rabbit, 1:5,000
GAPDH	1:10,000	Anti-mouse, 1:10,000

3.2 Treatment of primary cell cultures with MOs

Culture plates

Cover slips were added to 24-well culture plates (Becton Dickson, Franklin Lakes, NJ) for immunoflourescence imaging. Poly-D-lysine (500µl) (Sigma-Aldrich Canada LTD., Oakville, ON) was then added to each well for 5 minutes to facilitate cell binding. Well's were then rinsed 5 times each for 5 minutes with sterile water to remove excess poly-lysine, and to ensure an even coat.

Media

The following media solutions were used in the experiment; Hanks balanced salt solution (HBSS; autoclaved), HBSS with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), plating media [DMEM/F12 60:40, 10% FBS, 0.05% penicillin/streptomycin] and culture media [DMEM/F12 60:40, 5% B27 supplement, 0.05% penicillin/streptomycin]. All media solutions were prepared and filtered one day prior to the experiment. Solutions were stored at 4°C until needed, and were warmed to 37°C prior to use.

C-section

One pregnant female Sprague-dawley rat (Charles River, Wilmington, MA), gestational day E18, was anaesthetized with isoflourane prior to surgery. The rat was maintained on anaesthetic via a nose cone, and was placed on her back for the procedure. The abdomen was sprayed with 70% ethanol and wiped with gauze. One large incision was made down the center of the abdomen and the walls of the abdomen were opened with small haemostats. The uterus was pulled out of the abdomen and placed into a 250ml beaker containing 50ml of HBSS.

Dissection of embryos

The dissection surface was cleaned and sprayed with 70% ethanol prior to removing the embryos. On a large petri dish containing 20ml of HBSS on ice, the uterus was cut open and foetuses were removed one at a time. The foetuses head was cut off and placed into a 50ml falcon tube containing 20ml of HBSS. In a sterile culture hood, the heads were transferred to a small petri dish containing HBSS. One at a time, the skin from the top of the head was carefully removed using scissors and forceps, and was pealed back to reveal the brain. Brains were then transferred to a separate petri dish containing ice-cold HBSS. Using a dissecting microscope, the brains were then placed on the stage of the microscope and cortical and striatal tissue were removed from each brain. Dissections obtained from all embryos were pooled together for each brain region.

Processing of brain tissue

A sterile transfer pipette was used to remove HBSS from the two petri dishes containing cortical and striatal tissue. The tissue was then minced with two #10 scalpel

blades (scalpels were drawn across the tissue in the petri dish in opposite directions). Two millilitres of HBSS + 10% FBS was then added to the minced tissue. Using a large bore fire-polished Pasteur pipette, the cells were triturated by passing them through the pipette approximately ten times. The process was repeated again with a small bore fire-polished pipette. One millilitre of HBSS + 10% FBS was added to a 50ml falcon tube, and cells were then filtered through a 70μm filter into the falcon tube containing HBSS with FBS. This was repeated again with a 45μm filter. The petri dishes and filters were each rinsed with 1ml of HBSS +10% FBS to ensure all the cells were collected and the volume was brought up to 10ml with HBSS + 10% FBS.

Counting and plating cells

The cells were gently mixed and 100μl of each cell type (cortical and striatal) was transferred to separate 1ml eppendorf tubes for counting. 10μl of 0.02% trypan blue (Sigma-Aldrich Canada LTD., Oakville ON) was added to the 100μl aliquots to visualize the cells. Twenty microlitres of the sample was then added to a cell counter, and the number of cells in four random squares was counted. The number of cells per ml was calculated as follows: (# of cells counted in four random squares) x 4 x 10^4. The approximate number of cells was found to be 3,360,000 per ml (striatal) and 4,920,000 cells per ml (cortical). The falcon tube containing the remaining cells was then centrifuged at 800 rpm for 5 minutes. The supernatant was aspirated from the cell pellet, and the pellet was re-suspended in 5mls of HBSS + 10% FBS plus 5mls of plating media.

Cells were grown on both 24-well plates (for immunoflourescence) and 6-well plates (for western blot analysis). On 24-well plates, a 25µl 'island' of cells was seeded directly in the center of the well, which corresponded to a concentration of approximately 123,000 cells per well (cortical) or 84,500 cells per well (striatal). On 6-well plates, 1ml of media containing cells (4.92×10^6 cortical cells, 3.3×10^6 striatal cells) was added to each well. The cells were placed in a 37°C incubator for 45 minutes to allow the cells to adhere to the plates. Plating media was then replaced with culture media (500µl on 24-well plates and 2ml on 6-well plates) and the cells were returned to the incubator for 4 days. Once cell bodies and processes were visible, the cells were treated with either a synapsin II MO, a standard control MO or received no treatment (control).

MOs

A MO sequence [5' CGCCTCAGGAAGTTCATCATCTGGC 3'] complimentary to the translational blocking target of synapsin IIa and IIb was designed and produced by GeneTools, LLC (Philomath, OR). The sequence was received as 300nmol of lyophilized powder and was re-suspended in 300µl of sterile water to give a 1milliMolar (mM) working solution.

In order to rule out the fact that any observed changes in our protein was due to some feature of the MO itself (i.e. the backbone), a separate group of cells were treated with a standard control MO. The control MO chosen [5'-CCT CTT ACC TCA GTT ACA ATT TAT A] had no target and no significant biological activity (except in immature red blood cells of humans who suffer from thallasemia). One hundred nanomols of

lyophilized control MO was re-suspended in 100µl of sterile water to give a 1mM solution.

Endo-porter

In order to successfully deliver the target MOsequence into cells, a delivery moiety was used. Endo-porter was received as 1ml (1mM) in a 100% DMSO solution.

Treatments

Morpholino's are typically effective at inhibiting translation of a target gene at concentrations from 1microMolar (µM) to 10µM (Morcos, 2001). Since this technique has not previously been used to knockdown the synapsin II gene, a range of doses were tested in order to determine the most appropriate concentration. Thus, there were three treatment doses with the synapsin II MO (1nmol [2µM], 2nmol [5µM] and 5nmol [10µM] MO). In addition, it was necessary to test various doses of the delivery reagent since the most effective concentration to achieve delivery into cells was unknown. The recommended dose of end-porter was 6µl/ml [6µM] of media. However, since different cell lines can vary in their response to endo-porter, it was suggested that new experiments are designed with a range of doses, therefore 2, 4 and 6µl/ml was used. Control MO was also used in a range of doses (1, 2 and 5 nmol) with varying doses of endo-porter (2, 4 and 6µl/ml). The final treatment plan is summarized in Table 3.

Table 3: Primary cell culture treatment plan

Plate #	**Cell type**	**Treatment # 1-Control**	**Treatment # 2 Control MO**	**Treatment # 3 Synapsin II MO**	**Endo-porter**	**# of wells/ per treatment**
1 (24-well)	Cortical	0 nmol	1 nmol 2 nmol 5 nmol	1 nmol 2 nmol 5 nmol	2 µl/ml	3
2 (24-well)	Cortical	0 nmol	1 nmol 2 nmol 5 nmol	1 nmol 2 nmol 5 nmol	4 µl/ml	3
3 (24-well)	Cortical	0 nmol	1 nmol 2 nmol 5 nmol	1 nmol 2 nmol 5 nmol	6 µl/ml	3
4 (24-well)	Striatal	0 nmol	1 nmol 2 nmol 5 nmol	1 nmol 2 nmol 5 nmol	4 and 6 µl/ml	2
5 (6-well)	Cortical	0 nmol	2.5 nmol	2.5 nmol	6 µl/ml	2
6 (6-well)	Striatal	0 nmol	2.5 nmol	2.5 nmol	6 µl/ml	2

Note: MO=Morpholino's

Cells received either synapsin II MO, control MO or no treatment (control) for 48 hours. After treatment, cells on the 24-well plates were fixed with 4% formaldehyde. Briefly; media was aspirated out and 500µl of 4% formaldehyde was added to each well for 30 minutes. Following incubation, formaldehyde was removed and the wells were washed twice with phosphate buffered saline (PBS) for 5 minutes each. For 6-well plates, media was aspirated out and 2mls of 1mM EDTA in PBS was added to each well and left for 5 minutes. The bottom of each well was then scraped with a cell scraper (Sarstedt, Montreal, QC) and the cells from common treatment conditions were pooled together. The cells were centrifuged at 800 rpm for 5 minutes, the supernatant was

aspirated out and the cell pellet was re-suspended in 100µl of Tris-EDTA buffer with a protease inhibitor cocktail. Protein concentrations were then determined using the Bradford protein assay (Bio-rad, Hercules, CA) and western blotting was performed on the samples.

Immunocytochemistry

Immunocytochemistry was performed on cells from 24-well culture plates. Briefly; the wells were rinsed once for 5 minutes with PBS, followed by the addition of 250µl of blocking solution (4% donkey serum, Abcam, Cambridge MA, USA) for 1 hour at room temperature. Blocking solution was then removed and the cells were incubated in 1° antibody. Both synapsin II (1:200) and B-tubulin III (1:1000) were used as primary antibodies. Primary antibodies were diluted in PBS with 0.3% triton X (Amersham, Oakville, ON), and 200µl was added to each well. The plates were wrapped in tinfoil and kept at 4°C overnight. Following incubation with 1° antibody, the wells were rinsed 3 times each for 5 minutes with PBS. Secondary antibodies were then diluted in PBS. Secondary flourescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (1:200) (Jackson Immuno Research Laboratories, West Grove, PA) was used for synapsin II, and Cy 5-conjugated AffiniPure Goat Anti-chicken IgY (IgG) ,1:200 (Jackson Immuno Research Laboratories, West Grove, PA) was used for B-tubulin . All secondary antibody solutions were prepared in the dark. Two hundred and fifty microlitres of secondary antibody was added to each well and was left to incubate for 4 hours in the dark at room temperature. Following secondary antibody incubation, wells were rinsed 5 times each

for 5 minutes with PBS. After rinsing, slides were labelled and 3µl of ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene OR) was added to the center of each slide. Cover slips were placed face down on each slide and secured with nail polish. A Leica DMI 6000 B widefield deconvolution microscope (63x objective) was used to acquire immunoflourescence images.

3.3 Treatment of PC12 cells with MOs

Frozen PC12 cells were removed from -80°C and 300µl of horse serum (HS) was added to the tube while the cells defrosted. Ten millilitres of media [500ml RPMI, 10% HS, 5% FBS, 1% penicillin/streptomycin] was added to a 100x20mm cell culture plate (Sarstedt, Montreal QC, Canada) and placed in a 37°C incubator for 30 minutes. Once the cells were defrosted, they were transferred to a 15ml falcon tube and 6mls of media was added. The cells were then centrifuged for 5 minutes at 2000 rpm. The pellet was re-suspended in 5mls of media, and the cells were added to the flask containing 10mls of pre-warmed media. The cells were left undisturbed for 4 days at 37°C to grow. Once the plate appeared confluent, cells were split 1:4. Briefly; media was warmed in a 37°C water bath, a cell scraper was used to collect the cells and the media was split into new plates already containing (new) media. For example, 3mls of media containing the cells was added to four separate plates which contained 12mls of new media. New plates were returned to the incubator for 4 days to allow the cells to grow. One plate of cells was split again to continue the cell line, while 3 of the plates were pooled together in order to harvest the cells. To harvest the cells, plates were pooled in a 50ml falcon tube and the

cells were centrifuged for 5 minutes at 2000 rpm. The supernatant was then removed, the pellet of cells was re-suspended in 100µl of TE buffer and the sample was sonicated briefly. Protein concentration was then determined using the Bradford protein assay and a western blot was performed on the samples to confirm the presence of synapsin II. Refer to 'western blot' in objective one for a detailed explanation of the methods; all conditions were the same except 100µg of protein was loaded onto the gel. A subsequent western blot was performed to try and optimize the protein concentration from PC12 cells. Therefore, 20, 40, 60 and 80µg of protein were also tested. The procedure was the same as the previous western, however, this time the cells were re-suspended in a lysis buffer (CellLytic- Sigma-Aldrich, Oakville, ON) to try and break open the cells more efficiently.

Once a sufficient number of PC12 cells were grown, they were then collected, spun down and the supernatant was removed. The cells were then re-suspended in 20ml of new media and counted. Briefly, 20µl of media containing cells was added to a cell counter with a coverslip, and the number of cells in four random squares was counted. The number of cells per ml was calculated as follows; # of cells counted in four random squares x 4 x 10^4. The approximate number of cells was found to be 4,480,000 cells per ml. Next, 500ul of media containing the cells (approximately 2.25 million cells) was plated into each well of a 6-well plate and 1.5ml of new media was added to each well. The cells were then left for one day to grow and adhere to the plates. The following day, the cells were then treated according to the treatment plan in Table 4. The treatment lasted approximately 52 hours, and was performed in duplicates. After treatment, the

wells were scraped to collect the cells, the solution was spun down, the supernatant aspirated out and the cells were re-suspended in TE buffer with mini C tablet. Protein concentrations were then determined using the Bradford protein assay, and western immunoblotting was performed to determine if the MO had successfully knocked down synapsin II.

Table 4: PC12 cell treatment plan on 6-well plates

Well number	Treatment
1	Control (only received new media)
2	Endo-porter only (new media + 6µl/ml EP)
3	2 nmol synapsin II MO + 6µl/ml EP
4	5 nmol synapsin II MO + 6µl/ml EP
5	10 nmol synapsin II MO + 6µl/ml EP
6	Control (only received new media)

Note: EP= Endo-porter

4 Results

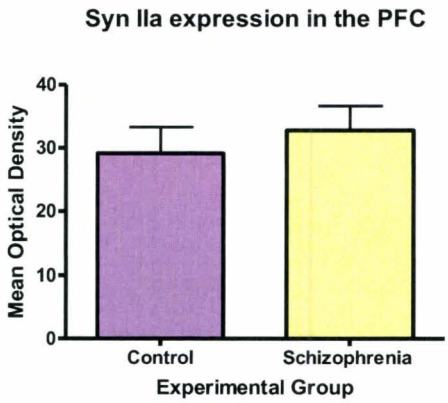
4.1 Synapsin II expression is not significantly altered in the PFC of patients with SCZ

The purpose of the present study was to examine the expression of synapsin II in the PFC, since a previous study found a significant reduction in synapsin II mRNA in this particular brain region of patients with schizophrenia (Mirnics et al, 2000). Thus, our goal was to try and replicate this finding by examining synapsin II at the protein level. Results obtained from post-mortem prefrontal cortex samples indicated that there was no

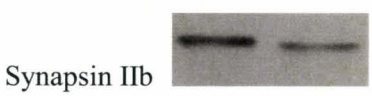
significant difference in synapsin IIa [t (38) = 0.61, p=0.54, d=0.2, Figure 3A] or synapsin IIb [t (38) = 0.76, p=0.45, d=0.05, Figure 3B] in patients with schizophrenia (n=24) when compared to healthy controls (n=16). In addition, no significant difference was observed in the housekeeping gene, GAPDH [t (38) = 0.68, p=0.49, d=0.23, Figure 3C].

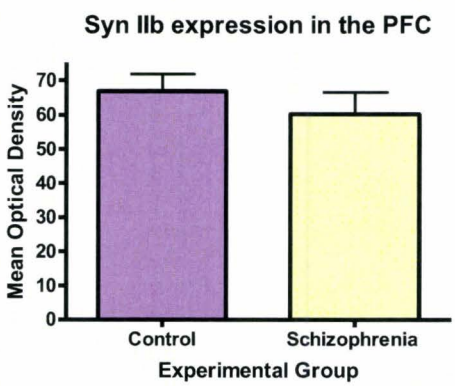
Figure 3. (A) Synapsin IIa expression in the PFC of patients with SCZ (n=24) and controls (n=16), p=0.54 **(B)** Synapsin IIb expression in patients with SCZ (n=24) and controls (n=16), p=0.45 and **(C)** GAPDH expression in patients (n=24) and controls (n=16), p=0.49 Representative immunoblots for synapsin IIa, IIb and GAPDH are shown to the left of the graph.

(A)

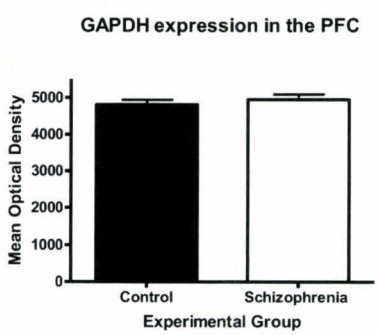


(B)





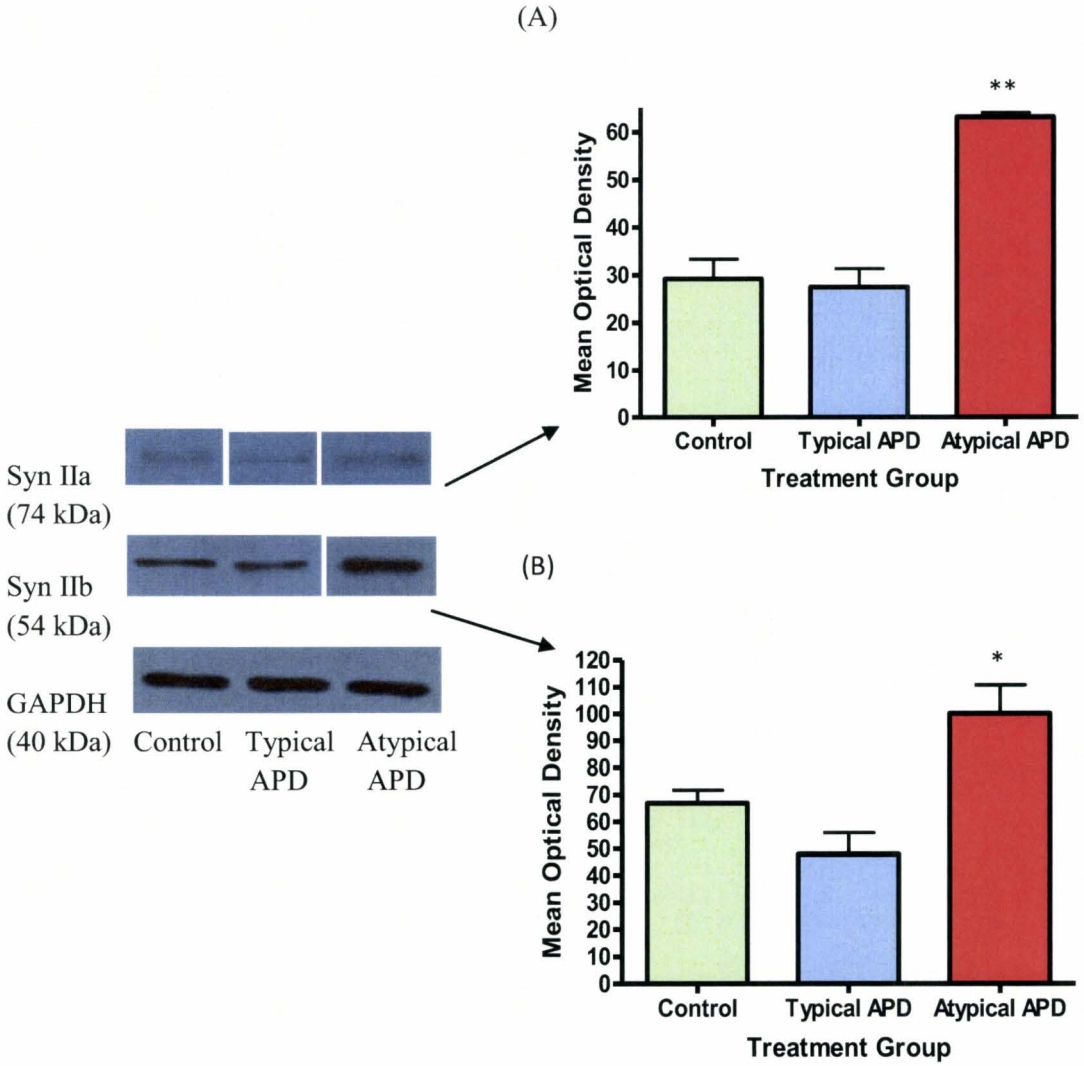
(C)



4.2 Atypical APDs significantly upregulate synapsin IIa and IIb in the PFC of patients with SCZ

When the present data were analyzed previously, the results showed that synapsin IIa and IIb expression were not significantly different between patients with SCZ and normal controls (Fig 3A & 3B). However, with the knowledge that APD treatment affects synapsin II (ex. Chong et al, 2006), the data was re-organized and re-analyzed according to the type of APD patients were previously receiving; typical APDs, atypical APDs and controls (no APDs); patients whose APD history was not available, or those who received a combination of typical and atypical APDs were excluded from the study to avoid potential confounds of drug-class interactions (n=7). Results from this study revealed that both synapsin IIa [t (16) = 4.84, $^{**}p<0.01$, d=2.63, Figure 4A] and synapsin IIb [t (16) = 2.30, $^{*}p<0.05$, d=1.81, Figure 4B] were significantly increased in the prefrontal cortex of patients who received atypical APDs (n=2) compared to both healthy controls (n=16) and patients who received typical APDs (n=15). Further, those patients receiving only typical APDs displayed synapsin IIa and IIb levels comparable to healthy controls. As expected, expression of the housekeeping gene GAPDH did not differ between controls and patients. Despite having a low sample size for the atypical APD group, the size of the experimental effect was significantly large.

Figure 4. (A) Synapsin IIa expression in post-mortem prefrontal cortical samples from controls (n=16) and patients with SCZ previously receiving typical APDs (n=15) or atypical APDs (n=2), $^{**}p<0.01$ **(B)** Synapsin IIb expression in post-mortem prefrontal cortical samples of controls (n=16) and patients with SCZ previously receiving typical APDs (n=15) or atypical APDs (n=2), $^{*}p<0.05$. Representative immunoblots for synapsin IIa, synapsin IIb and GAPDH are shown to the left of the graph.



4.3 Synapsin II is significantly reduced in the SN, but not the STR or PFC, of patients with Parkinson's disease

Aside from the role of α-syn in LBs and LNs, very few studies have examined other synaptic vesicle proteins and how they might be contributing to the etiology of PD. Synapsin II has previously been shown to be disrupted in SCZ (Mirnics et al, 2000), a psychiatric illness which involves dysregulated dopaminergic transmission, and since synapsin II is regulated by DA D1 and DA D2 receptors (Chong et al, 2006), then it is possible that this phosphoprotein might also be dysregulated in PD. Thus, the purpose of this experiment was to investigate the expression of synaptic vesicle proteins, including the synapsins, in patients with PD and normal controls. Expression of synapsin I, II and III, as well as synaptophysin was investigated in the SN, STR and PFC of post-mortem samples from PD patients. The results from these experiments showed that synapsin IIa [t (6) = 4.60, p<0.01, d=3.76] and IIb [t (5) = 2.0, p<0.05, d=2.28] were significantly reduced in the SN of patients with PD when compared to controls (Fig 5B). On the other hand, synapsin II expression did not differ significantly between the two groups in either the STR [IIa- t (4) = 0.94, p=0.39, d=0.94 & IIb- t (5) = 0.46, p=0.66, d=0.42, Fig 6B] or PFC [IIa-t (16) = 0.05, p=0.95, d=0.03 & IIb- t (18) = 0.60, p=0.55, d=0.28, Fig 7B]. In addition, synapsin III was found to be slightly increased in the SN of patients with PD, however, this was not statistically significant [t (6) = 1.46, p=0.19, d=1.2, Fig 5C]. Finally, there were no other differences observed in the other proteins (synapsin I, III, synaptophysin and GAPDH) in the STR (Fig 6) or PFC (Fig 7), and no changes in

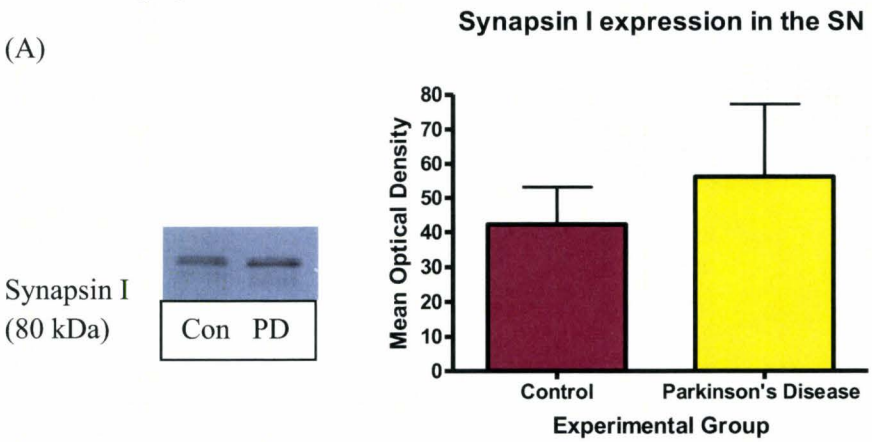
synapsin I, synaptophysin or GAPDH in the SN (Fig 5) (Please refer to Table 5 for other non significant t values and Cohen's D values).

Table 5: Summary of non-significant data from post-mortem studies of patients with PD

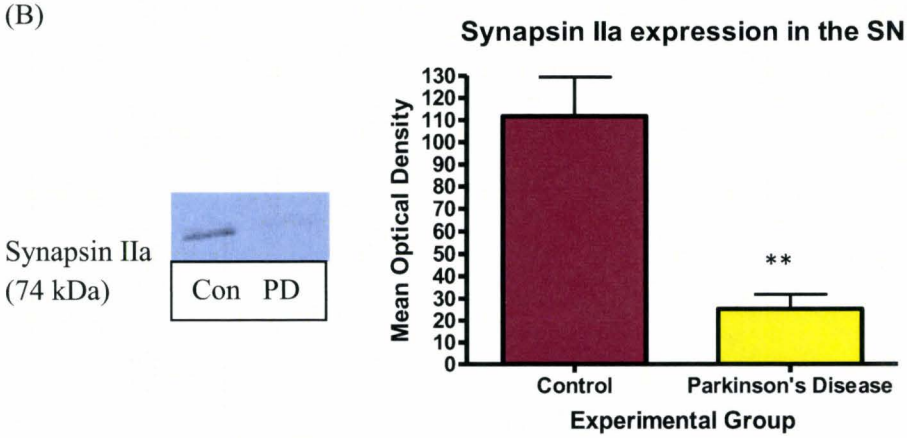
Protein	**Brain Area (PD vs. Con)**	**t value**	**Cohen's D value**
Synapsin I	SN	$t(6)=0.59, p=0.55$	0.48
Synaptophysin	SN	$t(6)=0.64, p=0.54$	0.53
GAPDH	SN	$t(6)=0.82, p=0.59$	0.31
Synapsin I	STR	$t(5)=1.48, p=0.19$	0.74
Synapsin III	STR	$t(6)=0.65, p=0.53$	0.54
Synaptophysin	STR	$t(6)=0.66, p=0.53$	0.54
GAPDH	STR	$t(6)=0.61, p=0.39$	0.18
Synapsin I	PFC	$t(17)=0.57, p=0.57$	0.28
Synapsin III	PFC	$t(18)=0.87, p=0.39$	0.41
Synaptophysin	PFC	$t(10)=0.01, p=0.98$	0.01
GAPDH	PFC	$t(18)=0.82, p=0.45$	0.31

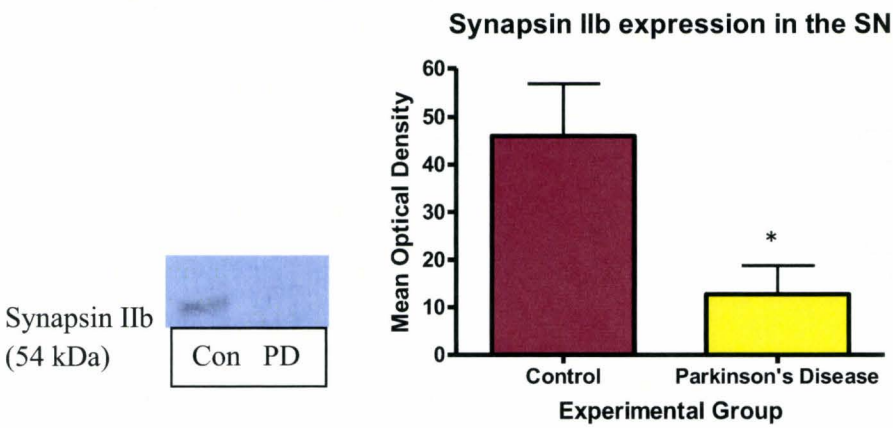
Figure 5. Western blot analysis of post-mortem **SN** samples from patients with Parkinson's disease (n=4) and normal controls (n=4) probed for **(A)** Synapsin I, p=0.57 **(B)** Synapsin IIa, p=<0.01 and IIb, p<0.05 **(C)** Synapsin III, p=0.19 **(D)** Synaptophysin, p=0.54 and **(E)** GAPDH, p=0.71. Representative immunoblot images are shown to the left of the graph.

(A)

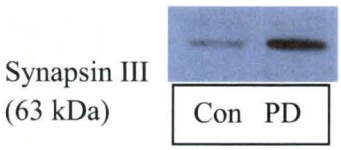


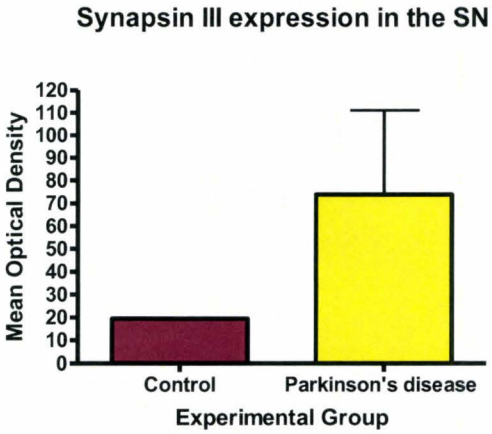
(B)



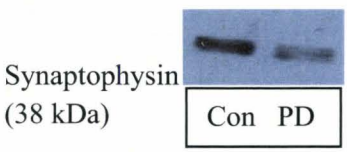


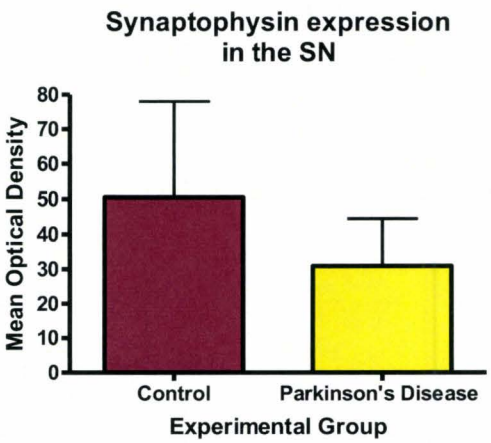
(C)


Synapsin III
(63 kDa)
Con PD

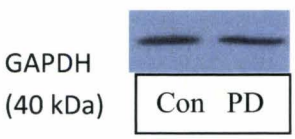

Synapsin III expression in the SN
Mean Optical Density
120
110
100
90
80
70
60
50
40
30
20
10
0
Control
Parkinson's disease
Experimental Group

(D)


Synaptophysin
(38 kDa)
Con PD


Synaptophysin expression
in the SN
Mean Optical Density
80
70
60
50
40
30
20
10
0
Control
Parkinson's Disease
Experimental Group

(E)


GAPDH
(40 kDa)
Con PD

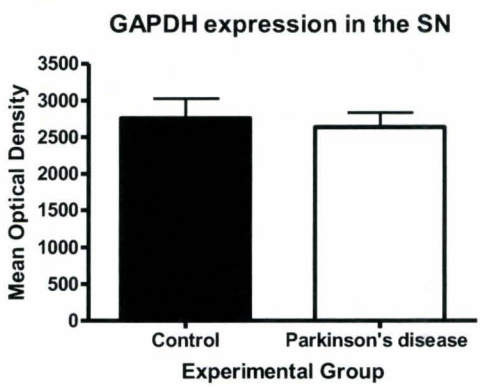
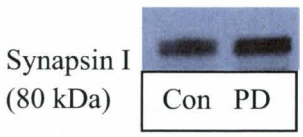
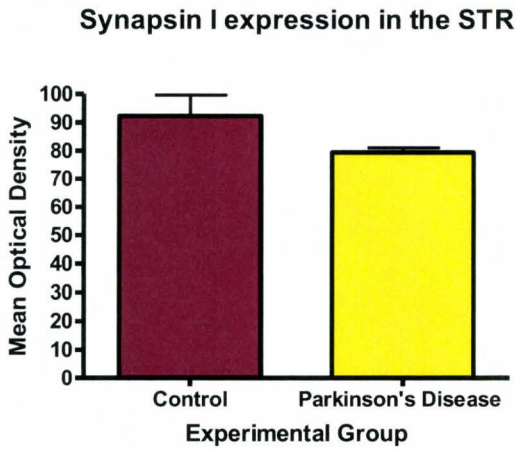

GAPDH expression in the SN
Mean Optical Density
3500
3000
2500
2000
1500
1000
500
0
Control
Parkinson's disease
Experimental Group

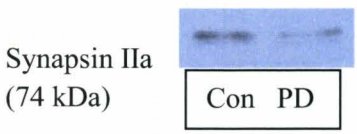
Figure 6. Western blot analysis of post-mortem **striatal** samples from patients with Parkinson's disease (n=4) and normal controls (n=4) **(A)** Synapsin I, p=0.19 **(B)** Synapsin IIa, p=0.39 and IIb, p=0.66 **(C)** Synapsin III, p=0.53 **(D)** Synaptophysin, p=0.53 and **(E)** GAPDH, p=0.83. Representative immunoblot images are shown to the left of the graph.

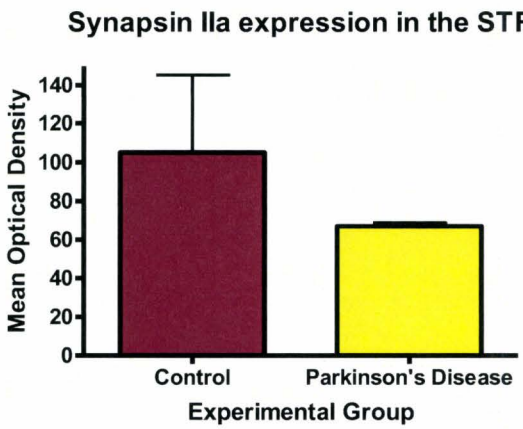
(A)

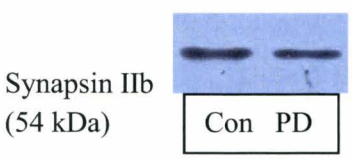


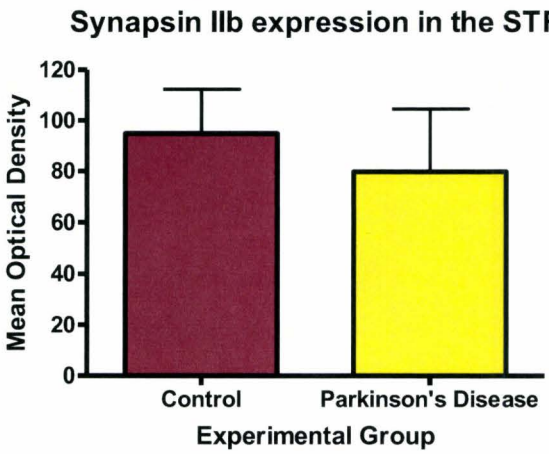


(B)

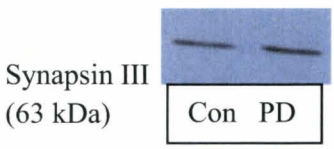


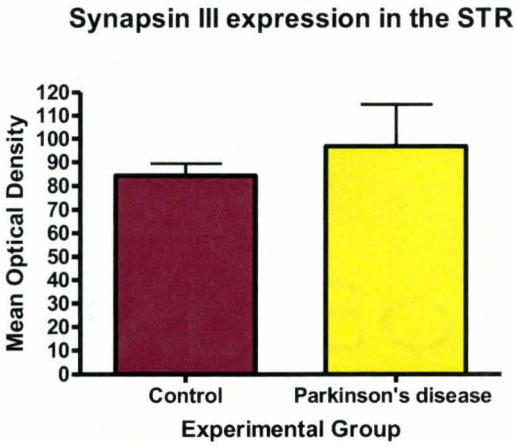




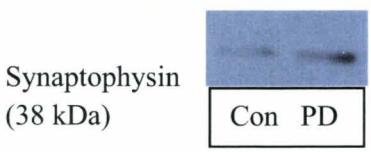


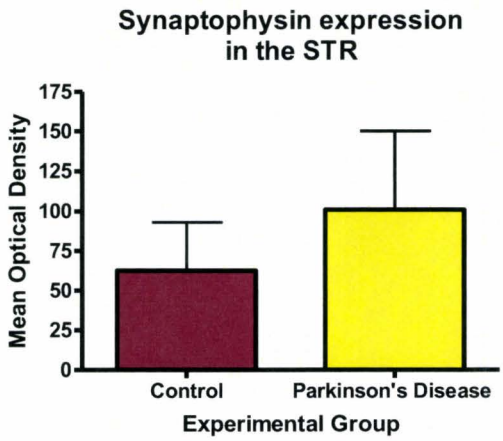
(C)


Synapsin III
(63 kDa)
Con PD

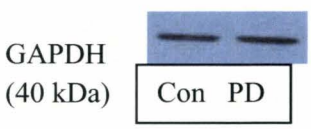

Synapsin III expression in the STR
Mean Optical Density
120
110
100
90
80
70
60
50
40
30
20
10
0
Control
Parkinson's disease
Experimental Group

(D)


Synaptophysin
(38 kDa)
Con PD


Synaptophysin expression
in the STR
Mean Optical Density
175
150
125
100
75
50
25
0
Control
Parkinson's Disease
Experimental Group

(E)


GAPDH
(40 kDa)
Con PD

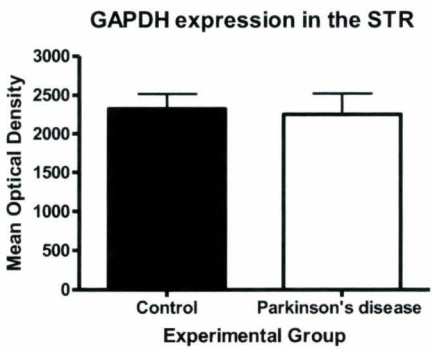
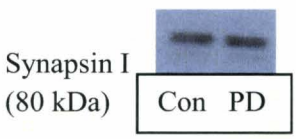
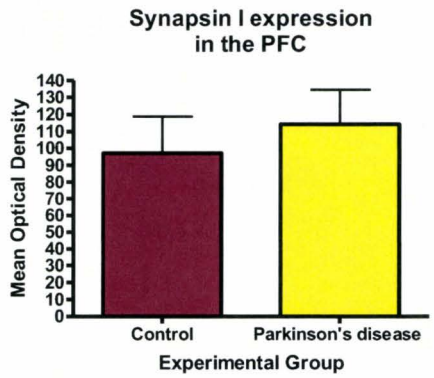

GAPDH expression in the STR
Mean Optical Density
3000
2500
2000
1500
1000
500
0
Control
Parkinson's disease
Experimental Group

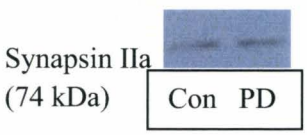
Figure 7. Western blot analysis of post-mortem **PFC** samples from patients with PD and normal controls (A) Synapsin I (PD n=10, control n=9), p=0.57 (B) Synapsin IIa (PD n=9, control n=9), p=0.95 and IIb (PD n=10, control n=10), p=0.55 (C) Synapsin III (PD n=10, control n=10), p=0.39 (D) Synaptophysin (PD n=6, control n=6), p=0.98 and (E) GAPDH, p=0.52. Representative immunoblot images are shown to the left of the graphs.

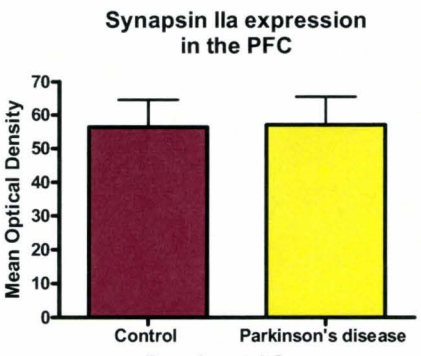
(A)

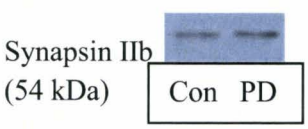


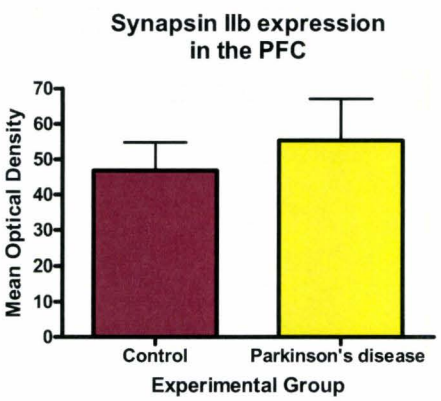


(B)

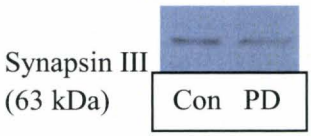


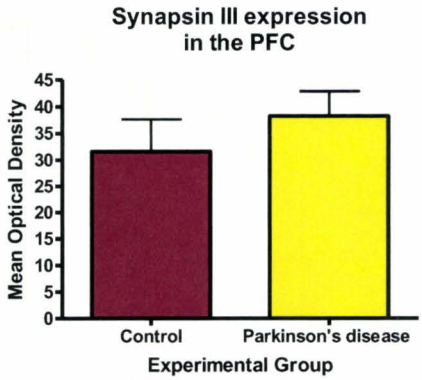




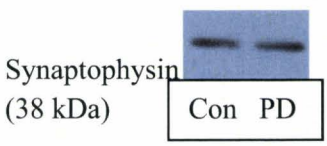


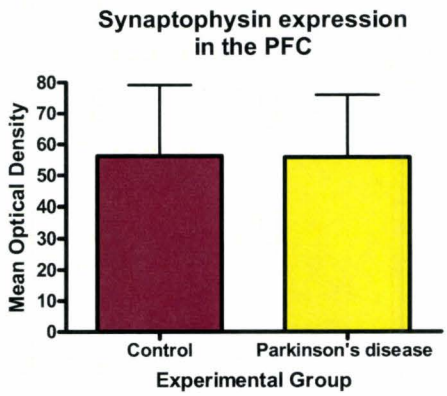
(C)


Synapsin III
(63 kDa)
Con PD

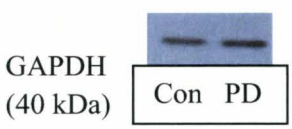

Synapsin III expression
in the PFC
Mean Optical Density
0
5
10
15
20
25
30
35
40
45
Control
Parkinson's disease
Experimental Group

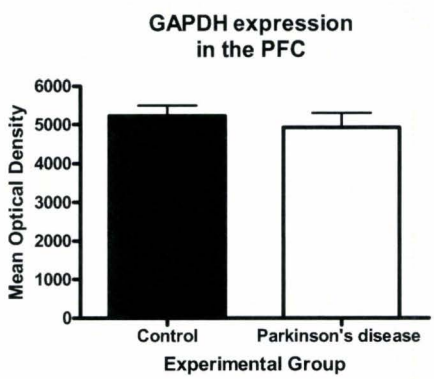
(D)


Synaptophysin
(38 kDa)
Con PD


Synaptophysin expression
in the PFC
Mean Optical Density
0
10
20
30
40
50
60
70
80
Control
Parkinson's disease
Experimental Group

(E)


GAPDH
(40 kDa)
Con PD


GAPDH expression
in the PFC
Mean Optical Density
0
1000
2000
3000
4000
5000
6000
Control
Parkinson's disease
Experimental Group

4.4 Morpholino's fail to knockdown the synapsin II gene in primary cortical and striatal cells

Synapsin II knockout mice have previously been shown to display a variety of behaviours indicative of SCZ (Dyck et al, 2007). Thus, the purpose of the present study was to investigate a novel technique for inhibiting expression of the synapsin II gene. The goal of this study was to try and create a stronger pre-clinical animal model of SCZ, one that displays a broader range of behavioural deficits, without the accompanying side effects (i.e. cell death) that is often caused by other knockdown reagents. Morpholino's are presumed to be superior to other knockdown techniques, since they do not cause significant off-target effect, and are more specific for their target RNA (Summerton, 2007).

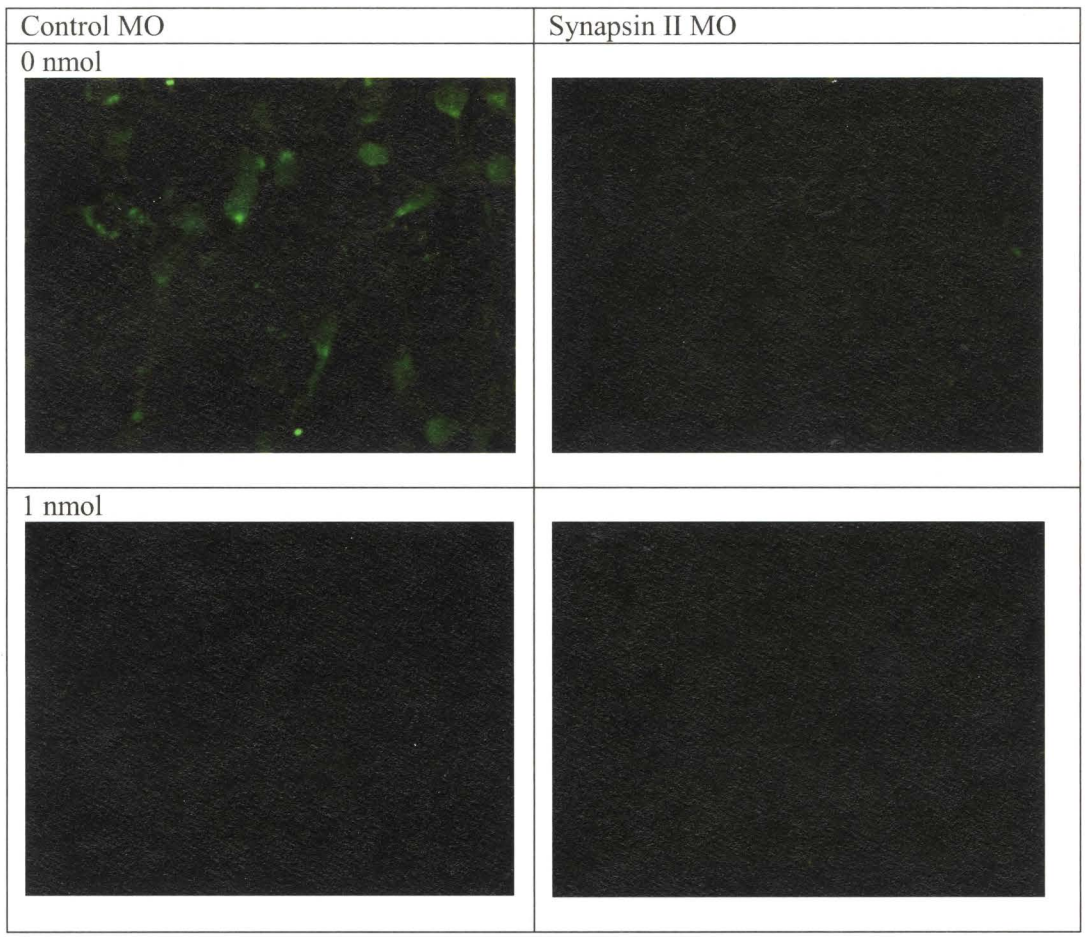
Immunoflourescence

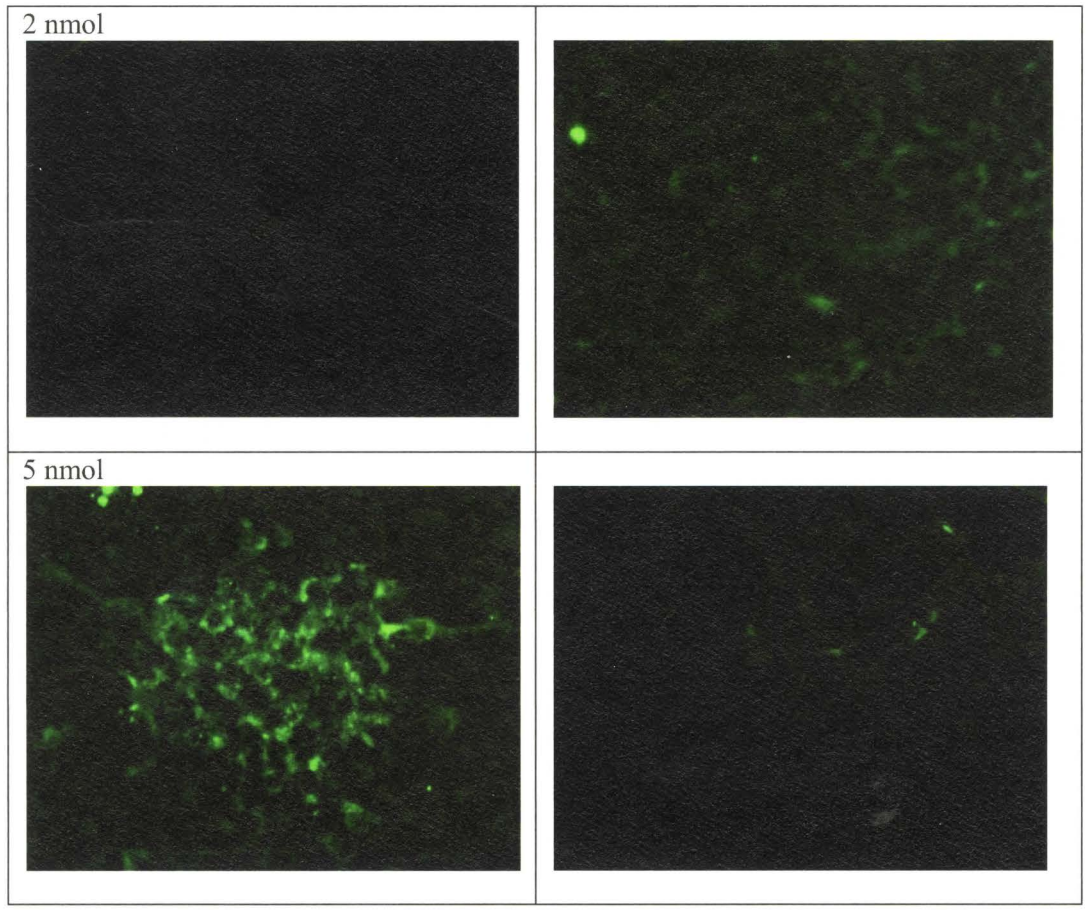
Results from the immunoflourescence experiment did not definitively suggest whether or not our MO sequence worked. In some images, it appeared that the synapsin II MO successfully knocked down synapsin II in the primary cell cultures (ex. Fig 10A, image 4 (5nmol)). However, several images were taken from each slide, and while some of the images appeared to have little (green) fluorescence (ex. Fig 8A, image 4 (5nmol)), others displayed relatively 'normal' fluorescence (ex. Fig 11D). Similarly, for the control MO and control slides (no treatment), some of the images had a fair amount of fluorescence (which would be indicative that the cells still express synapsin II) (ex. Fig 8A, image 3 (2nmol)), and others displayed less fluorescence. Since these results proved

to be unclear, the primary cortical and striatal cells were cultured and subjected to western blotting for a more direct measure of protein expression.

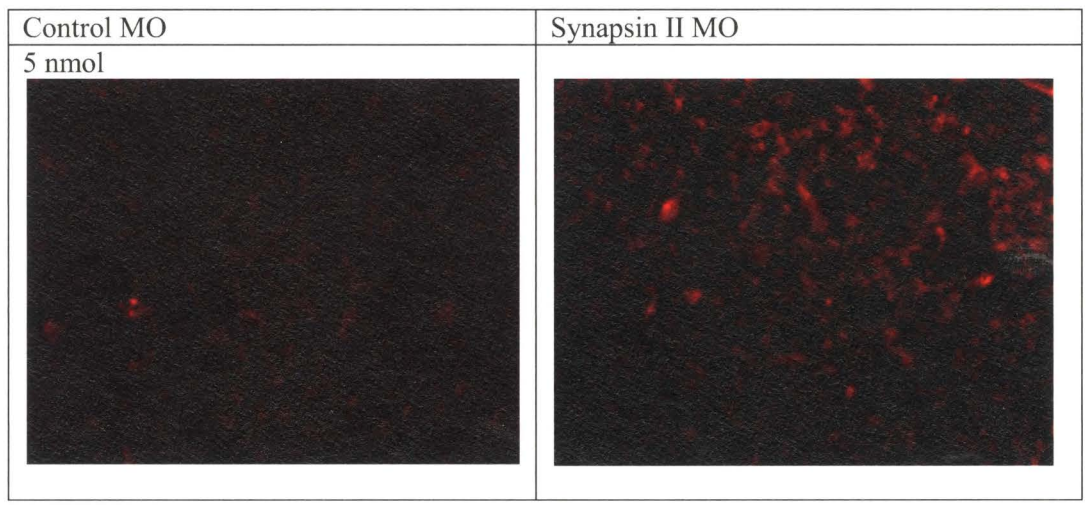
Figure 8. Immunoflourescence images of primary **cortical** cells labelled with synapsin II (1:200)/2°FITC (1:200) and B-tubulin III (1:10000)/2° CY5 (1:200), **plus 2 µl/ml Endo-porter**. Representative images show cells that were treated with **(A)** 0, 1, 2 or 5nmol (quantity indicated above image) synapsin II as well as **(B)** B-tubulin III expression **(C)** DAPI expression and **(D)** a composite image of synapsin II, B-tubulin and DAPI.

(A)

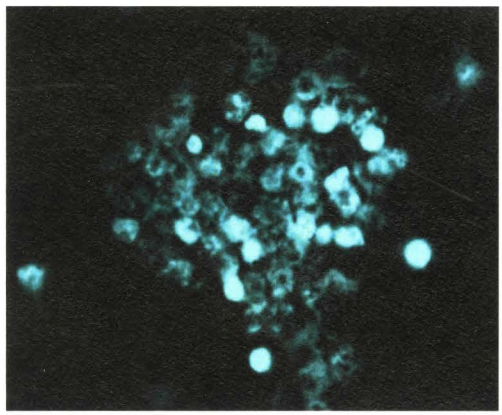
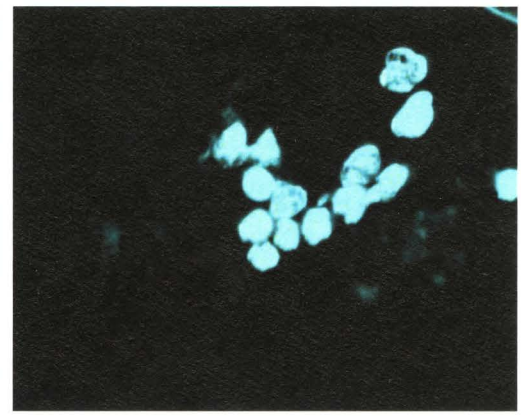




(B)



(C)

Control MO	Synapsin II MO
5 nmol 	

(D)

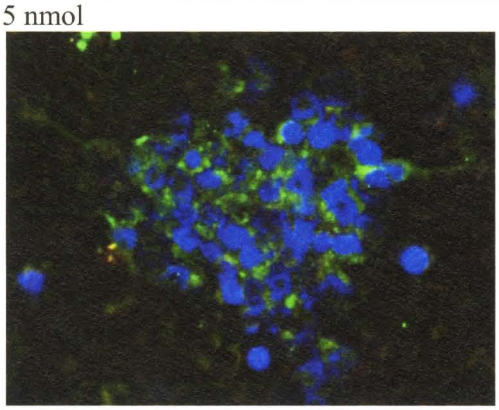
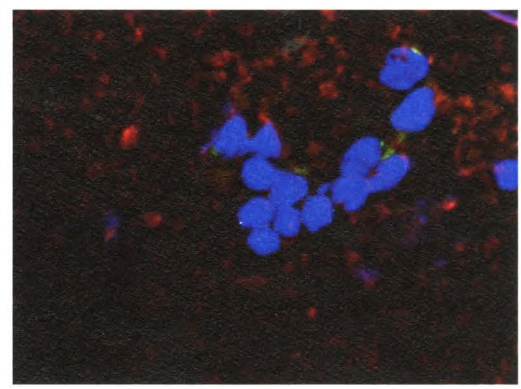
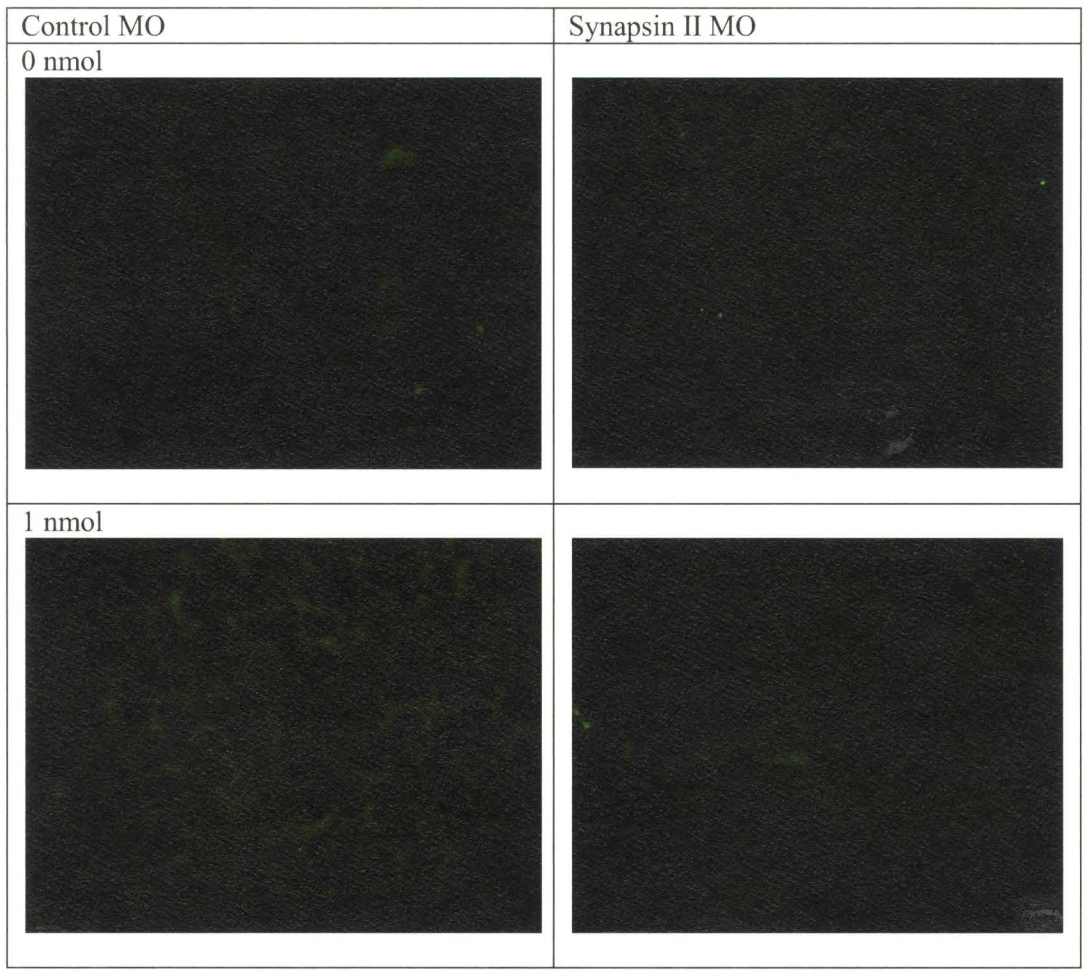
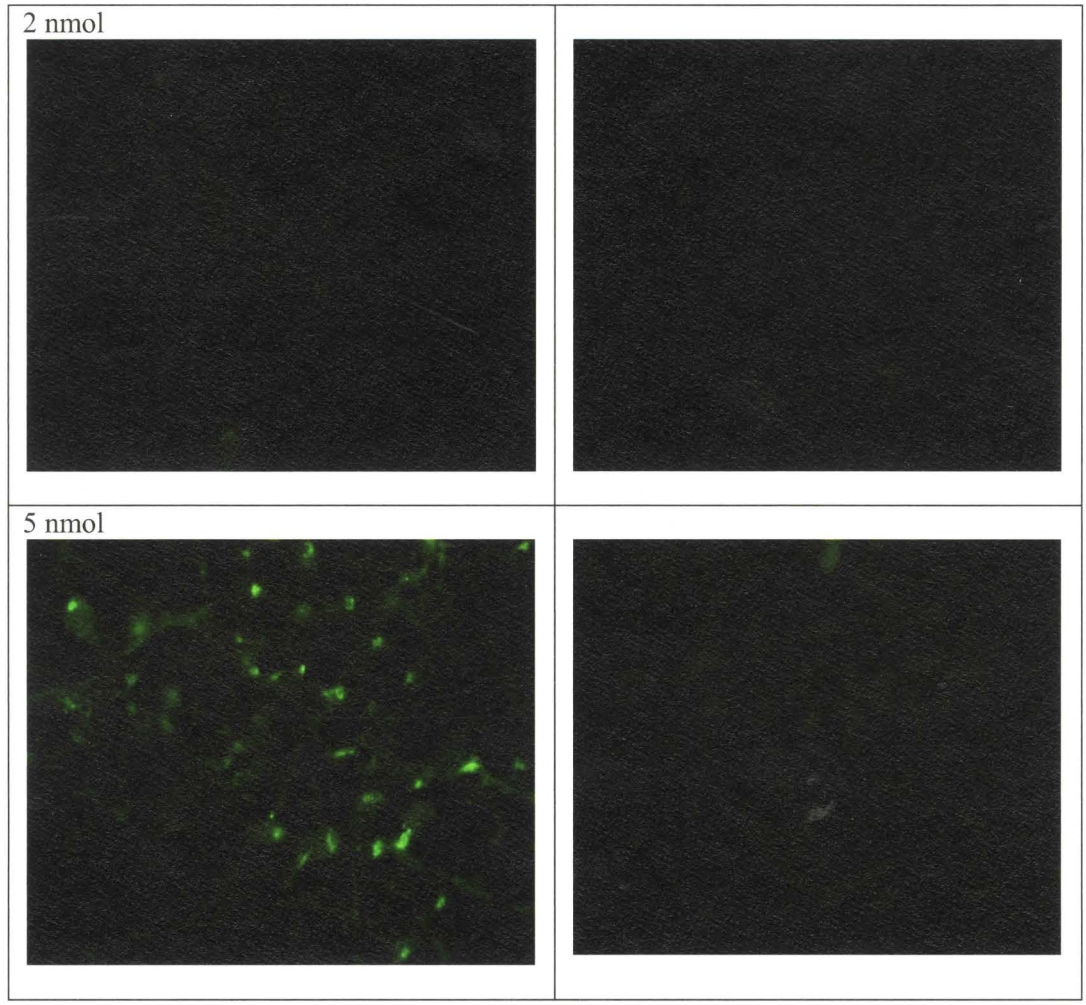
Control MO	Synapsin II MO
5 nmol 	

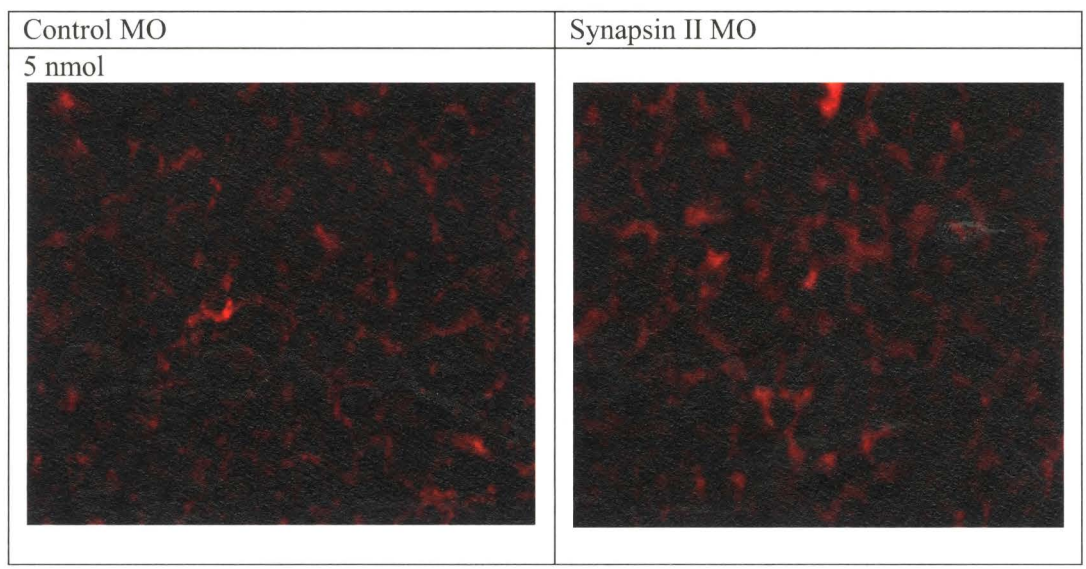
Figure 9. Immunoflourescence images of primary **cortical** cells labelled with synapsin II (1:200)/2°FITC (1:200) and B-tubulin III (1:10000)/2° CY5 (1:200), **plus 4 µl/ml Endo-porter**. Representative images show cells that were treated with **(A)** 0,1,2 or 5nmol (quantity indicated above image) synapsin II as well as **(B)** B-tubulin III expression **(C)** DAPI expression and **(D)** a composite image of synapsin II, B-tubulin and DAPI

(A)

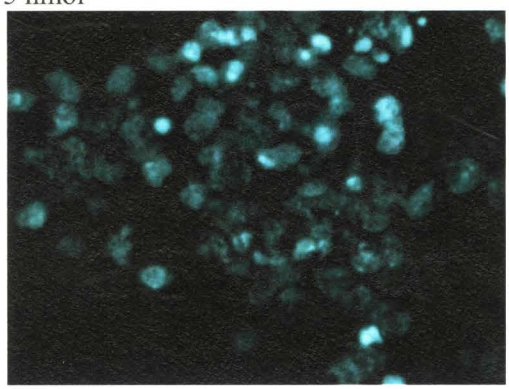
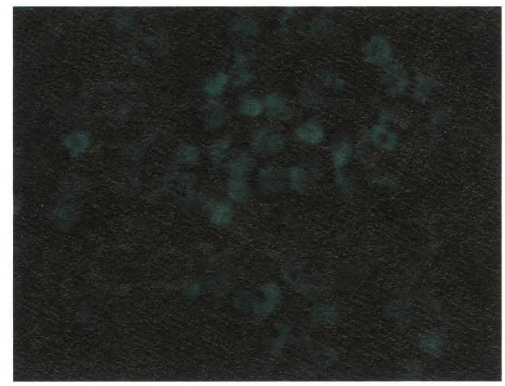




(B)



(C)

Control MO	Synapsin II MO
5 nmol	
	

(D)

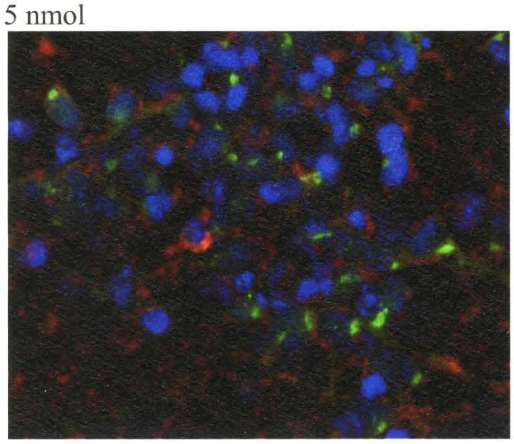
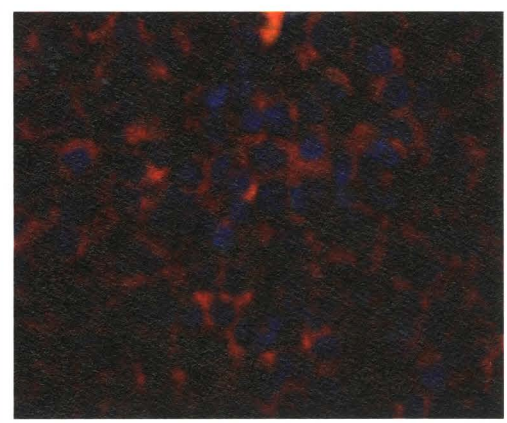
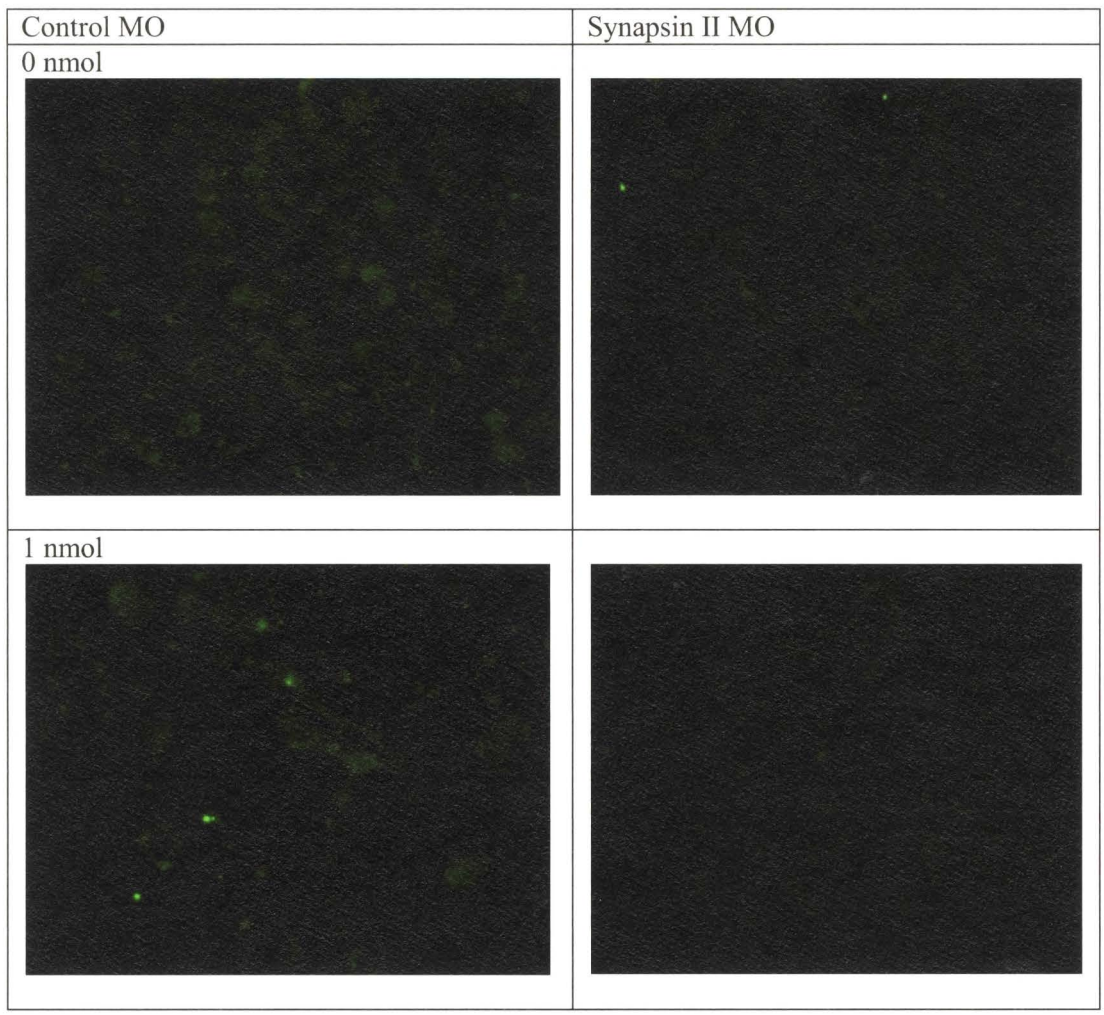
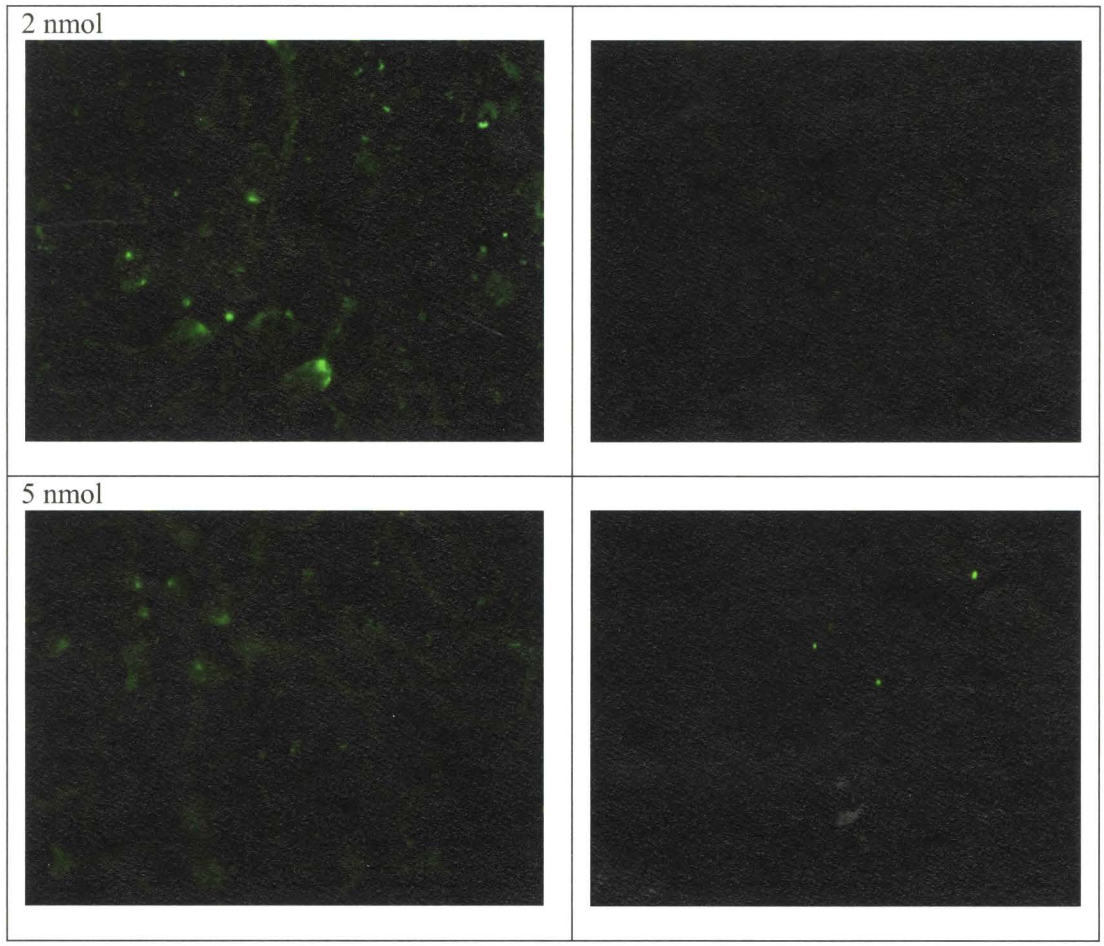
Control MO	Synapsin II MO
5 nmol	
	

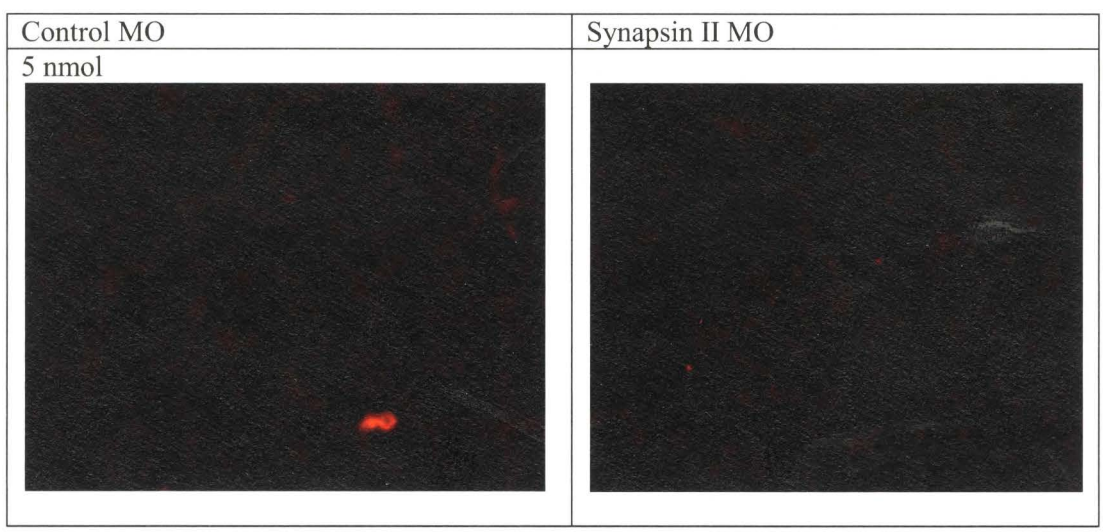
Figure 10. Immunoflourescence images of primary **cortical** cells labelled with synapsin II (1:200)/2°FITC (1:200) and B-tubulin III (1:10000)/2° CY5 (1:200), **plus 6 µl/ml Endo-porter** Representative images show cells that were treated with**(A)** 0, 1, 2 or 5nmol (quantity indicated above image) synapsin II as well as **(B)** B-tubulin III expression **(C)** DAPI expression and **(D)** a composite image of synapsin II, B-tub and DAPI.

(A)



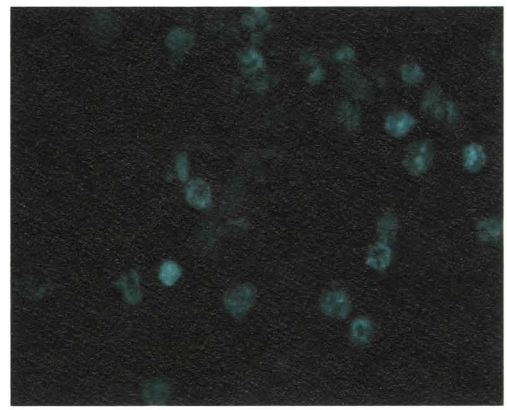


(B)



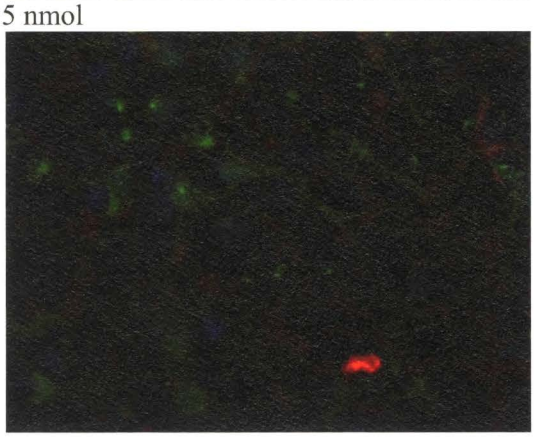
(C)

Control MO	Synapsin II MO
5 nmol	



(D)

Control MO	Synapsin II MO
5 nmol	



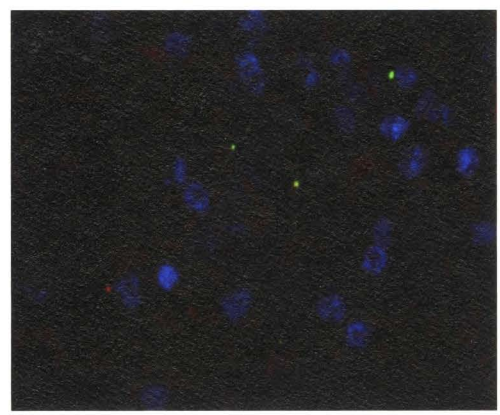
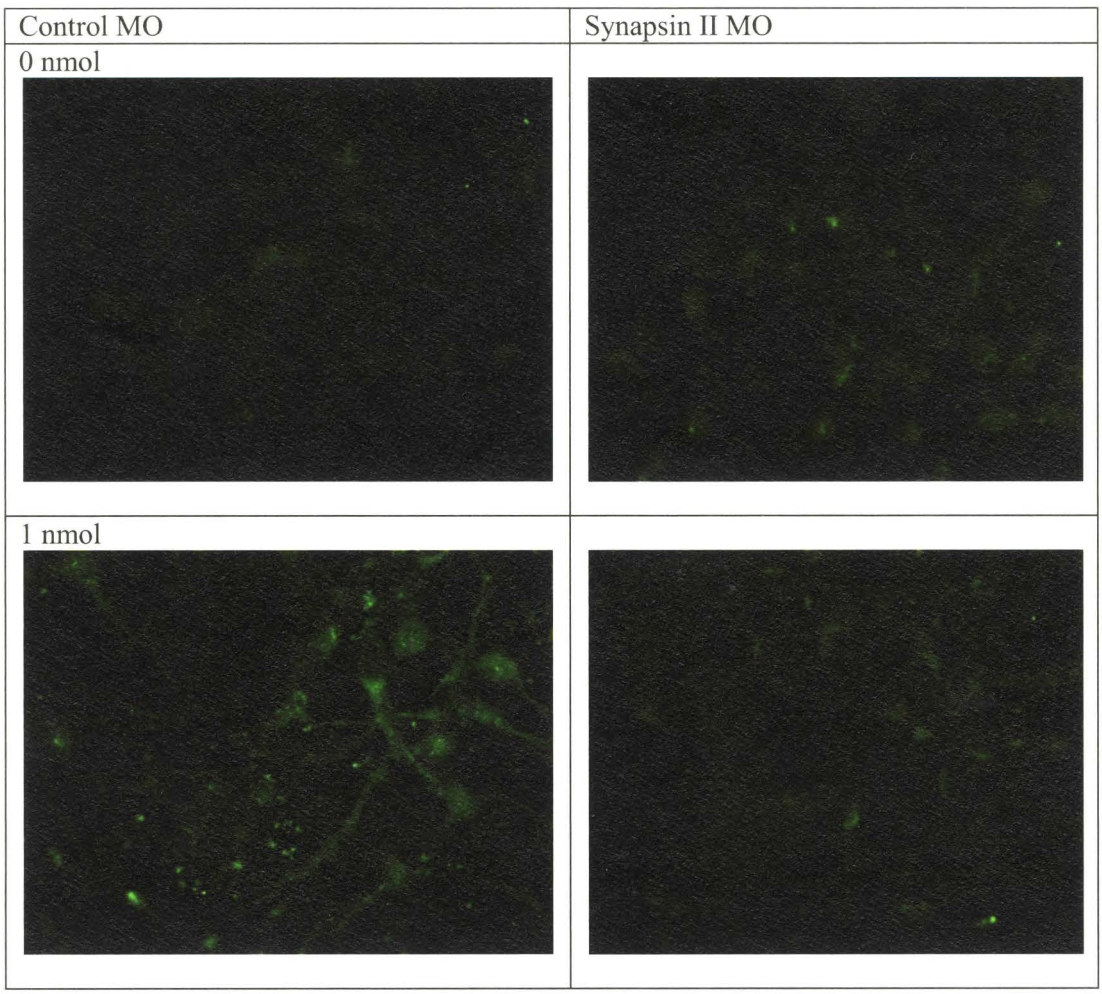
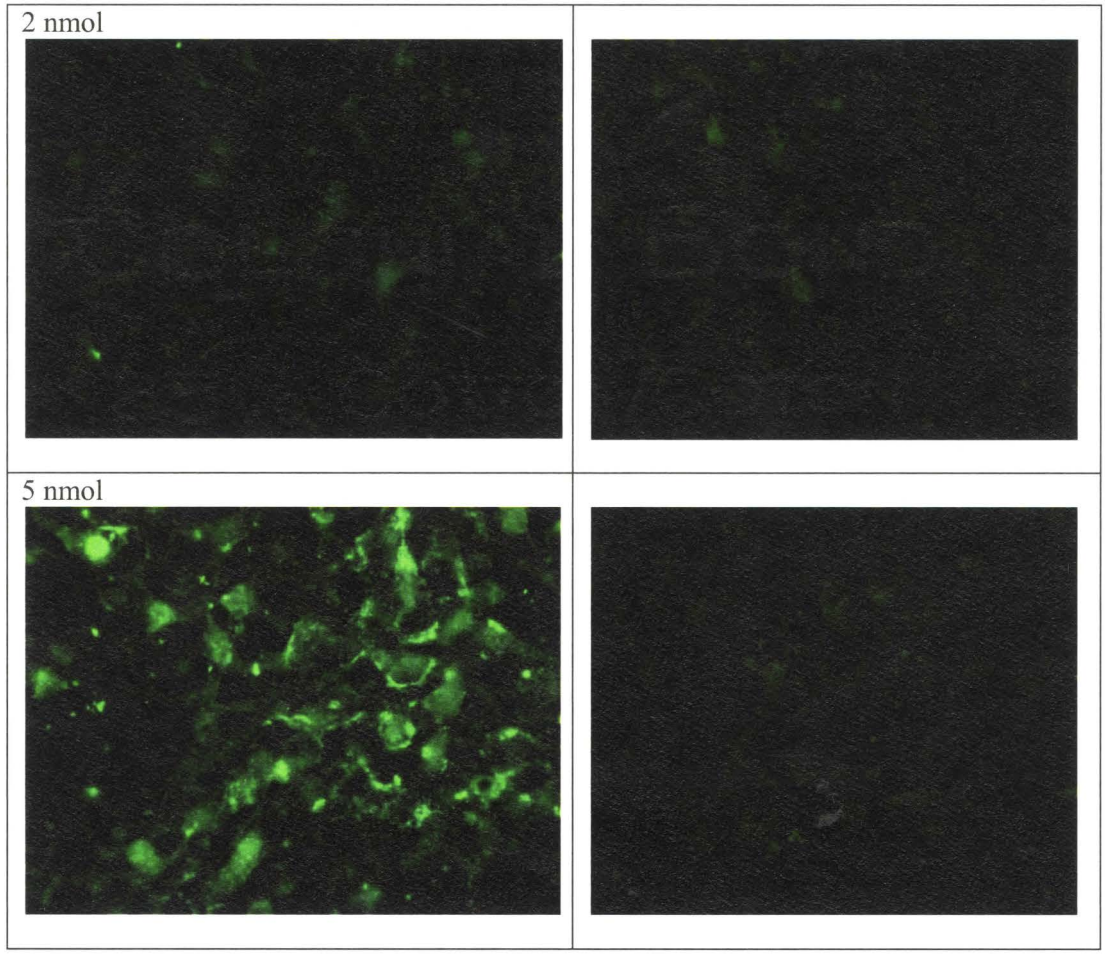


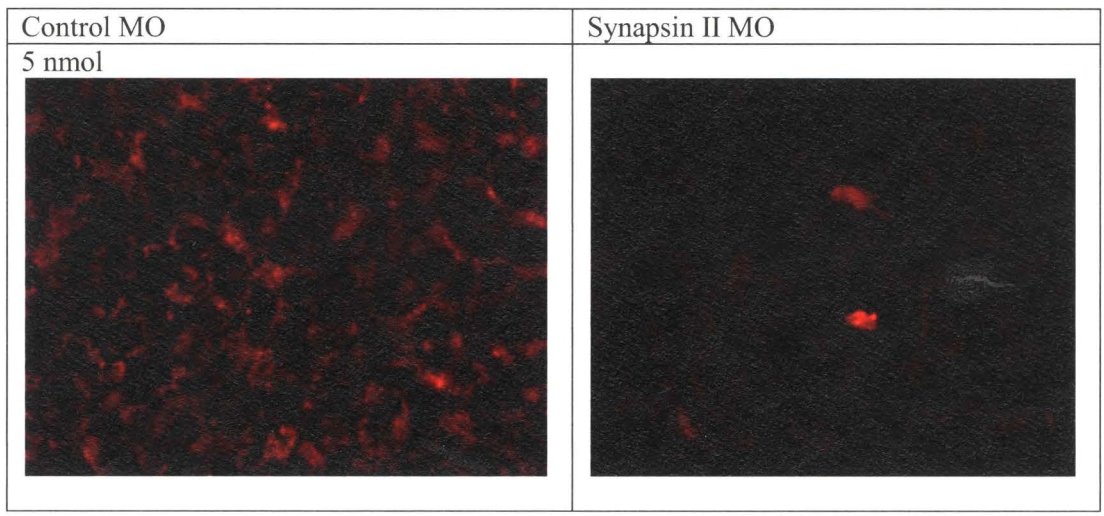
Figure 11. Immunoflourescence images of primary **striatal** cells labelled with synapsin II (1:200)/2°FITC (1:200) and B-tubulin III (1:10000)/2° CY5 (1:200), **plus 6 µl/ml Endo-porter**. Representative images show cells that were treated with **(A)** 0, 1, 2 or 5nmol (quantity indicated above image) synapsin II as well as **(B)** B-tubulin III expression **(C)** DAPI expression and **(D)** a composite image of synapsin II, B-tubulin and DAPI.

(A)



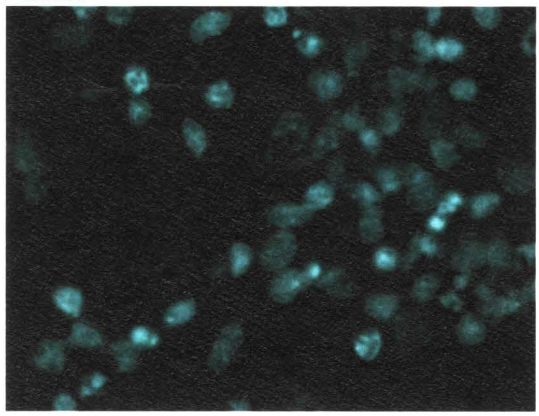


(B)



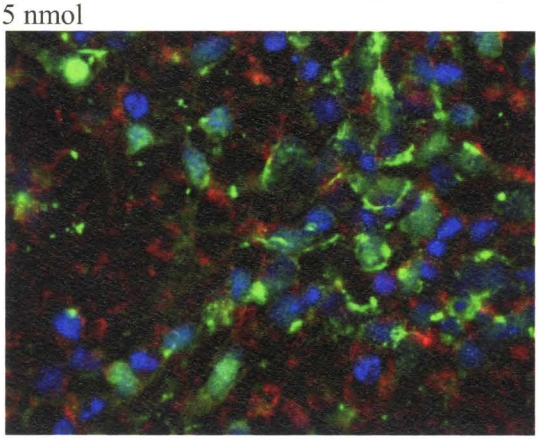
(C)

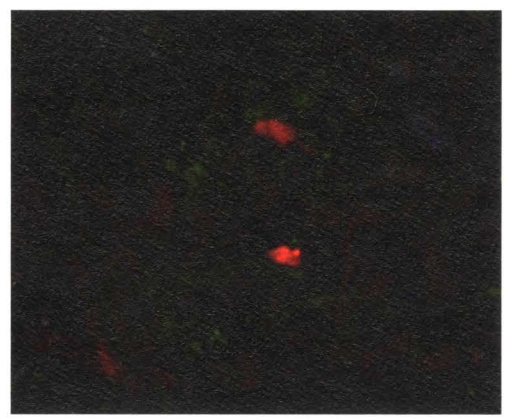
Control MO	Synapsin II MO
5 nmol	



(D)

Control MO	Synapsin II MO
5 nmol	



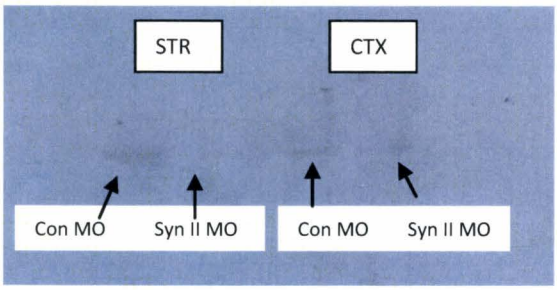


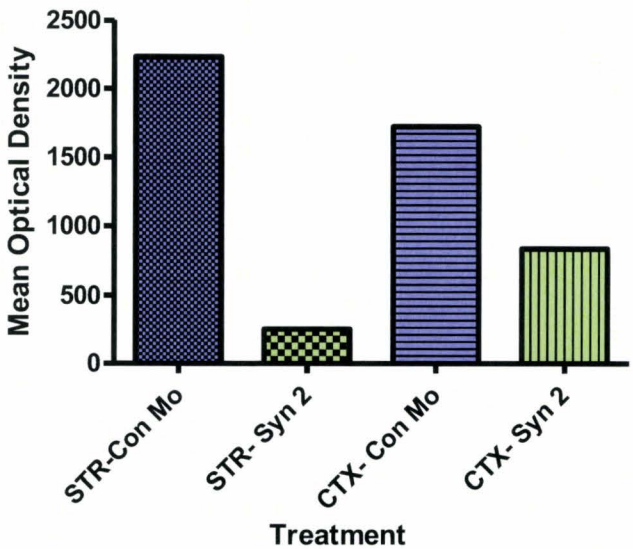
Western Blot

Results from the first western blot that was performed on samples from both cortical and striatal cells suggested that the synapsin II MO sequence conferred between 60-70% knockdown (Fig 12A). However, the antibody concentration that was initially used (1:10,000) was too low, as the protein bands were very faint. The same blot was then stripped and re-probed with the same synapsin II antibody, but at a higher concentration (1:2500) (Fig 12B). The second film revealed bands that were similar in intensity to the first one, suggesting that the synapsin II protein is expressed at a low concentration in primary cells. Alternatively, it is possible that the antibody stripping solution removed too much protein from the membrane. When the membrane was then stripped and re-probed for a second time with GAPDH, results revealed that the housekeeping gene also changed in the same way that the synapsin II protein changed, that is, it was significantly reduced in the synapsin II MO group (Fig 12C). These results were not expected, as the housekeeping gene is presumed to remain constant across all samples. Thus, to try and rectify this issue, a second (Fig 13A) and third (Fig 13B) western blot was performed on the same samples. The results from the second and third analysis suggested that our protein of interest did not change, that is, the synapsin II MO sequence did not cause a significant knockdown in either cortical or striatal cells.

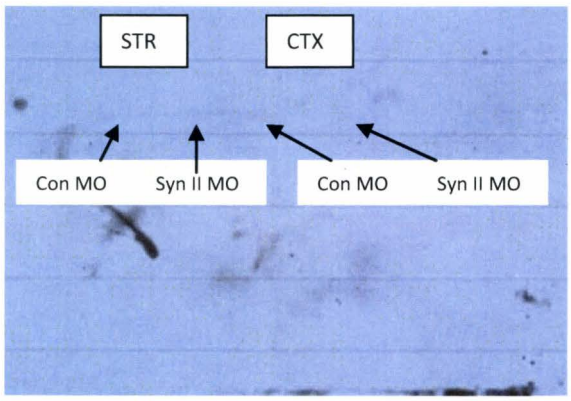
Figure 12. (A) Western blot analysis from primary cortical and striatal cells showing synapsin II expression following treatment with synapsin II MO or control MO (synapsin II 1:10,000) **(B)** Strip and re-probe of original blot using a higher primary antibody concentration (Syn II, 1:2500) and **(C)** GAPDH expression in primary cell cultures. [STR=Striatum, CTX=Cortex]

(A)





(B)


STR
CTX
Con MO
Syn II MO
Con MO
Syn II MO

(C)

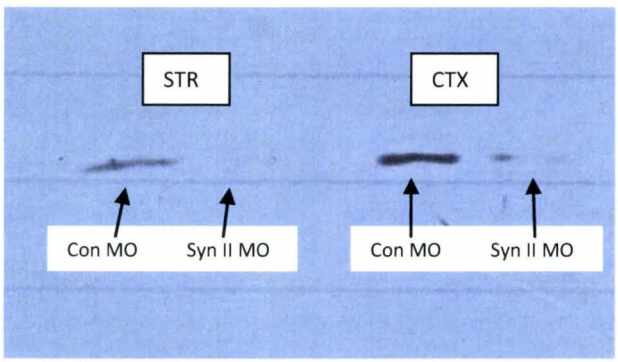
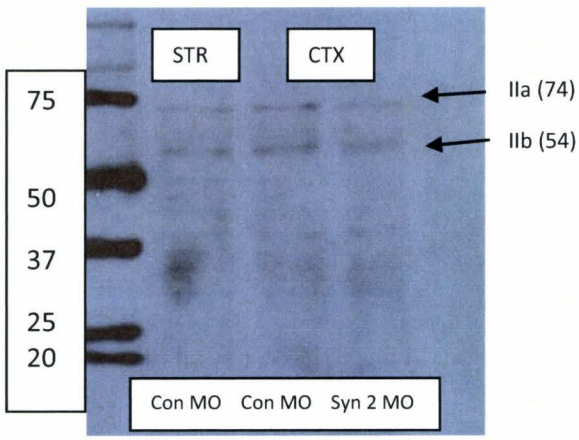
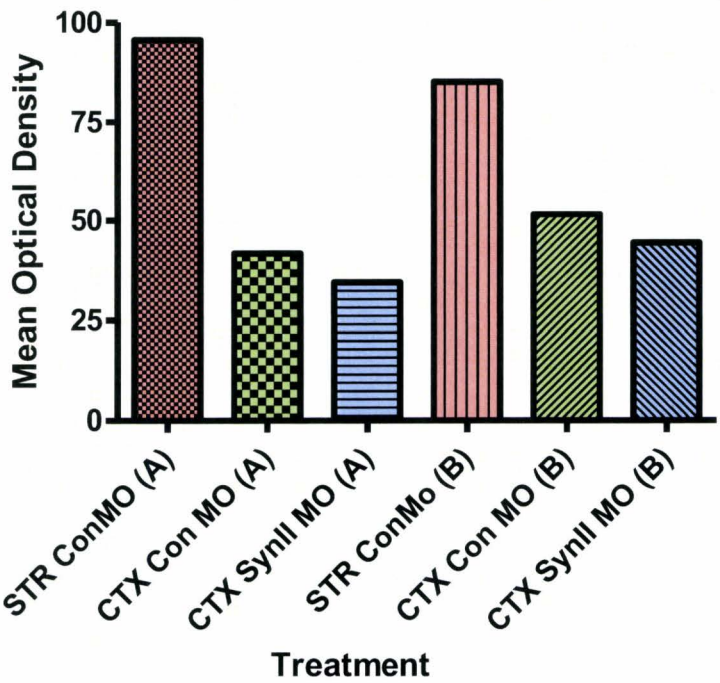

STR
CTX
Con MO
Syn II MO
Con MO
Syn II MO

Figure 13. (A & B) Additional western blot analysis of synapsin II expression in primary neuronal cell cultures (Syn II, 1:2500). (*Note:* There was an insufficient amount of sample from the striatal cells treated with the synapsin II MO to run on the second gel, and an insufficient amount of sample from both striatal samples (Con MO and Syn II MO) to run on the third gel).

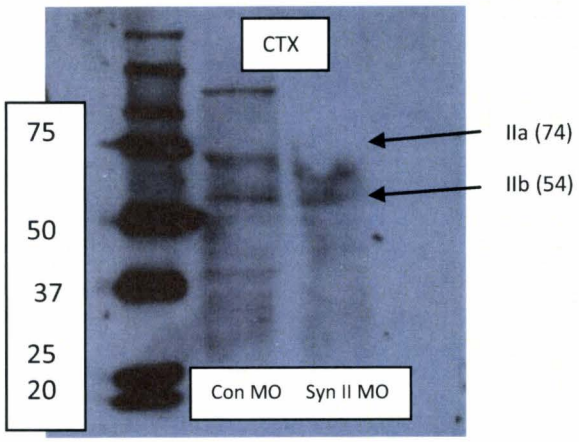
(A)

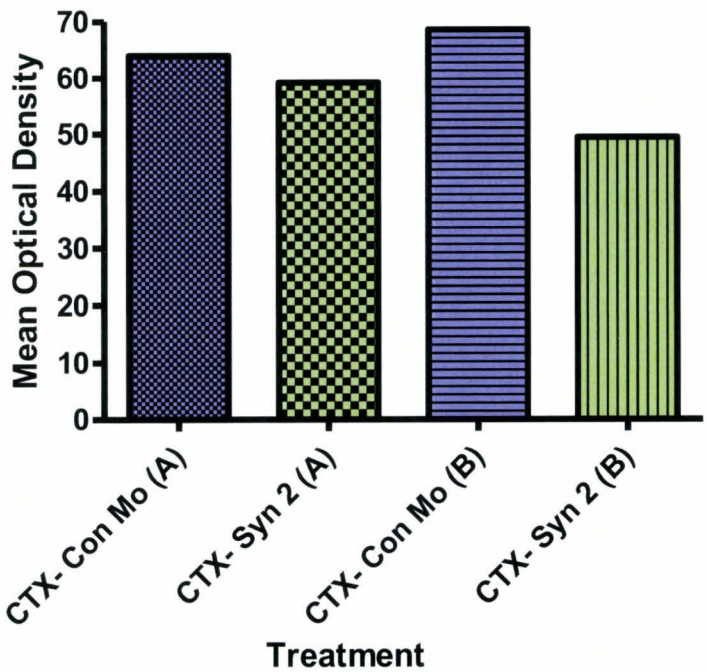




*Graph legend; (A) =Synapsin IIa expression (B) =Synapsin IIb expression

(B)


CTX
75
50
37
25
20
IIa (74)
IIb (54)
Con MO
Syn II MO

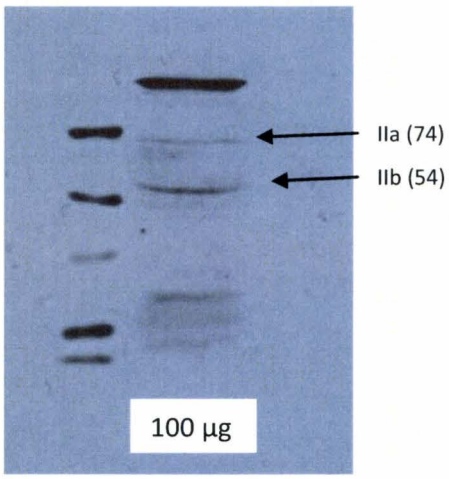

Mean Optical Density
70
60
50
40
30
20
10
0
CTX- Con Mo (A)
CTX- Syn 2 (A)
CTX- Con Mo (B)
CTX- Syn 2 (B)
Treatment

4.5 Morpholino's fail to knockdown the synapsin II gene in PC12 cells

Since the results from the previous experiments were inconclusive, the synapsin II MO sequence was tested in another cell line. The first part of this experiment involved confirming the presence of synapsin II in PC12 cells. After a sufficient number of cells were grown and collected, a western blot was performed and the results revealed that PC12 cells express both isoforms of synapsin II (IIa and IIb) (Fig 14A). The second part of this experiment involved optimizing the amount of protein from PC12 cells that would be needed to run future western blots. This experiment demonstrated that there is little synapsin II expression in 20μg of protein, but a sufficient amount present in concentrations of 40μg and up (Fig 14B). Finally, the third part of the experiment involved treating PC12 cells with the synapsin II MO (2, 5 and 10nmol) or a control (no treatment). Results from this part of the experiment revealed that the MO sequence failed to knockdown expression of the synapsin II protein (Fig 15 A & B).

Figure 14. Synapsin IIa and IIb expression in PC12 cells (Syn II, 1:10,000) with (A) 100µg of protein loaded and (B) 20, 40, 60 and 80µg of protein loaded.

(A)



(B)

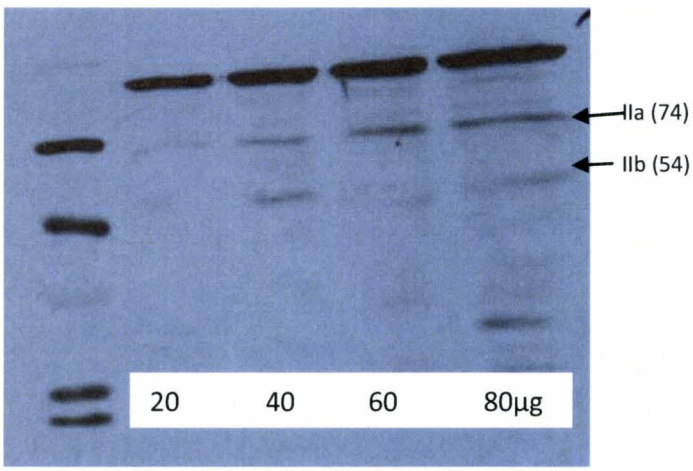
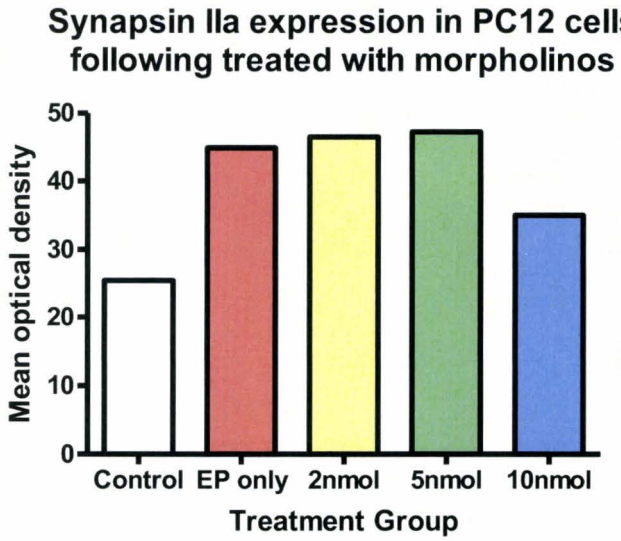
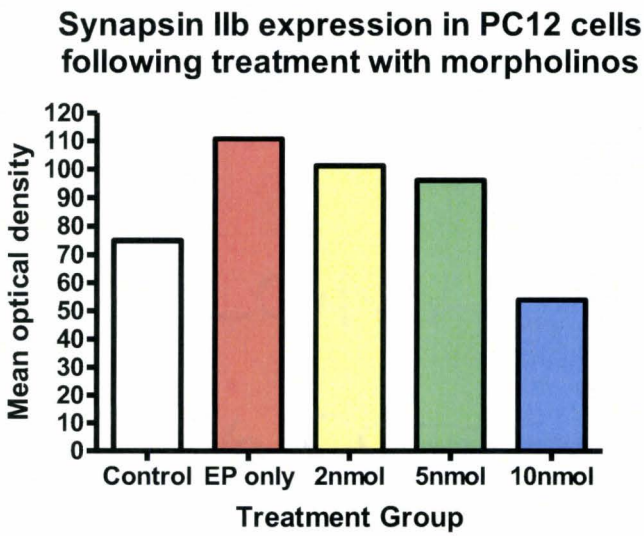


Figure 15. **(A)** Synapsin IIa and **(B)** synapsin IIb expression in PC12 cells following treatment with synapsin II MO (2,5 or 10nmol), endo-porter only (6µl/ml) or control (no treatment).

(A)



(B)



5 Discussion

5.1 Synapsin II protein expression is not significantly altered in the PFC of patients with SCZ

Schizophrenia is a psychiatric illness that is characterized by disruptions in several neurotransmitter systems, including DA and glutamate (Davis et al., 1999, Kim et al., 1980). A recent theory surrounding the etiology of SCZ proposes that this illness is caused by disruptions which originate at the synapse (Frankle et al, 2003). Although vague, this theory attempts to explain the converging factors which lead to synaptic dysfunction in patients with SCZ. In particular, it highlights abnormal connectivity of the PFC during the peri-pubertal and post-pubertal time period, a time when synaptic pruning and maturation is occurring (Frankle et al, 2003). According to this hypothesis, these events can cause the imbalances in excitatory and inhibitory neurotransmitters, and dysfunctions in dopamine transmission that is characteristic of SCZ.

Research on the role of synapsin II suggests that changes in this phosphoprotein may give rise to some of the behavioural and neurochemical deficits which are characteristic of SCZ. For example, synapsin II knockout rats display several behavioural abnormalities including deficits in PPI, social withdrawal, and disrupted performance on a memory task; indicative of the positive, negative and cognitive symptoms of SCZ, respectively. At the anatomical level, these animals show decreases in both glutamate and GABA, but no significant differences in DA (Dyck et al, 2009, submitted June 2010 to *Biological Psychiatry*). In addition, a previous study found that synapsin II mRNA was

significantly reduced in the PFC of patients with SCZ (Mirnics et al, 2000). In order to strengthen the hypothesis that synapsin II was involved in the pathophysiology of this illness, we attempted to show that synapsin II protein was also reduced in the PFC of patients. However, our results demonstrated that overall, synapsin II expression was not significantly different between patients compared to controls (Fig 3A & 3B). These results were surprising, since we expected that a decrease at the mRNA level would translate to a decrease at the protein level. However, previous research has demonstrated that treatment with typical and atypical APDs influence synapsin II expression in the rat MPFC and STR (Chong et al, 2006). Considering that all patients were on APDs prior to death, than it is possible that this may have had some influence on the results. Based on this, we hypothesize that we did not observe a difference between the two groups because APDs are acting to normalize synapsin II levels in patients. However, a previous study by Imai et al (2001), which measured both synapsin II mRNA and protein, also reported no significant difference between patients and controls.

There are other examples of discrepancies present in the literature in post-mortem studies of patients with SCZ. For example, while Vawter et al (2002) reported a significant reduction in synapsin II in the hippocampus of patients, Browning et al (1993) found a reduction in synapsin I, but not synapsin IIb in this brain region. These inconsistent results, however, are not entirely surprising given the complex nature of this disorder, and the high variability that is seen in patients at both the behavioural and neurochemical level. However, despite conflicting reports, the majority of the literature

does support a role for dysregulated synapsin II in the etiology of SCZ (e.g. Mirnics et al, 2000, Vawter et al, 2002, Dyck et al, 2007, Chen et al, 2004).

5.2 Atypical APDs significantly increase synapsin II in the PFC of patients with SCZ

Given the unexpected finding that synapsin II was not significantly altered in the PFC of patients, and given the knowledge that APDs can influence this protein, the data was re-grouped according to the type of APD patients were receiving (typical, atypical or no APDs 'controls'). The results revealed that both isoforms of synapsin II were significantly increased in patients who previously received monotherapy with atypical APDs (Fig 4A & 4B). In addition, patients receiving only typical APDs displayed synapsin II levels which were comparable to normal controls. The results from this study provide strong evidence that synapsin II expression is differentially regulated by treatment with typical and atypical APDs. Previous studies have found that the typical APD haloperidol significantly increases synapsin II mRNA and protein in the rat STR (Chong et al, 2002), and to a lesser extent in the rat MPFC (Chong et al, 2006). Following typical APD treatment, synapsin II expression has been shown to be almost 3 fold greater in the rat STR, than in the MPFC or nucleus accumbens (Chong et al, 2006). Therefore, it is possible that typical APD-induced upregulation of synapsin II is related to the drug's adverse side effects, since the STR is a region of the brain particularly important for motor functions, and has previously been shown to be implicated in extrapyramidal side effects (Farde and Nordstrom, 1992). On the contrary, since atypical

APDs cause significantly fewer motor side effects, these drugs may actually increase synapsin II to therapeutic levels in the PFC, while maintaining normal levels in the STR. This finding is also in agreement with the observation that atypical APDs exert their beneficial effects in the PFC, by increasing DA release. This increase in PFC DA is likely to underlie the improvement in cognition caused by atypical APDs (Moghaddam and Bunney, 1990). Further, the study by Mirnics et al (2000), which reported significant changes in synapsin II mRNA in the PFC of patients reported no significant change in synapsin II in monkeys following chronic haloperidol treatment, suggesting that the APD may have acted to normalize gene expression. Given the converging evidence which suggests a strong relationship between the synapsin II gene and SCZ, our recent findings may help in designing more effective treatment options which target synapsin II in the PFC.

5.3 Synapsin II is significantly reduced in the SN, but not the STR or PFC of patients with PD

One of the key pathological hallmarks of PD is the appearance of LBs and LNs which are composed of the presynaptic protein α-syn. Aside from the role of this protein in PD, there is limited data on other synaptic vesicle-associated proteins, and how they might be contributing to the etiology of this disorder. This is surprising given the important role of synaptic vesicle proteins in biological processes, including neurotransmitter release and the formation of synapses (Ferriera and Rapoport, 2002). Thus, our goal was to determine the expression pattern of various vesicular proteins, including the synapsins, in three separate brain regions of patients with PD. A previous

post-mortem study reported a reduction in both synapsin I and II in the STR of patients using microarray analysis (Miller et al, 2006). However, they did not report any results for synapsin I or II in the SN, so it is unclear whether or not they observed a change in this area of the brain. To our knowledge, our experiment is the first post-mortem study to examine expression of these particular synaptic proteins in the SN, STR and PFC of patients. These brain regions were selected based on the knowledge that they are all afflicted in PD (Braak et al, 2005).

Results from this study revealed that both isoforms of synapsin II are significantly reduced in the SN (Fig 5A), but not the STR (Fig 6A) or PFC (Fig 7A) of patients with PD compared to controls. No other synaptic vesicle proteins (synapsin I, III or synaptophysin) were significantly increased or decreased in any other brain region. Although synapsin III appears to be increased in the SN of patients, the results were not significant. This is likely due to the high variability and low sample size. However, this (non significant) increase suggests that synapsin III may be acting via a compensatory mechanism, whereby a decrease in synapsin II in the SN causes this increase. The finding that no other synaptic vesicle protein is significantly altered suggests that dysregulated synapsin II may be specific to disorders which involve deficits in dopaminergic neurotransmission, since a reduction in this phosphoprotein was previously observed in patients with SCZ (Mirnics et al, 2000).

A reduction in synapsin II in the SN, but not the STR or PFC, can partially be explained by a recent 'pathological staging' of PD known as Braak's staging. In this

model, Braak et al (2003), propose that early stages of the disease involve LB's in the anterior olfactory nucleus, medulla and pontine tegmentum, midstages involve the SN and later stages of the disease involve LB's in cortical areas. Thus, it is possible that in the subset of patients we studied, the pathological effects of PD has already affected the SN, but has not affected the STR or PFC to a significant extent. At present, this hypothesis cannot be ruled out and it would be interesting to determine if synapsin expression correlates with the stage of illness in PD. That is, if patients who are in later stages of the disease show a more significant decrease in synapsin.

The relationship between synapsin II and the pathophysiology of PD remains to be determined. Very few studies have examined synaptic vesicle proteins, other than α-syn, in post-mortem samples or preclinical animal models of PD. At present, it is difficult to determine whether the reduction in synapsin II contributes to the underlying etiology of PD, or is simply a by-product of the diseased state. For instance, a recent study found that over-expression of α-syn caused a 20-45% reduction in synapsin I and II in transgenic mice (Nemani et al, 2010), suggesting that the disruption in synapsin is secondary to the increase in α-syn. However, even if it is a by-product of the diseased state, synapsin II could still contribute to the synaptic dysfunction that is observed in PD (Bagetta et al, 2010), as this protein is important in synaptogenesis and synapse stabilization (Ferreira et al, 1995).

On the other hand, synapsin II could have an underlying causative role in PD. A recent study found that administration of mutant human α-syn into the SN of rats caused

significant DA cell loss by 17 weeks (Chung et al, 2009). However, at 4 weeks post infusion, and before neuronal loss, there were significant changes reported in several proteins which are important in synaptic transmission and axonal transport. Therefore, an alternative explanation is that synapsin II may actually show 'pre-degenerative changes', before cell death, and is therefore a cause of the disease and not simply a by-product. This hypothesis, however, is not supported by results from the Chung et al (2009) study, which found no change in synapsin II prior to DA cell death. Although synapsin II was reported to remain unchanged in this study, it is possible that changes were present, but were below the sensitivity of the researcher's assay. Likewise, the study by Nemani et al (2010) which over-expressed α-syn, also reported a significant inhibition in neurotransmitter release and synaptic transmission. Since synapsin II has previously been shown to be involved in both of these processes (Hilfiker et al, 1999, Ferriera & Rapoport, 2002), then it is possible that a reduction in synapsin II is actually causing these deficits. However, further studies are warranted to elucidate the role of reduced substantia nigral synapsin II in PD.

5.4 Morpholino oligonucleotides failed to significantly decrease synapsin II in vitro

Morpholino's are proposed to be more advantageous then existing knockdown agents, in that they provide high specificity and stability in biological systems, and produce no off-target effects (Summerton, 2005). Previous studies have shown that knocking down the synapsin II gene with antisense in pre-clinical models results in behavioural deficits that are characteristic of SCZ (Dyck et al, 2007). However, antisense,

as well as siRNA, have been shown to cause serious side-effects *in vivo*. In addition, these techniques only confer up to a 50% reduction in a target gene (Summerton, 2005). Morpholino's, on the other hand, can produce a 70 to 98% loss of protein, suggesting that the behavioural and/or neurochemical consequences would be much more pronounced. Therefore, the purpose of our experiment was to determine if a MO sequence that was designed to block translation of the synapsin II gene was effective in primary cell cultures and in PC12 cells. If MOs are capable of reducing synapsin II more than antisense, and without the accompanying side effects, then perhaps this will provide us with a novel tool to generate a more robust animal model of SCZ.

However, results from the synapsin II MO-treated primary cells were questionable, with some immunoflourescence images displaying a lot of fluorescence (indicative that the cells still expressed synapsin II) (ex. Fig 8A), while others displayed little fluorescence (ex. Fig 10A). Reasons for the inconclusive results could be due a procedural problem with the antibodies. Although they were optimized previously, it is possible that the primary and/ or secondary antibodies were distributed unevenly on the slides, which could have caused some areas to fluoresce more than others. Unfortunately, results from the western blot on the primary cortical and striatal cells were also inconsistent. Results from the first immunoblot suggested that our MO sequence caused a large reduction in our protein (Fig 12A), however, when our housekeeping gene displayed the same pattern of expression (Fig 12C), our results could not be relied on. Furthermore, results from additional immunoblots revealed that the synapsin II protein

did not differ significantly in MO-treated cells, compared to control cells (Fig 13A & 13B).

There are a few explanations as to why we might have observed these discrepancies in our results. It is possible that because our initial blot was stripped and re-probed twice, some of the protein that was present on the membrane was removed, and thus, led to the observation that GAPDH expression was decreased in synapsin II MO-treated cells. However, an alternative explanation is that these results are indicative that our MO sequence did not successfully target the synapsin II gene. Although GeneTools, LLC assert that MOs are more efficient and have higher specificity than other techniques, the chance that a designed sequence will work is typically 70-75% (Summerton, 2007), therefore it is entirely possible that our sequence did not work.

Since our results with the synapsin II MO were inconclusive, our next step was to test the sequence in another cell line to confirm whether or not we could use the MO in future *in vivo* experiments. The first part of this experiment confirmed that PC12 cells express synapsin IIa and IIb (Fig 14A). To our knowledge, this is the first experiment to confirm the presence of both synapsin II isoforms in this particular cell line. A previous study by Leoni et al (1999) was only able to detect the synapsin IIb isoform using both western blotting and immunoflourescence. This same study utilized 100µg of protein from PC12 cells for immunoblotting , however, our second experiment revealed that it may only be necessary to use about 40µg of protein (Fig 14B). Finally, the third part of this experiment involved treating PC12 cells with the synapsin II MO and measuring

expression of the phosphoprotein. Results from this experiment provided strong evidence that our MO sequence was not able to target the synapsin II gene (Fig 15). Although it appears as though synapsin II expression decreases with an increasing concentration of the MO, the highest dose of oligo (10 nmol) still displayed expression levels similar to the control.

A possible explanation for our insignificant results could have been due to an issue with delivery of the oligo into the cytosol. It is possible that the endo-porter did not effectively shuttle the MO into the cells. This issue could have been resolved by assessing delivery of the MO using fluoresceinated dextran (as a proxy for MOs), or by having our MO designed with a fluorescent tag. However, this additional experiment would have required far more resources (i.e. time and money), and given our results, we did not feel it was worth it to invest in this. Furthermore, we believe that this explanation is unlikely, considering there are several studies in the literature which use endo-porter as the delivery reagent, without using a fluorescent tag, and still get positive results (Summerton, 2005).

It is possible that the way in which our MO sequence was designed could have contributed to its 'inactivity'. As mentioned previously, MOs are 25 base pair sequences which are designed by targeting a region from the 5'cap through to the first 25-bases of the coding region. Therefore, MOs are designed to include the start codon. In theory, this will allow the MO sequence to prevent the initiation complex from forming, and halt translation of the gene (Summerton, 2007). One problem with designing a sequence to

target an ATG codon is that the gene may possess an upstream start codon which is the actual start of translation, and thus the MO would be ineffective. While the full length synapsin II gene does have more than one ATG site, it is unclear whether our MO sequence was targeted to the actual translation start site, but this explanation cannot be ruled out.

6 Conclusions and Future Directions

In conclusion, although we did not observe a significant difference in synapsin II expression between patients with SCZ and controls, we showed that atypical APDs significantly increase this phosphoprotein in the PFC of patients. This increase in synapsin II may underlie the beneficial effects of atypical APDs in treating the symptoms of SCZ, and may help in designing more effective treatment options for patients. Future experiments might attempt to correlate cumulative lifetime APD usage with synapsin II protein expression to determine if higher doses of APDs increase synapsin II more significantly. Further, it would be interesting to explore the expression of synapsin II in other brain regions that have been implicated in SCZ, such as the basal ganglia.

Our post-mortem analysis of patients with Parkinson's disease revealed significant decreases in both isoforms of synapsin II in the SN, but in not the STR or PFC of patients. This finding may provide evidence for Braak's (2005) pathological staging of PD, which proposes that lower parts of the brain are afflicted in earlier stages of the disease. However, it remains to be determined whether this synapsin dysregulation contributes to the etiology of PD, or is a by-product of the diseased state. In the future, it

would be interesting to consider if treatment with anti-parkinsonian drugs (i.e. L-DOPA) influences synapsin II levels, as APDs have been shown to increase this phosphoprotein. In addition, future experiments could determine if a synapsin II decrease in the SN is correlated with stage of illness, and if this effect is observed in other brain regions. That is, it would be interesting to determine if patients who are in later stages of the disease show disruptions in synapsin in the STR and/or PFC as well.

Finally, our experiment with MOs demonstrated that our sequence failed to knockdown the synapsin II gene in both primary cell cultures and in PC12 cells, suggesting that this novel technique may not be a valid tool for targeting this gene. However, in future experiments, the MO sequence could be re-designed to target a different portion of the synapsin II gene. This may provide a higher degree of knockdown, and thus, a novel technique for inhibiting expression of synapsin II.

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