



**The role of non-coding regions of the alpha-synuclein gene in protein
expression and the determination of alternatively spliced alpha-
synuclein isoforms in oxidatively stressed cells**

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Abstract

The role of non-coding regions of the alpha-synuclein gene in protein expression and the determination of alternatively spliced alpha-synuclein isoforms in oxidatively stressed cells

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Parkinson's disease (PD) is a neurodegenerative disorder caused by substantial loss of dopaminergic neurons and has been extensively linked to alpha synuclein (α -synuclein). This thesis has investigated the non-coding regions and alternatively spliced isoforms of α -synuclein and an improved cell model of PD in which this might be studied. The results indicate that there are several different 5' untranslated region isoforms of α -synuclein expressed and that these stimulate significantly different levels of protein expression when analysed using a luciferase assay. Real-time PCR data indicates that these isoforms are differentially expressed; particularly isoform 202 which was up-regulated in both oxidatively stressed cells and a sample of cingulate gyrus from a dementia with Lewy body (DLB) patient. Interestingly this α -synuclein 202 isoform stimulated the lowest levels of protein expression in the luciferase assay. Three of the four previously reported splice isoforms of α -synuclein were detected; 112, 126 and 140 and the expression levels determined by real-time PCR. Results indicate that isoform 112 expression is up-regulated in oxidatively stressed cells and a sample of cingulate gyrus from a DLB patient but this is not seen when levels of total α -synuclein are normalised. The three spliced isoforms were also cloned and used to determine that a novel antibody was able to detect specifically the 126 α -synuclein isoform by western blotting. Finally, progress was made in developing a new protocol for the differentiation of induced pluripotent stem cells (iPSCs) into dopaminergic neurons using modified messenger RNAs encoding dopamine-specific transcription factors (TFs). Five of required TFs were cloned and ligated to the UTRs needed to produce RNA. Additionally we discovered that iPSCs could not only survive plating onto neuronal substrate in neuronal medium with two daily transfections, but they start to express neuronal markers after four days of culture.

Declaration

I declare that this study consists entirely of my own work. I have not received help or plagiarised in the writing of this report and I declare that all ideas that are not my own have been cited correctly.

Sarah Williams

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Abbreviations

AD	Alzheimer's Disease
ADCC	Aromatic amino acid decarboxylase
Amp	Ampicillin
Ascl1	Achaete-scute homolog 1
ATP	Adenosine Triphosphate
BDNF	Brain derived neurotropic factor
BG	Basal Ganglia
BH ₄	Tetrahydrobiopterin
BSA	Bovine serum albumin
CBP	Co-activator protein
cDNA	Complementary Deoxyribonucleic Acid
CDS	Coding Sequence
CPE	Cytoplasmic poly adenylation element
CPEB	Cytoplasmic poly adenylation element-binding protein
CSP α	Cysteine string protein alpha
DAT	Dopamine transporter
DHPR	Dihydropteridine reductase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded DNA
eIFs	Eukaryotic initiation factors
ELK-1	E twenty-six (ETS)-like transcription factor 1
ERK	extracellular signal-regulated kinase
ETC	Electron transport chain
FGF	Fibroblast growth factor
FIH-1	Factor inhibiting HIF1 α
FoxA2	Forkhead box protein A2
GDP	Guanosine diphosphate
GIRK2	G-protein-coupled inwardly-rectifying potassium channel 2
GOI	Gene of Interest
GP	Globus pallidus
Gpe	Globus pallidus externa
GSP	gene-specific primer
GTP	Guanosine triphosphate
GTPCH1	GTP cyclohydrolase
GWAS	Genome wide association studies
HD	Huntington's Disease
HGB	Human genome browser
Hif1 α	Hypoxia-inducible factor 1 α
HRE	Hypoxia responsive elements
HSV	Herpes simplex virus
iPSCs	Induced pluripotent stem cells
IRE	Iron responsive element

IRP	Iron regulatory protein
Kb	Kilo-Base pairs
LAR-II	Luciferase assay reagent II
LB	Lysogeny broth
LB's	Lewy bodies
L-DOPA	L-3,4-dihydroxyphenylalanine
Lmx1a	LIM homeobox transcription factor 1 alpha
LRK2	Leucine-rich repeat kinase 2
MAPK	Mitogen-activated protein kinase
MCS	multiple cloning site
miRISC	miRNA-induced silencing complex
miRNA	Micro Ribonucleic Acid
MPP ⁺	Pyridine ion
MPTP	1, 2, 3,6-methyl-phenyl-tetrahydropyridine
mRNA	Messenger Ribonucleic Acid
MSA	Mutiple System Atrophy
mtDNA	mitochondrial DNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NAC	non-amyloid β component
NBM	Neurobasal medium
NOS	Nitric oxide synthase
Nurr1	Nuclear receptor related 1 protein
ORF	Open reading frame
PABP	Poly A-binding protein
PBS	Phosphate-buffered saline
PBS-T	PBS with 0.1% Tween-20
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PFA	Paraformaldehyde
PINK1	PTEN-induced kinase 1
PitX3	Pituitary homeobox 3
PKC	Protein kinase C
PolyA	Polyadenylation
PP2A	Protein phosphatase 2A
pVHL	E3 ubiquitin ligase complex containing VHL protein
ROS	Reactive oxygen species
RT	Reverse Transcription
SDS	Sodium Dodecyl Sulfate
SMA	Supplementary motor area
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment receptor
SNCA	Gene encoding α -synuclein
SNpc	Substantia nigra pars compacta
SNPs	Single nucleotide polymorphisms
SR	Seplapterin reductase

SV-40	Simian virus 40
TCA	Tricarboxylic acid
TFs	Transcription factors
TH	Tyrosine hydroxylase
tRNA	Transfer RNA
TSS	Transcriptional start site
UCHL1	Ubiquitin caryboyl-terminase esterase L1
UTP	Uridine triphosphate
UTR	Untranslated Region
VLo	Ventrolateral nucleus part of the thalamus that projects to the motor cortex
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
WT	Wild Type

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1 Introduction

Neurodegenerative diseases are relatively common, extremely debilitating conditions which impact heavily on the lives of individuals and their relatives. These typically age-related disorders are characterised by the loss of neurons within the central nervous system and include Parkinson's Disease (PD), Alzheimer's Disease (AD) and Huntington's Disease (HD). As the average age of the population increases the incidence of conditions such as these is expected to rise substantially (Lees, 2009) leading to an increased burden both on society and the economy (Chen 2010) . As several neurodegenerative diseases have similar symptoms to PD the only definitive diagnosis is via a post mortem analysis confirming the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of lewy Bodies (LBs) in the remaining SNpc neurons (Braak, Tredici et al. 2003). Current therapies are able to alleviate the symptoms of these neurodegenerative diseases, not prevent or treat their progressive nature. This is mainly due to a lack of understanding of the causes of PD and the molecular pathways involved.

One protein, α -synuclein, has been extensively linked to PD, with point mutations and duplications of the gene (SNCA) causing familial PD and genome wide association studies (GWAS) indicating a role in sporadic PD. It is also a major component of LBs. While the function of α -synuclein is still under investigation relatively little is known about the function of the non-coding regions contained within α -synuclein transcripts and the role of alternatively spliced protein. This report focuses on elucidating the role of the non-coding regions and alternative splicing of α -synuclein in a variety of cellular conditions.

Finally it is the lack of good models and molecular tools that hinders the understanding of PD pathophysiology. This report also aims to test 'in-house' antibodies developed to be

specific for the alternatively spliced isoforms of α -synuclein (not commercially available) and create the tools for using modified mRNAs as a method of differentiating iPSCs into dopaminergic neurons.

1.1 Parkinson's Disease

PD, a debilitating neurodegenerative disorder, was first described in 1817 as a “shaking palsy” by the physician James Parkinson, based on the observation of six people (Parkinson 2002 (Originally 1817)). Since then its prevalence has expanded exponentially and is now thought to affect 1:800 people in the UK with the numbers set to increase in the near future (Lees, Hardy et al. 2009). It is predominantly an age-related disease with studies showing that approximately 1% of all individuals over the age of 65 years will develop the condition (Dauer and Przedborski 2003).

PD is usually characterised with four movement-related features; tremor at rest, rigidity, bradykinesia and postural instability (Jankovic 2008). Patients also develop a variety of neurological symptoms such as sleep disturbances, depression, cognitive defects and anosmia (Lees, Hardy et al. 2009) as well as autonomic dysfunctions such as constipation. Many of these symptoms are shared with other neurodegenerative diseases so misdiagnosis is common. The only definitive way to diagnosis PD is via a post-mortem autopsy of the brain, showing substantial loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) of the midbrain and the presence of intra-cytoplasmic inclusion bodies, called Lewy bodies (LBs) (Braak, Tredici et al. 2003). The loss of these neurons results in a decrease in the levels of striatal dopamine, causing the cardinal motor symptoms of PD (Dauer and Przedborski 2003). This also explains why treatment with a dopamine precursor, L-DOPA (L-3,4-dihydroxyphenylalanine), the most common drug used in PD

patients, alleviates most of the symptoms. These nigrostriatal dopaminergic neurons are usually high in neuromelanin so their decline accounts for the “pale brain” observed at the post mortem of PD patients (Marsden 1983), as shown in figure 1. It is generally considered that at the time of diagnosis ~60% of the of SNpc dopaminergic neurons have already been lost (Dauer and Przedborski 2003).

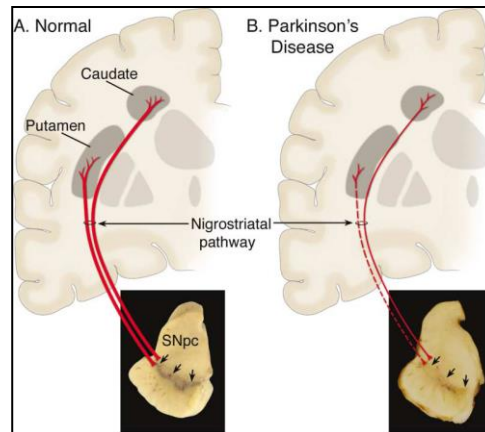


Figure 1: Schematic indicating the nigrostriatal pathway in normal brains and its degeneration in PD brains. Taken from (Dauer and Przedborski 2003)

Approximately 90-95% of PD cases are sporadic with no known genetic links, with the remaining cases familial linked to mutations at specific loci. These “PARK” genes include SNCA (or PARK1/PARK4) (Singleton, Farrer et al. 2003), LRRK2 (Paisán-Ruíz, Lang et al. 2005), and Parkin (Shimura, Schlossmacher et al. 2001). Sporadic PD usually presents at the age of 65 years (Wickremaratchi, Ben-Shlomo et al. 2009) whereas familial PD usually has an earlier onset, especially LRRK2 mutations which can affect individuals <45 years old (Yang, Wood et al. 2009). While only a small number of cases are hereditary it is these mutations that have provided an insight into the genes and proteins involved in the pathophysiology of PD and may eventually yield therapeutic targets.

Many studies have drawn conclusions between environmental factors and PD. The most highlighted environmental exposure was with herbicides and pesticides, based on the observation that they several share similarities to toxins used to create animal models of

PD. However it is unlikely that exposures like this could cause the majority of sporadic PD cases. There is also some controversial evidence that some environmental factors, including smoking and drinking coffee actually have an inverse effect on PD, and decrease the risk of developing the disease (Hernan 2002). It is likely, as with most diseases, that both genetics and environmental factors play a role in disease development.

1.2 Parkinson's Disease Pathophysiology

1.2.1 The Basal Ganglia Loop

The definitive diagnosis of PD is based on the selective loss of dopaminergic neurons within the SNpc and the presence of LBs within neuronal cell bodies (Gelb Dj 1999; Braak, Tredici et al. 2003). In that study the dopaminergic neurons affected in PD account for 10-40% of all neurons in the SNpc and are easily identified in healthy brains by the presence of black neuromelanin, thought to accumulate throughout normal aging in response to cellular stress (Sulzer, Bogulavsky et al. 2000). It is these neurons that are lost in PD patients (Dauer and Przedborski 2003).

The SNpc is part of the midbrain basal ganglia (BG) along with the caudate nucleus, putamen, globus pallidus (GP) and subthalamic nucleus. Information is thought to cycle from the cortex through the BG and thalamus and back to the cortex, especially to the supplementary motor area (SMA). Therefore one of the functions of the BG loop is thought to be the selection and initiation of voluntary movements. The cortex has excitatory projections to the putamen which activates inhibitory synapses on neurons in the globus pallidus. The neurons from the GP form inhibitory synapses with cells in the ventrolateral nucleus part of the thalamus that projects to the motor cortex (VLo) and the thalamocortical connection (VLo to SMA) is excitatory and 'discharges' movement-related neurons in the SMA (Alexander and Crutcher 1990).

SNpc dopaminergic neurons receive the majority of inhibitory GABAergic inputs from the globus pallidus externa (GPe) with smaller inhibitory signals from the neostriatum (Smith and Bolam 1989; Mendez, Elisevich et al. 1993; Misyed 2004). The dopaminergic neurons of the SNpc mainly project to the striatum (Schwab, Agid et al. 1977) (formed by the caudate and putamen) where their release of dopamine stimulates various dopamine receptors (D1-5) on the postsynaptic membranes of neurons in the striatum (Hemmings, Nairn et al. 1984; Nishi, Snyder et al. 1997; Fienberg, Hiroi et al. 1998).

At rest the neurons in the GP are spontaneously active and inhibit the neurons in the VLo. Cortical activation excites the neurons in the putamen, thus inhibiting the GP inhibitory neurons which allow excitation of the neurons in the VLo. Activated VLo neurons results in increased SMA activity and completing the positive feedback loop. An overview of the BG loop is depicted in figure 2, in both physiological (A) and PD (B) conditions.

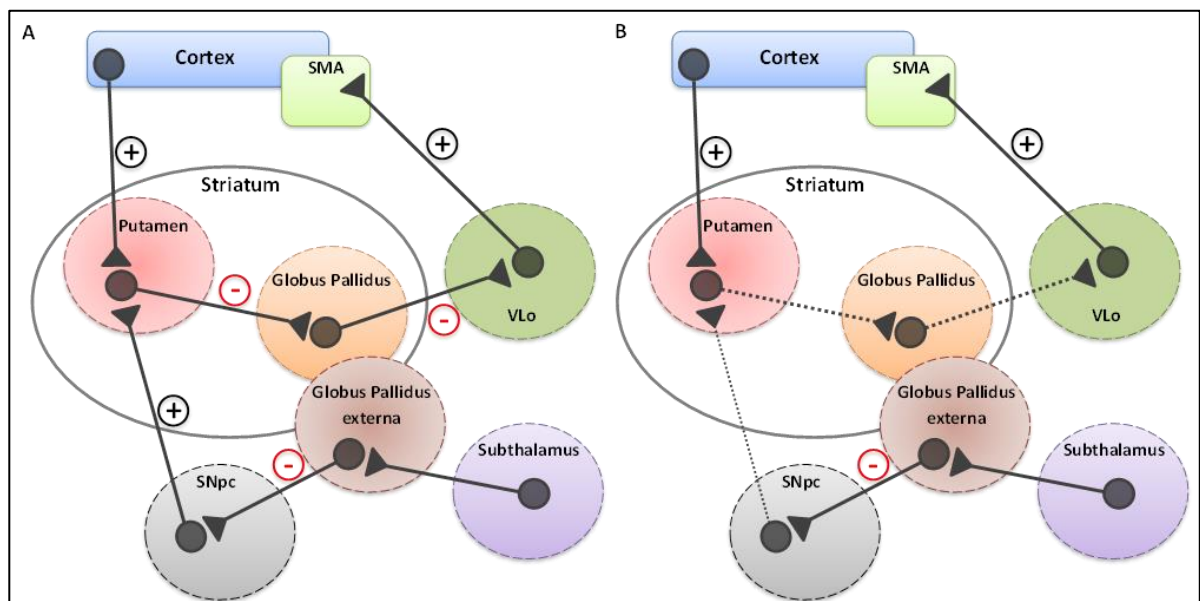


Figure 2: The basal ganglia loop in A) Normal physiological conditions and B) In Parkinson's Disease where the dopaminergic neurons of the Substantia Nigra pars compacta (SNpc) are lost. (VLo- the ventrolateral nucleus part of the thalamus that projects to the motor cortex, SMA- supplementary motor area)

In PD degeneration of the SNpc inputs to the striatum removes the activation of inhibitory neurons from the striatum, releasing the VLo from GP-induced inhibition (Fisone, Håkansson et al. 2007). This results in the classical PD hypokinetic symptoms.

The brain contains many different subtypes of dopaminergic neurons in many different areas but it is only those in the SNpc which are severely affected in PD. The possible reasons for this are discussed in the next section.

1.2.2 Why these dopaminergic neurons?

There are lots of different dopaminergic neuronal subtypes and yet only the D9 neurons suffer the extreme neurodegeneration seen in PD. There are several factors that could provide the answer to their susceptibility.

The first is their size; nigrostriatal dopamine neurons in the rat have been shown to have an extraordinary length (Matsuda, Furuta et al. 2009) and have the capacity to form many thousands of synapses in the striatum (Oorschot 1996; Arbuthnott and Wickens 2007; Venda, Cragg et al. 2010). Therefore the turnover and metabolic demands of these neurons might result in their weakness to survive oxidative stress (Venda, Cragg et al. 2010). Studies have also noted the presence of large numbers of mitochondrial deletions in SNpc neurons of PD patients and age-matched controls which were found to impair the mitochondrial respiratory chain (Bender, Krishnan et al. 2006; Kraytsberg, Kudryavtseva et al. 2006).

Dopamine itself is a highly dangerous oxygen-reactive neurotransmitter, so if its levels are not carefully maintained, for example sequestered for storage in vesicles by vesicular monoamine transporter 2 (VMAT2), it is easily oxidised to form reactive oxygen species

(ROS). Any defects in vesicle pathways could predispose neurons to increased cytotoxicity when faced with an oxidative insult.

The A9 dopaminergic neurons affected in PD are calbindin negative but G-protein-coupled inwardly-rectifying potassium channel 2 (GIRK2)-positive neurons (Damier, Hirsch et al. 1999) compared to the calbindin-positive GIRK2-negative relatively unaffected A10 dopaminergic neurons of ventral tegmental area (VTA) (Venda, Cragg et al. 2010). Another observed difference between the SNc and VTA dopaminergic neurons are the channels that drive the ‘pacemaker’ activity; in SNc neurons it is driven by L-type CaV1.3 calcium channels (Chan, Guzman et al. 2007) while the VTA neurons utilises sodium channels (Surmeier, Guzman et al. 2011). This extra requirement for calcium in combination with a lack of calbindin could mean that A9 neurons are subjected to increased calcium levels and have a lower capacity for calcium buffering leading to increased toxicity (Venda, Cragg et al. 2010). Finally, potassium channel activation has been suggested to be important in the neuronal vulnerability seen in PD. K-ATP channels in SNc neurons are activated by toxins used to induce Parkinsonism but the same effect was not seen in VTA neurons (Liss, Haeckel et al. 2005). They also have a higher dopamine transporter (DAT)/VMAT2 ratio than their VTA counterparts (Uhl 1998; Miller, Gainetdinov et al. 1999). This ratio is necessary to ensure that the dopamine levels are maintained; DAT transports dopamine into the neuron and VMAT2 ensures it is safely stored in vesicles, therefore lower VMAT2 levels can lead to increased cytosolic dopamine and the toxicity mentioned previously.

It is likely that there are many more factors that specify why the A9 dopaminergic neurons selectively undergo the neurodegeneration observed in PD, especially as these have been

determined for mainly *in vitro*. It is likely that the toxic molecules such as dopamine and calcium are subject to further layers of regulation within the brain which cannot be replicated in a tissue culture dish.

1.2.3 Parkinson's Disease Aetiology

1.2.3.1 Familial PD

Despite the incomplete knowledge surrounding the cause of sporadic PD over the last decade there has been many discoveries linking what have now become 'PARK' genes to familial PD. Initially PD was considered to be a completely idiopathic disease with no genetic link and caused by certain environmental triggers, specifically pesticides and head trauma (Seidler, Hellenbrand et al. 1996). A genetic link was not a favourable hypothesis until the initial mutation in α -synuclein was discovered in 1997 (Polymeropoulos, Lavedan et al. 1997). Currently there are 10 genes which directly cause or increase the risk of developing PD. These include PARK1/4 (α -synuclein), PARK2 (Parkin), PARK5 (ubiquitin caryboyl-terminase esterase L1 or UCHL1), PARK6 (PTEN-induced kinase 1 or PINK1), PARK7 (DJ-1), PARK8 (Leucine rich repeat kinase 2 or LRRK2), PARK9 (ATPase type 13A2), PARK13 HTRA-serine peptidase 2, PARK14 (phospholipase A2 group VI) and PARK15 (F-box only protein 7) and are covered in in a review by Martin and Dawson et al. (Martin, Dawson et al. 2011). However, there always seems to be new genes awaiting confirmation of links to PD and there is no doubt that the number will increase in the near future.

In addition, GWAS have confirmed variability around the loci of α -synuclein and LRRK2 indicating that genetics may also play a role in sporadic PD (Pankratz, Wilk et al. 2009; Satake, Nakabayashi et al. 2009; Simon-Sanchez, Schulte et al. 2009).

As with most complex diseases it is likely that both genetics and environmental factors play a role in developing PD. One of the factors most commonly associated with PD is oxidative stress.

1.2.4 Oxidative stress and PD

Mitochondrial dysfunction and Parkinson's disease were first linked when

1,2,3,6-methyl-phenyl-tetrahydropyridine (MPTP) was found to induce parkinsonism in drug abusers (Lin and Beal 2006). The MPTP is metabolised by monoamine oxidase- β (MAO- β) (Maguire-Zeiss, Short et al. 2005) in glial cells in the brain into the active metabolite, a pyridine ion (MPP^+). MPP^+ is then taken up via DAT, particularly by dopaminergic neurons, and actively transported into mitochondria where it acts as a complex I inhibitor (Maguire-Zeiss, Short et al. 2005). This is now exploited to produce animal models for PD.

Mitochondria are organelles present in all cells of the body (except red blood cells) and are maternally inherited. They contain their own circular double stranded (ds) mitochondrial DNA (mtDNA) (together with the nuclear DNA these encode the peptides required for the mitochondrial systems (Schon and Przedborski 2011). Mitochondria perform “house-keeping” functions including the biosynthesis of amino acids and steroids, β -oxidation of fatty acids and play a role in apoptosis (Schon and Przedborski 2011). But it is their capacity to produce adenosine triphosphate (ATP), via the tricarboxylic acid (TCA) cycle and electron transport chain (ETC), which gives mitochondria their function as the “powerhouse” of the cell. Briefly the ETC is a complex of subunits (I, II, III, IV) and two electron carriers which uses the energy created by food catabolism to create a proton gradient and transfer electrons to oxygen, creating water (Schon and Przedborski 2011).

This gradient also drives ATP synthesis via complex V. They contain electron carriers that can produce ROS but also a variety of antioxidants capable of “mopping” them up (Lin and Beal 2006).

As we age mtDNA develops mutations due to the accumulation of ROS, which are thought to contribute to the aging process (Lin and Beal 2006). As many neurodegenerative diseases carry increased risk with age it stands to reason that mitochondria could play a role in either disease initiation or progression. There is a close relationship between protein accumulation and aggregation, oxidative stress and mitochondrial dysfunction (Lin and Beal 2006) but it is unclear which is the causal element. Unfortunately mitochondrial dysfunction creates a cycle whereby mitochondrial damage results in decreased antioxidants, leading to increased ROS and more mitochondrial damage (Lin and Beal 2006). This dysfunction in the mitochondria could be contributing to the damage of vulnerable genes within the aging brain leading to neurodegenerative disorders (Lin and Beal 2006). Additionally accumulation of mtDNA mutations have been found in the substantia nigra of sporadic PD patients (Bender, Krishnan et al. 2006).

Several of the genes implicated in PD, including α -synuclein, have links to mitochondrial dysfunction. For example α -synuclein overexpression in mice leads to impaired mitochondrial function, increased oxidative stress and enhanced pathology when MPTP is applied (Song, Shults et al. 2004). α -synuclein-null mice are resistant to MPTP (Fornai, Schlüter et al. 2005), while overexpression of WT- α -synuclein has been shown to cause fragmentation of mitochondria (Kamp, Exner et al. 2010).

1.3 Translational regulation

1.3.1 Alternative splicing

In complex organisms the protein-coding sequences (exons) are surrounded by non-coding segments (introns) that are included in the pre-mRNA but not in the resulting mRNA that is translated. These introns are removed during pre-mRNA splicing, a precise process mediated by the spliceosome; a complex of five small nuclear RNAs, RNA-binding proteins and enzymes (Cooper, Wan et al. 2009). During this process “alternative splicing” can occur where exons are differentially spliced. The advantage of alternative splicing is that many different mRNAs can be produced from a single gene, allowing humans to produce >100,000 proteins from a relatively low number of genes (25,000-30,000). These mRNAs differ either in coding regions or UTRs (as depicted in figure 3). This allows the regulation of altered proteins either in different cell types, developmental stage or upon environmental changes.

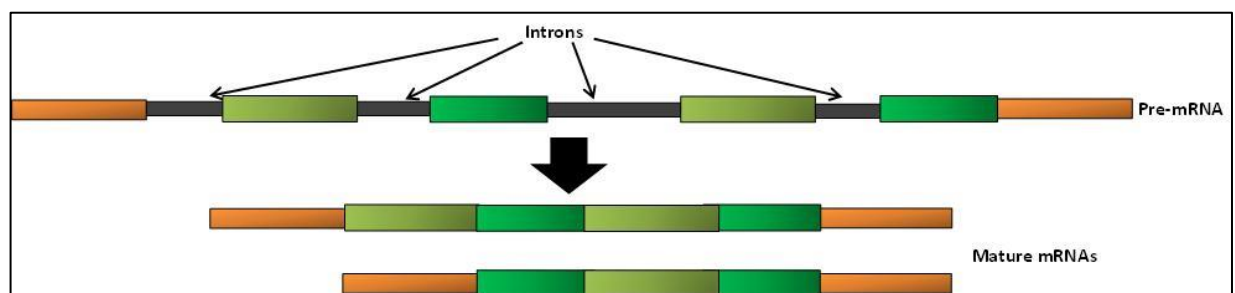


Figure 3: An example of a pre-messenger RNA being alternatively spliced during the removal of introns to produce two different mature messenger RNAs which contain different exons.

In eukaryotes the translation of mature mRNA to produce protein can be divided into three separate sections; initiation, elongation and termination. While the second two stages are determined by a host of specific eukaryotic initiation factors (eIFs) of which the expression levels can influence the levels of translation in a non-specific way (Jackson, Hellen et al. 2010) initiation is more complex. It involves the interaction of many proteins and RNA sequences and as a result can be highly regulated in a manner specific to each mRNA.

Briefly, cap-dependent initiation (which is the mechanism by which 95% of mRNAs are translated in eukaryotic cells (Merrick 2004) involves the recruitment of a complete 80S ribosome to the mRNA start codon AUG. In order for this to occur the 40S ribosomal subunit binds to the 5' end of the mRNA and scans it in a 5' to 3' direction until the start codon is identified. The large 60S subunit then associates with the 40S to form the active 80S ribosome.

These initial steps can be affected by several components within the mRNA including the 5' cap, 3' poly A tail, secondary structures, protein binding sites, upstream open reading frames (ORF) and micro RNA (miRNA) sites. These are depicted in figure 4 and regulation can be broadly separated into two groups; global non-specific, affecting all translation in a similar fashion regardless of the mRNA, or mRNA-specific.

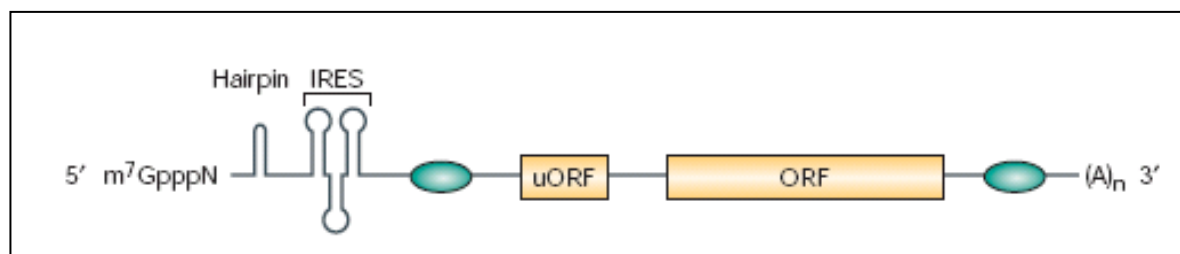


Figure 4: An overview of the regulatory elements acting on messenger RNA translation. IRES-internal ribosome entry site, uORF-upstream open reading frame. Taken from (Gebauer and Hentze 2004).

1.3.2 Global non-specific regulation of translation

Translation initiation is a complex process involving many different factors. An overview of cap-dependent initiation is shown in figure 5.

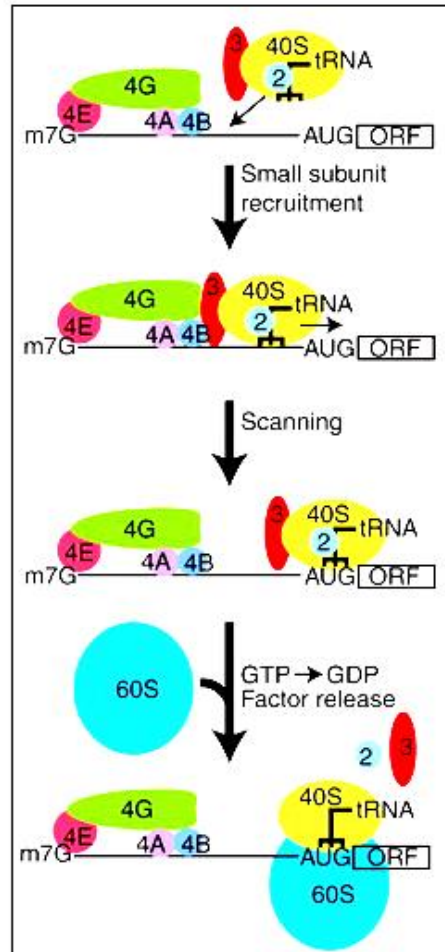


Figure 5: Overview of cap-dependent translation initiation. Eukaryotic initiation factors (eIFs) 4A, B, G and E bind to the 5' cap and facilitate the recruitment of the 43S pre-initiation complex (40S subunit with eIFs 2 and 3). The 43S pre-initiation complex scans until the AUG start codon is reached, eIFs 2 and 3 are released and the 60S subunit binds. Taken from (Pfingsten, Costantino et al. 2006).

The eIF4F complex contains several proteins, including eIF4E, which binds to the m7GpppN cap structure; eIF4A, a RNA helicase that is believed to unwind secondary structures in the 5' UTR so that the 40S subunit can bind and scan the mRNA; and eIF4G, which functions as a scaffold protein by interacting with eIF4E, eIF4A and eIF3 (Jackson, Hellen et al. 2010).

The 40S subunit, together with eIFs 3, 1, 1A and 5, a methionine initiator tRNA that will recognize the AUG start codon and eIF2 that is coupled to GTP, forms a 43S pre-initiation complex that binds to the mRNA. In mammals, binding of the 43S pre-initiation complex

to the mRNA is thought to allow interactions between eIF3 and the eIF4F protein complex, which associates with the 5' cap structure of the mRNA (Lamphear, Kirchweyer et al. 1995).

eIF4G also interacts with the poly(A)-binding protein (PABP), and the simultaneous interaction of eIF4E and PABP with eIF4G is believed to circularize the mRNA, which brings the 3' UTR in close proximity to the 5' end of the mRNA (Wells, Hillner et al. 1998). As most of the well-defined translational regulatory sequences are found within 3' UTRs this proximity highlights the functional connection between the mRNA ends during translation (Jackson, Hellen et al. 2010).

As mentioned previously the later stages of translation relies on the recruitment of eIFs which lies beyond the scope of this thesis. For a thorough overview see the review by Jackson et al. (Jackson, Hellen et al. 2010). However through decreased expression levels, altered phosphorylation states and proteolytic cleavage the availability and activity of these factors can be altered thus impacting on the overall translation levels (Jackson, Hellen et al. 2010). Specific examples include the phosphorylation of eIF2 which results in reduced dissociation of the utilised GDP for GTP (Rowlands, Panniers et al. 1988) and cleavage of certain factors during viral infection results in reduced cellular translation thus freeing the 'machinery' for viral translation (Schneider and Mohr 2003).

1.3.3 mRNA-specific regulatory elements

mRNA transcripts themselves contain many sequences and structures that can function to regulate its translation.

1.3.3.1 Secondary RNA structures

The necessity of a helicase as part of the 43S initiation complex supports the reasoning that the more complex the secondary structure of the 5'UTR the lower the levels of translation

initiation. In addition to the canonical cap-dependent translation initiation described there is an alternative that is cap-independent and mediated by internal ribosome entry sites (IRESs). These are RNA structures that help to recruit ribosomal complexes to internal sites of the 5' UTR, either directly or near, the start codon (Pestova 2001). Although IRES-dependent translation is vital in viral replication, it is not thought to occur regularly in mammalian cells but there is some evidence that facilitate translation of stress response genes such as heat shock proteins. For a an overview see the review by Spriggs and Bushell et al. (Spriggs, Bushell et al. 2010).

1.3.3.2 RNA Binding Proteins

The recruitment of the 43S complex can also be regulated by the presence of RNA binding proteins. These can affect recruitment in two different ways; either by steric blocking or preventing eIF4F binding to the cap. The best characterized example of an RNA-binding protein in the 5'UTR of an mRNA is within ferritin mRNA. Ferritin is an iron storage protein and in iron-depleted cells iron regulatory proteins (IRP) 1 and 2 bind to a stem-loop motif in the 5'UTR, located within 40 nucleotides of the 5' cap, termed the iron-responsive element (IRE) (Gebauer and Hentze 2004). The IRE blocks the association of the 43S complex with the eIF4F complex, therefore preventing translation initiation (Gray and Hentze 1994; Muckenthaler, Gray et al. 1998). An overview of this is shown in figure 6.

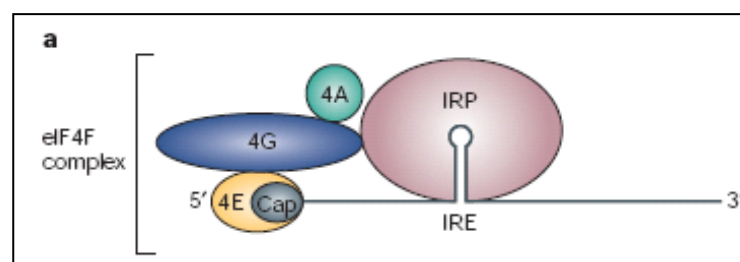


Figure 6: Steric blocking of 43S ribosome subunit recruitment in ferritin messenger RNA by iron regulatory protein (IRP) binding to iron-responsive elements (IRE) in iron-depleted cells. Taken from (Gebauer and Hentze 2004).

Although IRPs allow the eIF4F complex to bind the 5'cap (Muckenthaler, Gray et al. 1998) there are some regulators that target the formation of this complex.

During oocyte maturation and early development in vertebrates cytoplasmic-polyadenylation-element-binding protein (CPEB) regulates the expression of maternal mRNA by binding to a uridine-rich sequence called the cytoplasmic polyadenylation element (CPE) which is located in the 3'UTR of target mRNAs (Mendez and Richter 2001). CPEB binds the protein Maskin which contains a eIF4E binding-domain, similar to the one eIF4G, suggesting that Maskin competes with eIF4G and inhibits translation initiation (Stebbins-Boaz, Cao et al. 1999).

1.3.3.3 Upstream ORFs

Upstream ORFs are generally thought to reduce levels of translation as they reduce the number of 40S subunits available to bind the 'real' ORF (Gebauer and Hentze 2004). This may seem counter intuitive as if the ribosome complex is already bound why wouldn't it read through to the next ORF? However, it is thought that during translation termination at the stop codon of the upstream ORF the 60S subunit dissociates while the 40S keeps scanning (Gebauer and Hentze 2004). But, the 40S requires the factors to form an active 80S complex, and the likeliness of this occurring increases the further it moves from the upstream ORF (Gebauer and Hentze 2004). Therefore the further away the upstream ORF from the start codon the less inhibitory it is.

1.3.3.4 miRNAs

MicroRNAs are a class of small non-coding RNAs approximately 22 nucleotides in length. They display only partial complementarity to their target sequence, most commonly located in the 3'UTRs of mRNA. Several thousand miRNAs have been identified in plants and animals which have been shown to regulate the translation of genes involved in many

processes including differentiation, cell growth and apoptosis. miRNAs are transcribed as large primary miRNA molecules usually containing several miRNA sequences, and processed in the nucleus by the RNase Drosha (Lee 2003) to form hairpin structures termed pre-miRNAs. These pre-miRNAs are transported into the cytoplasm by Exportin-5 and further processed by another enzyme, Dicer. The resulting mature miRNA is loaded into the miRNA Induced Silencing Complex (miRISC) and guides RISC to a small target region in the 3' UTR of target mRNA. The miRNA binds to the mRNA, via a 5' 'seed region' (6-8nts) (Gottwein and Cullen 2008) leading to translation inhibition. Studies indicate that miRISC binding inhibits the recruitment of the 60S ribosome and prevents translation initiation, inhibiting ribosome elongation and/or facilitates the deadenylation and degradation of nascent peptides (Fabian, Sonenberg et al. 2010).

1.4 Alpha Synuclein

The human gene encoding alpha synuclein (α -synuclein) (known as SNCA or PARK1/4) is located on chromosome 4q21.3-q22 and was the first gene to be identified in familial PD (Polymeropoulos, Lavedan et al. 1997). The α -synuclein transcript (SNCA) comprises of 7 exons, 5 of which are protein coding and highly conserved between vertebrates (Venda, Cragg et al. 2010). The α -synuclein protein is made up of three regions: the highly conserved N-terminal domain containing 6 imperfect 11aa repeats with the consensus sequence KTKEGV (similar to the lipid-binding domain of apolipoproteins) which can form amphipathic helices; a central hydrophobic region that contains the non-amyloid β component (NAC) which is associated with fibril formation; and an acidic C-terminal tail containing negatively charged residues and is mostly unfolded (Venda, Cragg et al. 2010), (Moore, West et al. 2005). The three known α -synuclein missense mutations known to cause familial PD are located in the N-terminal region.

α -synuclein, until recently was thought to exist in healthy cells as monomers that are intrinsically unstructured and unfolded but, depending on the environment can interact to form protofibrils that then aggregate further to produce fibrils, such as those found in LBs (Venda, Cragg et al. 2010). A generalised overview of α -synuclein monomer aggregation is shown in figure 7.

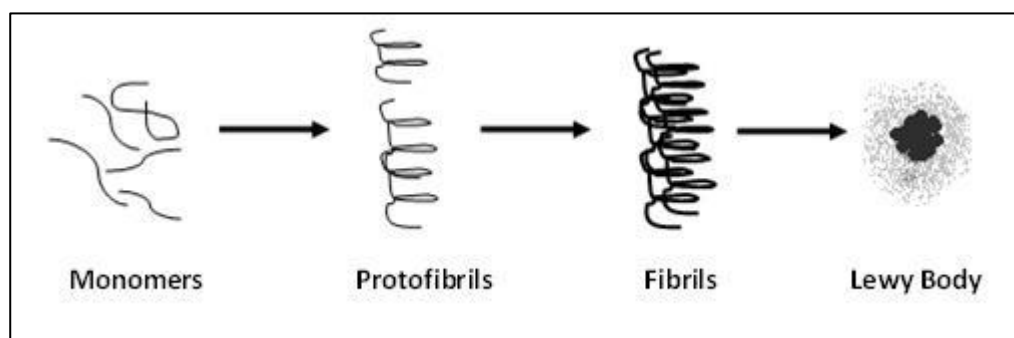


Figure 7: The assembly of α -synuclein monomers into oligomers, fibrils and Lewy bodies. (Adapted from (Moore, West et al. 2005))

It is generally accepted that protofibrils of α -synuclein are cytotoxic whereas fibrillar aggregates are cytoprotective (Caughey and Lansbury 2003; Dev, Hofele et al. 2003; Sharon, Bar-Joseph et al. 2003). Whether there is a catalyst or inhibitor that allows or prevents fibril formation is unknown.

However, a new study by Bartels et al., describes α -synuclein as a folded tetramer in healthy human cells, made up of four monomeric chains with α -helical structures. Whereas naively unfolded monomers appear to easily aggregate *in vitro* these tetramers appear surprisingly stable and unlikely to form amyloid-like fibrils (Bartels, Choi et al. 2011). This suggests that the tetramers would first have to dissociate and then aggregate into the toxic protofibrils that form LBs (Bartels, Choi et al. 2011).

α -synuclein is known to undergo alternative splicing. Within the literature three well defined isoforms of the α -synuclein transcripts and protein are described; 140, 126 and 112. α -synuclein 140 is the full length transcript while -126 and -112 are missing exons 3 and 5 respectively (Beyer 2006). Most recently a fourth alternatively spliced transcript was identified: α -synuclein 98 which is missing both exons 3 and 5 (Beyer, Domingo-Sábat et al. 2008) but to our knowledge has not been confirmed by other research groups and is not annotated in any expressed sequence tag (EST)-databases. These alternatively spliced isoforms are shown in figure 8.

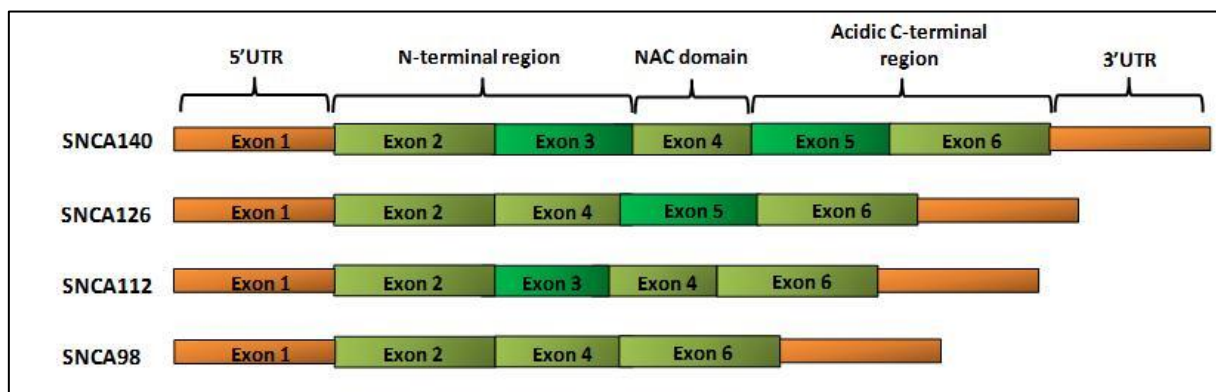


Figure 8: The alternatively spliced isoforms of α -synuclein; full length 140, 126 minus exon 3, 112 minus exon 5 and 98 minus exons 3 and 5. The three main region and untranslated regions (UTRs) are also annotated. NAC; non-amyloid β component.

Several of the α -synuclein alternatively spliced isoforms appear to have different aggregation properties to the full length 140aa protein. α -synuclein 126 is missing exon 3 which interrupts the membrane-protein interaction domain. As membrane interaction is thought to be involved in the steps leading up to aggregation (Lee, Choi et al. 2002) it potentially leads to a diminished capacity for aggregation and indicates a possible neuro-protective role (Beyer 2006). In contrast α -synuclein 112 is missing exon 5 causing a shortening of the C-terminus and aggregation is expected to be increased (Beyer, Lao et al. 2004; Beyer 2006; Beyer, Domingo-Sábat et al. 2008). As such it has been implicated in LB formation and neurotoxicity (Beyer, Domingo-Sábat et al. 2008).

The splicing factors regulating alternative splicing in α -synuclein mainly remain unknown. However, mechanistically, oxidative stress may play a role. The stressing of cells with oxidative compounds such as MPP^+ and rotenone resulted in the production of the alternatively spliced 112 α -synuclein transcript (Kalivendi, Yedlapudi et al. 2010). The authors attribute the switch in splicing to extracellular signal-regulated kinase (ERK- also known as mitogen-activated protein kinase-MAPK) which has been shown in other studies to affect the skipping of exons in other genes (Weg-Remers, Ponta et al. 2001). Therefore

a decrease in phosphorylation of Erk-MAP kinase could result in increased production of 112 α -synuclein (Kalivendi, Yedlapudi et al. 2010).

As mentioned there are several point mutations and different splice isoforms of α -synuclein that have been shown to or could potentially lead to the development of PD. However, WT α -synuclein protein has also been shown to be pathogenic when over-expressed. Duplications and triplications of the α -synuclein loci has been shown to cause familial PD disease, which increases in severity as the level of α -synuclein increases (Singleton, Farrer et al. 2003; Moore, West et al. 2005).

1.4.1 Functions of α -synuclein

As knockout models of α -synuclein show relatively few abnormalities the specific functions of α -synuclein remain unclear. This could indicate a level of redundancy and other members of the synuclein family could be compensating. However, triple knockout models of the entire synuclein family (α , β and γ) still have few defects, displaying only small disturbances in synaptic function and no sign of neurodegeneration (Buchman and Ninkina 2008).

Therefore most data collected on the roles of α -synuclein has been collected during *in vitro* studies using knockdown or over-expression techniques and any results should be interpreted with that limitation in mind. α -synuclein has been reported to be deeply involved in the regulation of dopamine homeostasis at a number of key stages including the regulation of tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC), two enzymes that are crucial in dopamine synthesis, as well as affecting vesicle cycling and dopamine uptake.

1.4.1.1 Dopamine synthesis

In order to produce dopamine tyrosine is first converted to L-DOPA by TH. L-DOPA is then used to produce dopamine, catalysed by the enzyme ADCC. The short term activity of TH is regulated by phosphorylation of serine residues, especially serine 40 (Ramsey, Hillas et al. 1996). α -synuclein modulates TH activity by binding it and preventing its phosphorylation (Perez, Waymire et al. 2002) as well as increasing the activity of protein phosphatase 2A (PP2A) (Peng, Tehranian et al. 2005). Luciferase data collected in α -synuclein overexpressing dopaminergic cell line indicates that α -synuclein may act as a trans-acting factor on the TH promoter, decreasing its activity (Gao, Li et al. 2007). Additional studies carried out using western blot and northern blot analysis determined that both TH protein and mRNA levels were reduced (Yu, Zuo et al. 2004) as a result.

There is also evidence that α -synuclein interacts with AADC which converts L-DOPA to dopamine. Over expression of both wild type and A53T-mutant α -synuclein in MN9D cells resulted in decreased dopamine production compared to controls (Tehranian, Montoya et al. 2006). Like TH, there was a decrease in the activity of AADC, resulting from a lower phosphorylation rate (Tehranian, Montoya et al. 2006). As with TH phosphorylation of AADC is paramount for its activation and it is interesting to speculate that this also results from the increased activity of PP2A in high levels of α -synuclein (Tehranian, Montoya et al. 2006).

Baptista and colleagues took these investigations further by carrying out transcriptional analysis by microarray and discovered that several genes involved in dopamine synthesis were down regulated in neuroblastoma cells line (BE(2)M17s) that were stably transfected with WT but not with mutated (A30P, A53T) α -synuclein. In addition to the previously

reported TH and ADCC there was also down regulation of GTP cyclohydrolase 1 and sepiapterin reductase (SR) , in cells over-expressing WT α -synuclein but not the mutated forms (Baptista, O'Farrell et al. 2003). The authors speculated that α -synuclein could be affecting the expression of Nuclear receptor related 1 protein (Nurr1) as its levels were also decreased upon WT α -synuclein over-expression. Nurr1 could be the 'master regulator' of all the mentioned dopamine synthesis genes as there are consensus binding sequences in upstream regions of these genes. This poses the question, how could α -synuclein affect the activity of Nurr1?

Over-expression of α -synuclein has been shown to impact on the phosphorylation of MAPK and possibly complex with ERK (also known as MAPK) or E twenty-six (ETS)-like transcription factor 1 (ELK-1). This affects the amount of active MAPK available thus affecting the phosphorylation of downstream MAPK targets resulting in increased dopaminergic cell death (Iwata, Maruyama et al. 2001; Iwata, Miura et al. 2001). Nurr1 is known to be regulated by MAPK pathways (Kovalovsky, Refojo et al. 2002; Baptista, O'Farrell et al. 2003). Studies indicate that α -synuclein may act directly on Erk or indirectly by increasing the expression of caveolin-1, a protein which forms the scaffolding onto which signalling molecules such as Erk assemble (Hashimoto, Takenouchi et al. 2003). Dysregulation of caveolin-1 could lead to changes in ERK expression. A summary of the modulation of dopamine synthesis by α -synuclein and the possible regulation of Nurr1 is shown in figure 9.

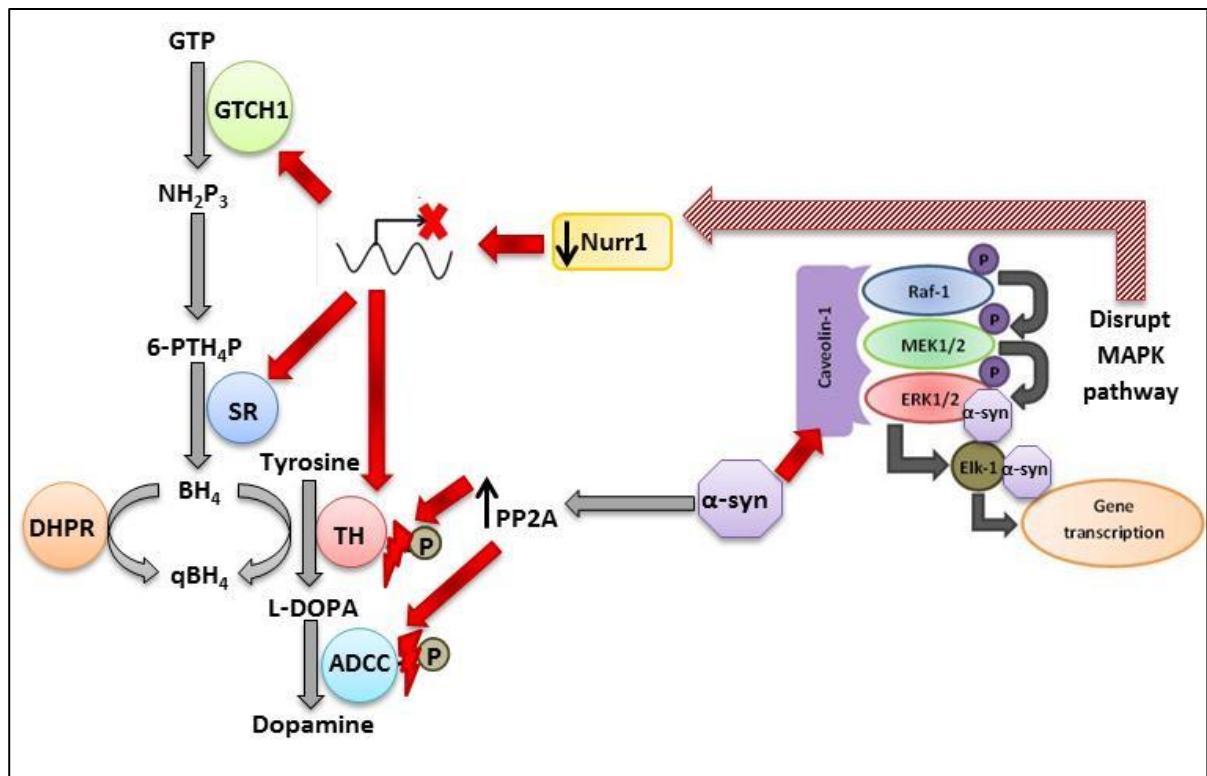


Figure 9: α -synuclein regulation of dopamine synthesis and its hypothesised interactions with the mitogen-activated protein kinase (MAPK) pathway and nuclear receptor related 1 protein (Nurr1).
 GTCH1; GTP cyclohydrolase, SR; Seplapterin reductase, TH; Tyrosine hydroxylase, ADCC; Aromatic amino acid decarboxylase, DHPR; Dihydropteridine reductase, BH₄; Tetrahydrobiopterin, PP2A; protein phosphatase 2A; 6-PTH₄P; pyruvoyltetrahydropterin

As dopamine is thought to affect the toxicity of α -synuclein (Xu, Kao et al. 2002) possibly by direct modification (Conway, Rochet et al. 2001) it maybe that by decreasing the levels of dopamine the cell is trying to reduce cytotoxicity.

1.4.1.2 Synaptic vesicle function

The majority of evidence linking synaptic vesicle function and α -synuclein comes from investigations carried out on non-dopaminergic hippocampal neurons (Venda, Cragg et al. 2010). However, as α -synuclein is widely expressed throughout different neuronal types it does give us insights into how it affects vesicle trafficking. Over-expression of α -synuclein has been shown to inhibit neurotransmitter release by specifically decreasing the size of the synaptic vesicle recycling pool (Nemani, Lu et al. 2010). Investigations carried out in dopaminergic neurons of α -synuclein knockout mice by Yavich and colleagues indicate

that α -synuclein inhibits the refilling of the 'readily releasable' vesicle pool (Yavich, Tanila et al. 2004; Venda, Cragg et al. 2010). Therefore an absence of α -synuclein promotes movement of vesicles from the reserve pool to the readily releasable pool (Yavich, Tanila et al. 2004).

It appears that α -synuclein inhibits the release of dopamine by interacting during late stage exocytosis, somewhere between the docking and fusion of the vesicles, as there was an increase in the number of docked vesicles in α -synuclein over-expression (Larsen, Schmitz et al. 2006).

1.4.1.3 α -synuclein as a chaperone

Interestingly α -synuclein over-expression rescued the phenotype observed in cysteine string protein alpha (CSP α) knockout mice (Chandra, Gallardo et al. 2005). CSP α is a presynaptic chaperone vesicle protein and knockout mice express a variety of abnormalities including an impaired SNARE complex (Chandra, Gallardo et al. 2005). Both are phospholipid binding proteins. While it is not thought that α -synuclein substitutes CSP α , WT expression but not that of the A30P mutant does rescue a proportion of the SNARE impairment (Chandra, Gallardo et al. 2005). As the SNARE complex is required for vesicle docking and fusion it is no surprise that α -synuclein is able to influence vesicle availability.

The chaperone-activity of α -synuclein was lost when the c-terminal acid domain was removed (Souza, Giasson et al. 2000). The N-terminal has been observed to bind to proteins forming high molecular weight complexes whereas the C-terminal domain is reported to be involved in solubilising these complexes (Park, Jung et al. 2002). N-terminal region has homology with chaperone protein 14-3-3 (Ostrerova, Petrucelli et al.

1999) and has the potential to interact with the same proteins which includes protein kinase C (PKC), Erk and TH (Ostrerova, Petrucelli et al. 1999; Perez, Waymire et al. 2002).

1.4.1.4 *DAT-mediated uptake*

Several studies carried out *in vitro* implicate α -synuclein in the regulation of the dopamine transporter (DAT). RNAi silencing of α -synuclein was found to reduce the number of DAT molecules on the surface, as well as decrease the total number of vesicles, but those vesicles did express more VMAT2 (Fountaine, Venda et al. 2008). Additionally in cells with diminished α -synuclein levels a decrease in the activation of nitric oxide synthase (NOS) was observed after treatment with MPP⁺ (Fountaine, Venda et al. 2008). NOS activation is a key step in the development of MPP⁺-dependent neurodegeneration.

Point-mutated α -synuclein, that does not affect gene expression of molecules involved in dopamine synthesis, has been implicated in other aspects of dopamine homeostasis, specifically by decreasing the expression of VMAT2 (Lotharius, Barg et al. 2002). This would result in decreased dopamine uptake into vesicles and an increased presence in the cytosol leading to increased toxicity (Baptista, O'Farrell et al. 2003).

1.4.1.5 *The role of alternatively spliced α -synuclein*

As point mutations in α -synuclein have been indicated to alter its molecular functions (Chandra, Gallardo et al. 2005; Yavich, Oksman et al. 2005; Tehranian, Montoya et al. 2006) it poses the question, what effect would deletions of entire exons have? There has been little investigation into these alternatively spliced isoforms other than the expression levels in certain cellular models and diseased brains. This is probably due to the fact that there are few molecular tools to differentiate between the isoforms. For example, there are no specific antibodies commercially available and thus there is little indication whether the

isoforms are localised to the same areas of neurons or whether they interact with other cellular components. As mentioned previously there is one study carried out in a model of oxidative stress, where they detect the 112 isoform of α -synuclein (Kalivendi, Yedlapudi et al. 2010). The authors report that the 112 isoform has an altered localisation, based on the observation that it was concentrated in the 'particulate' fraction and not in the cytosolic fraction like full length α -synuclein (Kalivendi, Yedlapudi et al. 2010). It was also reported that α -synuclein 112 inhibits proteasome function, specifically the trypsin-like proteasome activity of 20S (Kalivendi, Yedlapudi et al. 2010). This inhibition would reduce the cellular capacity for degrading potentially harmful proteins such as α -synuclein and could account for the increased cell death seen in cells over-expressing α -synuclein 112 (Kalivendi, Yedlapudi et al. 2010). It also appears that the over expression of α -synuclein 98 decreases the ability of dopaminergic neurons to cope with oxidative stress (Ma, Yuan et al. 2011).

Inappropriate splicing is now known to be instrumental in the development of several diseases (Cooper, Wan et al. 2009) and could prove equally important in the development of PD.

1.4.2 Potential regulation of α -synuclein expression

There are potentially several layers to the regulation of α -synuclein expression. Firstly, on a transcriptional level the amount of gene product could be regulated by a polymorphic dinucleotide repeat site, REP1, located 10 kb upstream of the α -synuclein transcription start site. Several different alleles have been identified (Xia, de Silva et al. 1996; Touchman, Dehejia et al. 2001). The second is the alternative splicing mentioned previously. The regulation of this alternative splicing is currently unknown but it has been

speculated that PD-associated polymorphisms could play a role (Venda, Cragg et al. 2010) as well as possible links to the MAPK pathway (Weg-Remers, Ponta et al. 2001).

Finally the 3'UTR of α -synuclein transcripts are known to contain miRNA binding sites for two miRNAs, miR7 and miR153, which have been shown to down-regulate α -synuclein expression in neurons (Junn, 2009 and Doxakis, 2010). Within the European population this region of the 3'UTR is known to contain many single nucleotide polymorphisms (SNPs) (Simon-sanchez, 2009) and these may modify disease susceptibility.

1.4.3 Differences in the 5'UTR of annotated α -synuclein transcripts

In addition to the alternatively spliced α -synuclein isoforms there are several different 5'UTR isoforms, which vary either in length or sequence, annotated within the human genome. Figure 10 shows the different 5'UTR isoforms annotated in either Ensembl (black) or the Human Genome Browser (HGB) (blue).

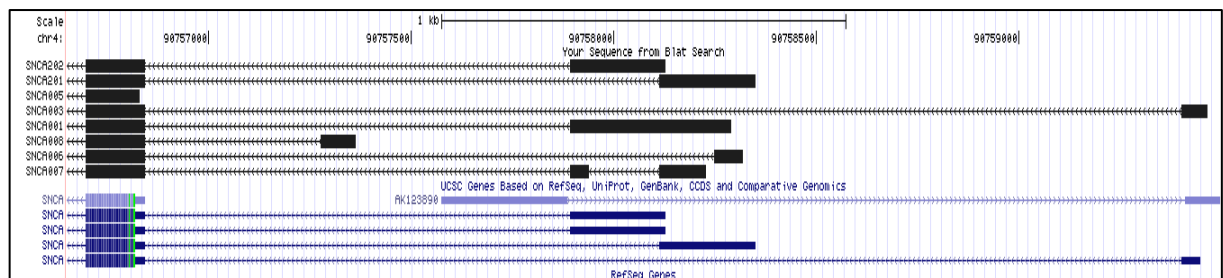


Figure 10: Snapshot of the α -synuclein transcripts (SNCA) annotated in the human genome browser (blue) and Ensembl (black), focused on the 5' untranslated regions (UTRs).

To the best of our knowledge no investigations have been made into the effect of these different 5'UTRs on translation of α -synuclein. Friedlich et al. did identify a potential IRE sequence within the 5'UTR sequence of an α -synuclein isoform, and postulated that it could be involved in translational regulation of the transcript (Friedlich, Tanzi et al. 2007). The mechanism of IRE regulation was covered in section 6.3.3. However this was based on an *in silico* prediction and more research is needed to confirm this. There is evidence

that iron is involved in PD, post mortem patient tissues analysed showed increased iron and ferritin levels in the SNpc (Lee and Andersen 2010) and iron levels have been shown to regulate α -synuclein mRNA association with polysomes and therefore its translation (Febbraro, Giorgi et al. 2012). To our knowledge no other regulatory elements have been identified within the 5'UTR of α -synuclein.

1.4.4 α -synuclein 3'UTR

The 3'UTR of α -synuclein transcript has been more widely studied than its 5' counterpart. Several papers confirm the presence of two miRNA binding sites; miR7 and miR153 within this region that are both highly expressed in neurons and have been shown to down-regulate α -synuclein expression (Junn, Lee et al. 2009; Doxakis 2010). There appears to be a range of 3'UTR lengths annotated within the human genome (shown in figure 11A), the longest of which contains several polyadenylation (PolyA) termination sites, as shown in figure 11B.

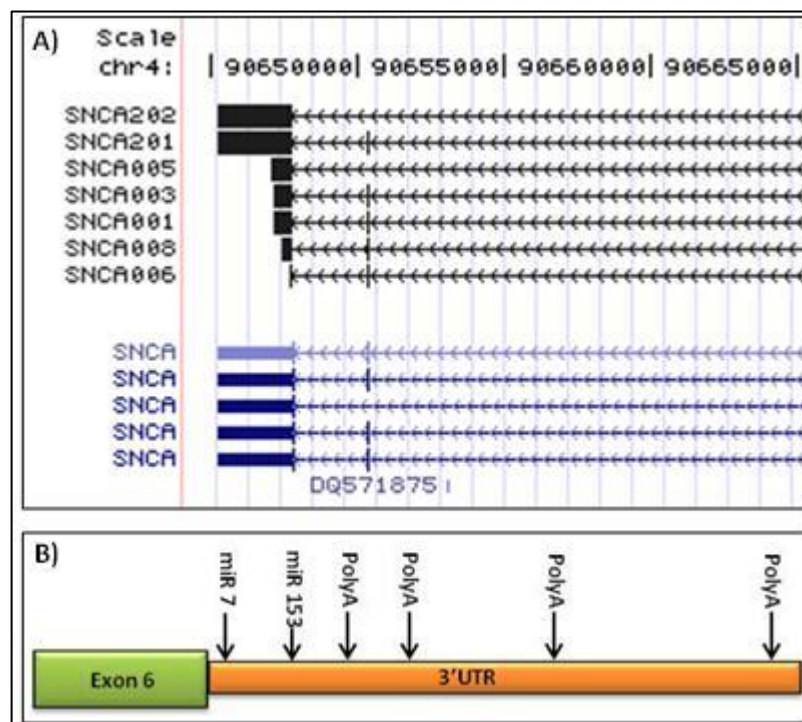


Figure 11: A) Snapshot of α -synuclein transcript annotation in the human genome browser (in blue) aligned with the α -synuclein transcript annotation of Ensembl (black), focused on the 3' untranslated regions (UTRs). B) Schematic of the longest α -synuclein 3'UTR annotated, indicating the location of the PolyA sites and microRNA binding sites for miR7 and miR153.

Interestingly the known miRNA binding sites, for miR7 and miR153, fall before the first PolyA site, so the regulatory elements within a longer 3'UTR are undetermined.

1.5 Modelling PD in a dish

As human neurons are not readily available to study and most animal models fail to replicate the pathology seen in PD patients there are several ways to produce PD models in a dish. As the field of stem cell biology is rapidly expanding it provides arguably the best tools for developing PD models *in vitro* through the reprogramming of somatic cells to iPSCs which can be differentiated into mature functional cells.

1.5.1 Induction of pluripotency to form iPSCs

Within the last decade researchers have been developing methods to study dopaminergic neurons in the lab so they can screen novel compounds and search for the pathways involved in their decline during PD. One area that has been instrumental in achieving this is the use of stem cells, specifically iPSCs. It is now possible to collect skin biopsies from patients and reprogram these into iPSCs, see figure 12.

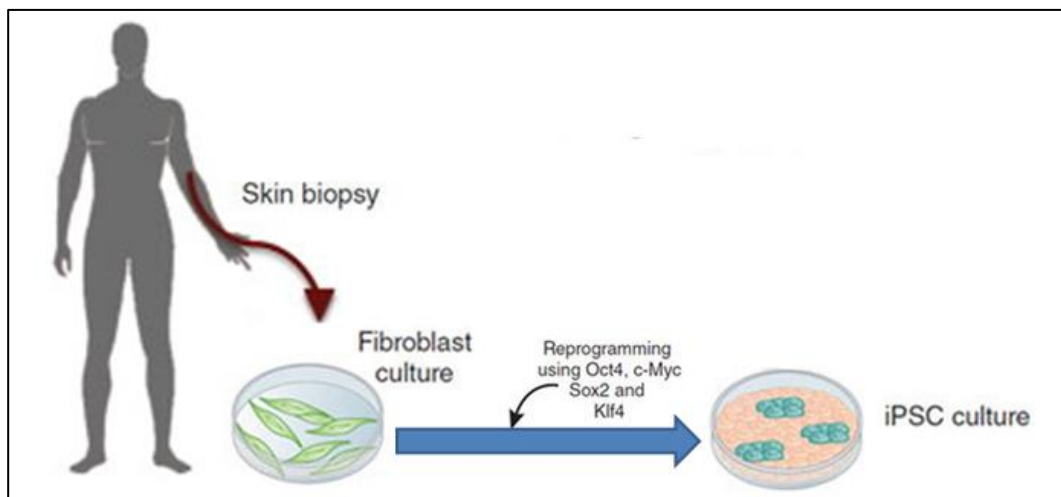


Figure 12: Schematic depicting the reprogramming of fibroblasts from a skin biopsy to induced pluripotent stem cells (iPSCs) using reprogramming factors Oct4, cMyc, Sox2 and Klf-4.

Yamanaka and colleagues first achieved induction of pluripotency via the forced expression of four transcription factors; cMYC, OCT4, KLF4 and SOX2 which are now known as the ‘Yamanaka factors’ (Takahashi and Yamanaka 2006). While this was a major breakthrough within the stem cell field, there were obstacles to the usefulness of this technology. The factors were carried within retroviral vectors which integrate at random

into the genome resulting in unpredictable genetic change. To this end since the initial publication in 2006 the focus has been on the induction of pluripotency using alternative vectors such as lentiviruses and adenoviruses (Stadtfield, Nagaya et al. 2008; Chang, Lai et al. 2009).

Most recently there has been an increase in the use of Sendai virus, an RNA virus that is non-integrative (Fusaki, Ban et al. 2009; Nishimura, Sano et al. 2011). However, it is unlikely that any protocol which involves the use of viruses will be useful as a clinical therapy. A promising novel method involving no viral vectors was published by Warren et al. in 2010 and is achieved via the transfection of modified mRNAs encoding the Yamanaka factors (Warren, Manos et al. 2010).

Warren et al., synthesised mRNA that was modified by incorporating pseudo (Ψ)-Uridine Triphosphate (Ψ TP) and methyl cytidine Triphosphate (mCTP) which bypasses the cells innate antiviral immune responses (Warren, Manos et al. 2010) They manufactured modified mRNAs encoding the Yamanaka factors supplemented with mRNA for Lin-28, which was shown to increase efficiency (Yu, Vodyanik et al. 2007; Hanna, Saha et al. 2009), and transfected them into fibroblasts daily, maintained in 5% O₂ conditions. Within two weeks they observed hESC-like colonies with the reported efficiency of 4.4% which is far superior to other reprogramming protocols (Warren, Manos et al. 2010).

1.5.2 Differentiation into Dopaminergic neurons

Although iPSCs do have the potential to spontaneously develop into any cells of the three germ layers; mesoderm, endoderm and ectoderm, they require specific signals to differentiate towards a specific lineage. There have been many protocols detailing which combination of growth factors are required to produce the highest yield of dopaminergic

neurons but these are often time-consuming, expensive and ultimately non-reproducible in different laboratories. These protocols also produce a mixed population of neurons and immature neuronal precursors which is not ideal for applications such as transcriptome analysis because any results are ‘diluted’ by the contaminating cells. In order to overcome this we have been trying to develop a method for differentiating iPSCs using modified mRNAs. This is based on the publication by Warren in 2010 where they show differentiation of their iPSCs into multinucleated myofibres via transfection with modified mRNA encoding the transcription factors (TF) MYO-D (Warren, Manos et al. 2010). Using this as proof of principle we determined to try and develop a protocol for differentiating iPSCs into dopaminergic neurons by transfecting modified mRNAs encoding TFs involved in dopaminergic neuronal development.

1.5.2.1 TFs

During brain development dopaminergic precursors originate from progenitors in the floorplate of the midbrain (Jacobs, Smits et al. 2007). The differentiation of these precursors into mature dopaminergic neurons is the result of a finely coordinated set of TFs and signalling molecules. Dopaminergic precursors specifically express TFs such as FoxA2, Otx2, Nurr1, PitX3 and Lmx1a (van den Munckhof, Luk et al. 2003; Andersson, Tryggvason et al. 2006; Smidt and Burbach 2007; Chung, Leung et al. 2009; Lin, Metzakopian et al. 2009). These factors then induce the expression of mature dopaminergic neuronal markers including VMAT2, TH and DAT. Several groups have tested these factors via the transduction of stem cells with viral vectors carrying the TFs (Palma, Lim et al. 2005; Martinat, Bacci et al. 2006; Friling, Andersson et al. 2009). More excitingly it was shown in 2011 that fibroblasts can be directly converted to dopaminergic neurons using early neuronal TFs in combination with dopaminergic specific TFs (Caiazzo, Dell'Anno et al. 2011; Pfisterer, Kirkeby et al. 2011). These results, while

promising, still involve the use of lentiviruses which is less than desirable for stem cell therapies.

We propose to develop the molecular tools for creating modified mRNAs for the dopaminergic TFs Achaete-scute homolog 1 (Ascl1 also known as Mash1), LIM homeobox transcription factor 1 alpha (Lmx1a), forkhead box protein A2 (FoxA2), Nurr1 and Pituitary homeobox 3 (PitX3). An overview of the potential protocol is shown in figure 13.

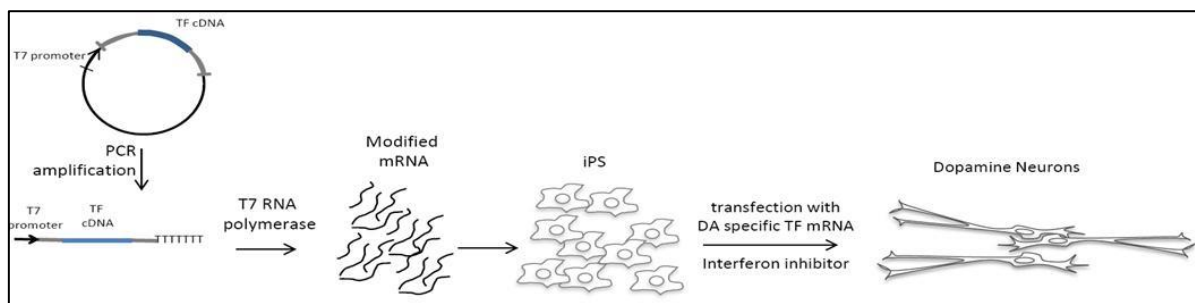


Figure 13: Production of modified messenger RNAs for differentiation of induced pluripotent stem cells (iPSCs) into dopaminergic neurons.

The ORFs of the transcription factors are cloned and a T7 promoter and poly A tail added for *in vitro* transcription. The advantage of this protocol is that it is possible to transfect different TFs on different days of the transfection regime. For example Mash1 has been implicated in early neuronal development (Vierbuchen, Ostermeier et al. 2010) so is used only in the initial stages while late dopaminergic TFs such as FoxA2 and PitX3 can be introduced towards the end of the protocol. This allows us to utilise the fact that some TFs such as FoxA2 and Nurr1 or PitX3 work in a synergistic manner (Martinat, Bacci et al. 2006; Lee, Bae et al. 2010).

As low O₂ conditions are reported to increase the efficiency of differentiation (Caiazzo, Dell'Anno et al. 2011) we will try and recapitulate this by creating a modified mRNA encoding mutated Hypoxia-inducible factor 1 α (Hif1 α). HIF1 α activity is regulated in an oxygen-dependent manner. In normoxic conditions Hif1 α is hydroxylated at proline

residues p402 and 564 by E3 ubiquitin ligase complex containing VHL protein (pVHL) (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001) and at asparagine residue N803 by factor inhibiting Hif1 α (FIH-1) (Dames, Martinez-Yamout et al. 2002). These hydroxylations result in ubiquitination and subsequent degradation by the 26S proteasome (Maxwell, Wiesener et al. 1999) and attenuation of transcription of Hif1 α target genes as it prevents the recruitment of the transcriptional co-activator protein (CBP) and p300 (Dames, Martinez-Yamout et al. 2002). In hypoxic conditions neither the proline or asparagine residues are hydroxylated so Hif1 α is not recognised by the proteasome and becomes stabilised (Fu, Wang et al. 2011). Because of the quick turnover of Hif1 α transfection of a non-mutated transcript would not be effective. However a transcript with mutations in the three residues vital for hydroxylation enables the expression of Hif1 α which is resistant to degradation by inhibiting its interaction with pVHL and preventing its targeting to the proteasome complex and leading to constitutive expression of hypoxic genes (Fu, Wang et al. 2011).

The aim of this thesis is to start to clone the dopaminergic-specific TFs and prepare them for *in vitro* transcription to create mRNA. In the future these TF tools will be utilised in various combinations to develop and optimise a protocol for differentiating iPSCs into dopaminergic neurons. This protocol has several advantages over the publications preceding it; it requires no generation of lentiviral constructs or stable cell lines, there is no possibility for integration artefacts, no embryoid body or neurosphere formation and hopefully producing a purer population of neurons.

1.6 Aims of the thesis

There are three main aims of this thesis. The first is to investigate the untranslated regions of α -synuclein, explore their role in protein expression and determine their expression levels in oxidatively stressed cells. The second aim is to determine the expression levels of the alternatively spliced α -synuclein transcripts and clone them in order to test novel, isoform specific antibodies. Finally, in order to create a new protocol for differentiating iPSCs into dopaminergic neurons using modified mRNAs, the open reading frames of various dopaminergic-specific transcription factors will be cloned and ligated to the UTRs required to carry out *in vitro* transcription. We will also start preliminary studies to determine the appropriate substrate and medium for the iPSCs during the mRNA transfections.

2 Materials and Methods

2.1 General Protocols

Ribonucleic acid (RNA) extraction: All RNA was extracted using an RNAeasy kit (Qiagen) according to the manufacturer's instructions. RNA was eluted from the column using 50µl H₂O. RNA concentration was determined using a Nanodrop and DNase-treated using TURBO DNA-free™ (Ambion) according to the manufacturer's protocol.

Complementary Deoxyribonucleic acid (cDNA) synthesis: All cDNA was created from RNA (1ug) using 1µL 500µg/mL oligo(dT), 1µL Deoxynucleotide Triphosphates (dNTPs) (10µM) and 200 units of SuperScript II (Invitrogen) in a 20µL reaction. Negative reverse transcription (RT) reactions, containing all the components above except SuperScript II, were included at least once per control group to test for DNA contamination.

Polymerase Chain Reaction (PCR): Unless otherwise stated, all PCRs were performed in 25µl reactions using 1µl of RT reaction as template. Primers were used at a final concentration of 0.2µM, dNTPs at a final concentration of 0.2mM and Taq polymerase (New England Biolabs-NEB) at 0.1 units/µl. The PCR program was 95°C for 5 minutes, 35 cycles of 94°C for 20 seconds; 55°C 20 seconds and 72°C for 1 minute followed by 72°C for 3 minutes. PCR products were visualised on a 1% agarose gel. If cloning was required bands were excised, extracted and the DNA purified using the Wizard® SV Gel and PCR Clean-Up extraction kit (Promega) following the manufacturer's protocol.

Phusion PCR: The cloning of the 5'UTRs for Luciferase assays used Phusion DNA polymerase (NEB) to amplify the target region (lower error rate). cDNA was added to a 20µL reaction mixture containing final concentrations of 1x Phusion GC buffer, 200µM dNTPs, 0.5µM primers (each) and 0.2µL Phusion DNA polymerase (2000 units/mL).

pGEM-T-easy Cloning: Individual α -synuclein 5'UTR isoforms and other inserts of interest were cloned pGEM-T-easy vector (Promega) using A/T overhang. The pGEM-T-Easy plasmid map is shown in appendix 1.

Ligations were carried out at a ratio of 3:1 using 1 μ L T4 DNA ligase (Promega) in a total reaction volume of 10 μ L. Following transformation cultures were spread onto Lysogeny broth (LB) plates with 50 μ L 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-Gal) (20mg/mL) and 100 μ L Isopropyl β -D-1-thiogalactopyranoside (IPTG, 100mM), to allow blue/white selection. The next day a number of white positive colonies from each plate were picked and grown up for mini prep.

Transformations and DNA amplification: For bacterial transformation, ~5ng of relevant plasmid were transformed into DH5 α subcloning efficiency *Escherichia coli* (Invitrogen), via heat shock at 42°C for 30 seconds. Transformed cultures were grown up on antibiotic ampicillin (AMP)-containing LB agar culture plates overnight at 37°C.

Mini preps: Positive colonies were identified and used to inoculate 5mL LB and were grown up overnight at 37°C, 250 RPM. Plasmids were isolated and purified from bacteria using commercially available mini-prep DNA extraction kits (Qiagen) according to the manufacturer's instructions. All constructs were subsequently verified by restriction digestions and/or sequencing using a T7F primer or other suitable primer (LifeScience, Oxford UK).

Colony PCR: Colony PCRs were used to confirm positive colonies and insert orientation before restriction digests and mini prep sequencing. The tip used to pick positive colonies for inoculation were placed in 50 μ L ddH₂O and heated to 95°C for 5 minutes. 5 μ L of inoculated H₂O was used as a template for PCR amplification (reaction according to the Taq polymerase PCR method) using T7 and SP6 promoter (pGEM-T easy), or T7 and

PCR product specific (psiCHECK) primers (detailed in the relevant methods sections). Following amplification PCR products were run on a 1% agarose gel at 130V.

pGEM-T-easy T7 forward primer- TAATACGACTCACTATAGGG

SP6 reverse primer- ATTTAGGTGACACTATAG

PsiCHECK2.2 T7 forward primer- TAATACGACTCACTATAGG

Midi preps: Should the plasmids be required in a larger concentration ~2mL of the remaining culture used for mini prep was used to inoculate 100ml of Amp selective LB medium and incubated overnight at 37°C, 250 RPM, before purification using a midi prep kit (Qiagen).

Restriction digests: Purified plasmid mini preps and vectors were digested either with NheI or EcoRI enzymes. 5µl of mini prep was digested with 10 units NheI or 20 units EcoRI in 2µl Buffer 2 (NheI) or EcoRI buffer and 0.5µL BSA (NheI only) and ddH₂O added to a total volume of 20µl. The reactions were incubated for 30 minutes at 37°C and a 10µl sample was then run on a 1% agarose gel.

Site Directed Mutagenesis: To correct any mutations in the sequences cloned site directed mutagenesis was used. Complementary forward and reverse primers were designed over the mutation, generally 10bp either side, with a melting temperature of 78°C. The input plasmid was diluted to 5ng/µl and used at concentrations of 5, 10, 25 and 50ng in 50µl reactions containing final concentrations of 1x Pfu buffer, 200µM dNTPs, 2.5ng/µl primers (each) and 1µL Pfu DNA polymerase (Promega) (3 units/µL). The PCR program was 95°C for 30 seconds, with cycles of 95°C for 30 seconds, 55°C for 60 seconds and 68°C for 2 minutes/kb of plasmid length. The number of cycles varied depending on the mutation; 12 cycles for point mutations, 16 for single amino acid changes and 18 for multiple amino acid deletions or insertions. The reactions were then cooled to 37°C when 1µl of DpnI (NEB) was added and incubated for 1 hour, to remove

the original plasmid. Finally 1µl of the reactions were transformed into DH5α (as previously described). Colonies were then picked from the plate with the lowest template input.

2.2 Oxidative stress of BE(2)M17 cells using MPP⁺

In order to stress the BE(2)M17 cells they were treated with varying concentrations of MPP⁺ (toxic but unable to cross the blood-brain barrier). Cells were seeded at 5x10³ cells per well in a 96 well plate and 1.5x10⁵ per well of a 6-well plate. The following day MPP⁺ solutions of the required concentration in OptiMEM were added to the cells. Following a 24 hour incubation RNA and protein was extracted using an RNeasy kit (Qiagen) and lysis buffer (containing 50mM Tris-HCl, pH7.4, 100mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) respectively. 96-well plates were used to perform an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (Promega), to determine the inhibition of proliferation due to oxidative stress. Briefly 20µL of MTS reagent was added to wells containing 100µL of OptiMEM. The plate was incubated at 37°C for 2 hours and read using a Wallac-victor 2 plate reader at 490nm. The absorbance is proportional to the number of viable cells.

MPP⁺-treated cells were also analysed for apoptosis using Annexin V-FITC and Propidium Iodide (both Beckman Coulter). Cells were seeded at 1x10⁵ cells/well of a 24 well plate in OptiMEM medium supplemented with 10% FBS. The following day 2mM MPP⁺ solution in OptiMEM with 10% FBS was added to the cells. After 24 hours the medium was removed and the wells rinsed with OptiMEM to remove traces of MPP⁺. 100µl 1x binding buffer (Beckman Coulter) and 5µl Annexin V-FITC was added to untreated and treated wells and incubated in the dark at room temperature for 15 minutes. 5µl Propidium Iodide and 400µl 1x binding buffer was added per well and the cells imaged immediately. Three random fields were captured using fluorescent and light microscopy.

2.3 Identification and cloning of different SNCA 5'UTR TSS isoforms

Based on the different 5'UTR sequences, sets of primers were designed to amplify either individual or dual isoforms that could be separated by size. One set of primers were designed to detect all isoforms.

α -synuclein isoform	Forward Primer	Reverse Primer	Annealing Temperature (°C)	Extension Time (minutes)	Expected Product Size (bp)
All	GACTTTCAAAGG CCAAGGAG	GGCTTCAGGTTCGTAGTCTTG	55	1	317-401
001/201	GCGGAAGTGAGG TGCGTGCG	TGCTGAGTGGGGGCAGGTACA	N/A*/58.9	1	982/721
003	TGGTGTGATCCA GGAACAGCTGTC	TGCTGAGTGGGGGCAGGTACA	56.3	1	657
008	ACCATCACACTT GGCCTGCTG	GAAATGCTGAGTGGGGGCAGG TA	N/A*	1	669
201trunc.	GTGGCCATTCGA CGACAGTGTG	TGCTGAGTGGGGGCAGGTACA	55/59	1	634
202	CATTCGACGAC AGGTTAGC	CTCTCTGCTAGCCCTACATA GAGAACACCCTCTTTTGTC	55	1	391

Table 1: Primers used to detect the different 5'UTR TSS isoforms untreated BE(2)M17 cells; All, 201, 003 and 201 truncated and MPP⁺-treated BE(2)M17 cells; 202. (* unable to amplify SNCA001 and 008).

The PCR products were initially sub-cloned into pGEM-T-Easy vector (Promega) using A/T overhang. Purified plasmids were digested with EcoRI and confirmed by sequencing.

2.4 Cloning of α -synuclein 5'UTRs into a PsiCHECK2.2 reporter construct

The synuclein- α 201 and 202 pGEM-T-easy constructs were used as templates for PCR amplification, creating NheI restriction sites at both ends. Isoforms 003 and 201-truncated were amplified directly from BE(2)M17 cDNA. For isoform 201 stepwise amplification with three forward primers (reactions a, b and c, see the schematic shown in figure 14), was required to overcome the 5'UTR repetitive nature. The primers are shown in Table 2.

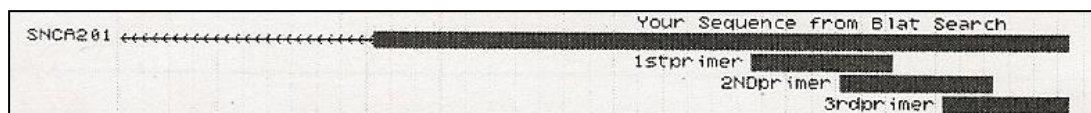


Figure 14: Stepwise amplification by PCR primers creating the entire 5'untranslated region of α -synuclein 201.

α-synuclein isoform	Forward Primer	Reverse Primer	Annealing Temperature (°C)	Extension Time (minutes)	Expected Product Size (bp)
201a	GAGAGGGGGCGAGCGACCG AGCGCCGCGACGCGGAAGT GAGGTGCGTG	CTCTCTGCTAGCC CTACATAGAGAAC ACCCTCTTTGTC	55	1	336
201b	GAGGAGGAGGACGGCGACG ACCAGAAGGGGCCCAAGAG AGGGGGCGAGCGACC	CTCTCTGCTAGCC CTACATAGAGAAC ACCCTCTTTGTC	55	1	371
201c	CTCTCTGCTAGCAGGAGAAG GAGAAGGAGGAGGACTAGG AGGAGGAGGACGGCGAC	CTCTCTGCTAGCC CTACATAGAGAAC ACCCTCTTTGTC	55	1	409
003	CTCTCTGCTAGCGGGAGGAG CTTGCTTCTCCATTCTGGTGT GATCCAGGAACA	CTCTCTGCTAGCC CTACATAGAGAAC ACCCTCTTTGTC	55	1	235
201truncated	CTCTCTGCTAGCGGAGTGGC CATTGACGACAGTGTG	CTCTCTGCTAGCC CTACATAGAGAAC ACCCTCTTTGTC	55	1	191
202	CTCTCTGCTAGCGCCATT CGACGACAGGTTAG	CTCTCTGCTAGCC CTACATAGAGAAC ACCCTCTTTGTC	55	1	405

Table 2: Primers used to clone α -synuclein 5'UTR isoforms into a PsiCheck luciferase vector

The yield from gel purification was so low that the full length 5'UTR isoforms were sub-cloned into pGEM-T-Easy (Promega). The 5'UTR α -synuclein inserts were digested from the pGEM-T-easy constructs and ligated into PsiCHECK 2.2 vector (adapted in the lab, originally Promega and shown in appendix 1) via the NheI restriction site.

During digestion with NheI PsiCHECK2.2 was dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIP) (NEB) to prevent self-ligation. 0.5 μ l CIP was added to the digestion reaction and tubes returned to 37°C for 30 minutes. The entire digestion reactions for inserts and plasmids were run on a 1% Agarose gel, the appropriate bands cut and purified using Wizard® SV Gel and PCR Clean-Up extraction kit (Promega) following the manufacturer's protocol.

Ligations were carried out at a ratio of 60:180fmol vector: insert with 2 μ l T4 DNA Ligase Reaction Buffer (10X), 1 μ l T4 DNA Ligase and H₂O to 20 μ l. Reactions were incubated at room temperature for approximately 1 hour before transformation into DH5 α .

2.5 Amplification of α -synuclein 3'UTR from undifferentiated BE(2)M17 cells

According to the α -synuclein sequences annotated in the Human Genome Browser and Ensembl α -synuclein transcripts contain 3'UTRs of differing lengths, the longest containing several polyA sites. As the sequences of the 3'UTR are identical, up until the point where they terminate, primer pairs were designed to amplify full length, intermediate and short UTRs. A schematic of the polyadenylation sites and the primers designed within the α -synuclein 3'UTR is shown in figure 15.

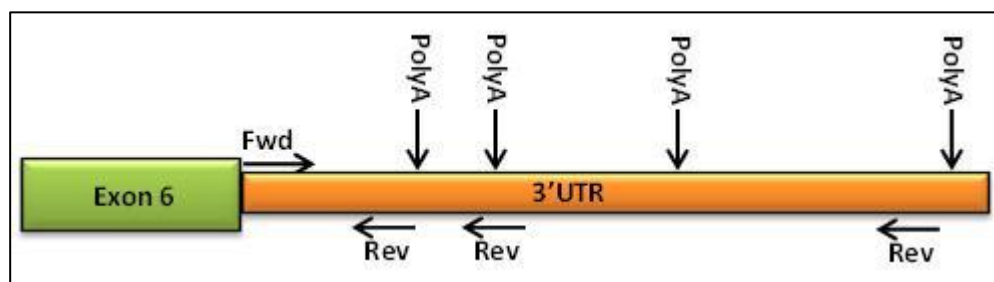


Figure 15: Polyadenylation sites within the α -synuclein 3'UTR and the primer pairs designed.

α -synuclein 3'UTR location	Forward Primer	Reverse Primer	Annealing Temperature (°C)	Extension Time (minutes)	Expected Product Size (bp)
Short-Before 1 st PolyA	GAAATATCTT TGCTCCCAGT T*	CTCACCATTAT ATACAAACAC	55	2.5	548
Intermediate-Over 2 nd PolyA	GAAATATCTT TGCTCCCAGT T*	CATGGTCGAATA TTATTTATTGTC*	55	2.5	1074
Long- Before 4 th PolyA	GAAATATCTT TGCTCCCAGT T*	TTGGTAAAGCCG ACCGTGGAGTC	55	2.5	2327

Table 3: Primers, annealing temperature, extension time and expected product size of the α -synuclein 3'UTR PCR products for undifferentiated BE(2)M17 cells, iPSC-derived neurons and differentiated BE(2)M17 cells. * Taken from (Junn, Lee et al. 2009)

Several cDNAs were tested including undifferentiated BE(2)M17s, iPSC-derived neurons (courtesy of Richard Wade-Martins laboratory) and differentiated BE(2)M17s. The primers and conditions used for the PCRs are annotated in table 3.

The cDNA was amplified using Taq DNA polymerase as described previously.

2.6 Transfection of BE(2)M17 and HEK293 cells for luciferase reporter assay

Cloning different α -synuclein 5'UTRs into the psiCHECK 2.2 Luciferase reporter created three constructs: psiCHECK2.2-201, psiCHECK2.2-201trunc. and psiCHECK2.2-003. These were transfected in triplicate along with a psiCHECK2.2 control vector (no insert) into HEK293 and BE(2)M17 cells. Cells were seeded at 1×10^5 in a 24-well plate and incubated at 37°C with 5% CO₂ overnight. At 80-90% confluency the cells were transfected with the psiCheck-5'UTR reporter constructs using 1 μ L Lipofectamine 2000 transfection reagent (Invitrogen) per 500ng of the construct.

2.7 Luciferase Assay

24 hours post-transfection the medium was removed. If also treated with MPP⁺ 500 μ L of 2mM MPP⁺ (diluted from 20mM stock in OptiMEM medium) was added per well (in triplicate) and returned to the incubator for a further 24 hours. Medium was removed and 100 μ L of lysis buffer (Promega) added to the wells. The plate was incubated for 20 minutes at room temperature while gently rocking. 20 μ L of the lysate was transferred to a 96-well Luciferase reading plate. 25 μ L of Luciferase Assay reagent II (LARII) (Promega) was added per well and an initial Firefly luciferase reading was measured on a Wallac-victor 2 plate reader. 25 μ L of Stop and glo reagent (Promega) was then added to each plate and the second Renilla luciferase reading was taken.

2.8 Western blotting

Cells were lysed using an in-house protein lysis buffer (containing 50mM Tris-HCl, pH7.4, 100mM NaCl, 1% NP-40, 0.1% SDS and 0.5% sodium deoxycholate) and protein content determined using Bradford reagent. BSA standards were prepared in 1 in 10 lysis buffer in ddH₂O to concentrations of 500, 250, 125, 61.25 and 30.625 μ g/ml. Samples were diluted 1 in 10 in ddH₂O. 5 μ L of samples and standards were added in triplicate to wells of a 96-well plate and 250 μ L of Bradford reagent (at room temperature) was added per well.

The plate was incubated at room temperature for 15 mins before reading at 595nm using a Wallac-victor 2 plate reader.

Appropriate amounts of protein lysate for each assay were diluted in 20µl and mixed with 4X NuPage loading buffer (Invitrogen). Samples were denatured at 70°C for 10mins before being loaded on 4-12% Bis-Tris gels and run at 200mV for 50 minutes. Protein was subsequently transferred to Polyvinylidene fluoride (PVDF) membrane at 30mV for 1 hour using the XCell II™ transfer system (Invitrogen). Membranes were fixed in 0.4% PFA as it was shown to improve detection of α -synuclein (Lee and Kamitani 2011). It was then blocked in 5% milk for 1 hour, washed for 3 x 5 minutes in phosphate-buffered saline (PBS) supplemented with 0.1% Tween-20 (PBS-T) (Sigma Aldrich, USA), and then incubated with desired primary antibody in 5% milk for 1 hour 1 (Table 4). Membranes were washed for 3 x 5 minutes in PBS-T followed by a 1 hour incubation of fluorescent secondary antibodies (Licor) diluted in 5% milk (Table 5.2). Following 3 x 5 minute washes in PBS-T, membranes were imaged using a Licor Western Imaging System.

Antibody	Origin	Dilution
α -synuclein (BD Bioscience)	Mouse	1:1000
B-Actin (Abcam)	Rabbit	1:3000
126- α -synuclein (Gift from Katrin Beyer's lab)	Rabbit	1:1000
112- α -synuclein (Gift from Katrin Beyer's lab)	Rabbit	1:1000 1:500
Anti-mouse 800 (Licor)	Goat	1:10000
Anti-rabbit 680 (Licor)	Goat	1:10000

Table 4: Antibodies used for Western blotting experiments

2.9 Splicing control PCR

To determine that the α -synuclein 5'UTR-PsiCHECK luciferase constructs were correctly splicing the intron (annotated in figure 17 in section 1.4.) a PCR was performed. BE(2)M17 cells were transfected as for a luciferase assay (section 1.6) and incubated overnight at 37°C. The following day medium was removed and RNA extracted using an RNAeasy kit (Qiagen). cDNA was synthesised as described in section 1.1 with RNA input of 200ng per reaction and using 1 μ l 10 μ M gene-specific primer (GSP) instead of Oligo(dT). Negative RTs were included for all constructs. Primers were designed in the SV-40 promoter and the ORF of Renilla luciferase. The sequences are shown below.

GSP for reverse transcription reaction- CCAAGCGGTGAGGTACTTGT

Forward primer- CCAGAAGTAGTGAGGAGGCTT

Reverse primer- CGTGCTTCTCGGAATCATAG

2 μ l of cDNA were added per reaction with an annealing temperature of 55°C, an extension time of 1 minute and a total of 35 cycles.

2.10 Detection of alternatively spliced α -synuclein transcripts in MPP⁺-treated BE(2)M17 cells

The RNA extracted from cells treated with MPP⁺ was used to synthesise cDNA as described previously (section 1.1). Four junction primers were designed to be used in different combinations to amplify the four reported alternatively spliced α -synuclein transcripts; 140, 126, 112 and 98. Figure 16 shows the alternatively spliced α -synuclein isoforms and location of the primers. Primer sequences are shown in Table 5.

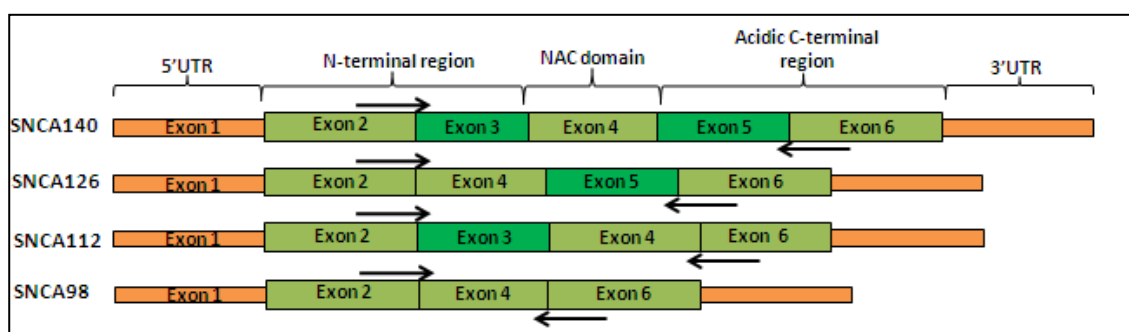


Figure 16: Schematic showing the alternatively spliced α -synuclein isoforms (140, 126, 112 and 98) and the location of the amplifying primers. Arrows indicate the location of primers and orange boxes represent untranslated regions (UTRs).

α -synuclein Isoform	Forward Primer	Reverse Primer	Expected Product Size (bp)
140	GGTGTTCCTCTATGTAGGCTC	GGCTCCTTCTTCATTCTTGC	216
126	GGTGTTCCTCTATGTAGTGGCT	GGCTCCTTCTTCATTCTTGC	174
112	GGTGTTCCTCTATGTAGGCTC	GTCTTGATACCCTTCCTTGC	216
98	GGTGTTCCTCTATGTAGTGGCT	GTCTTGATACCCTTCCTTGC	174

Table 5: The different primer combinations used to amplify the alternatively spliced α -synuclein transcripts and the expected product size.

The PCR was carried out using Taq polymerase at 55°C as previously described.

2.11 Cloning of alternatively spliced α -synuclein isoforms

A previous student in the lab had cloned the full length 140 isoform into the pEGFP-N1 plasmid (shown in appendix 1) using SacII and XhoI restriction sites within the multiple cloning site (MCS). The alternatively spliced α -synuclein isoforms 126 and 112 were amplified using a junction primer and an Exon 2 or Exon 6 common primer respectively. Therefore the entire coding sequence (CDS) with SacII/SalI cloning sites was created using stepwise amplification with forward and reverse primers, as shown in figure 17. The primers are shown in table 6.

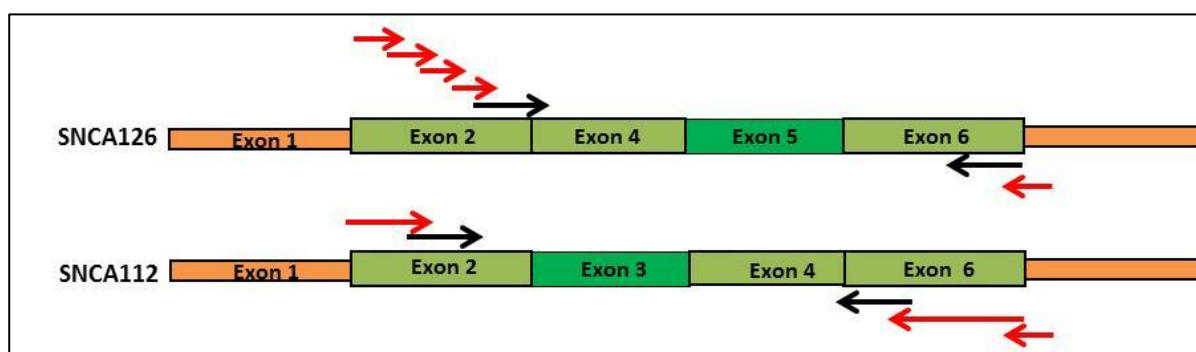


Figure 17: Schematic demonstrating the stepwise PCR amplifications used to create the entire coding sequence (starting in exon 2) of the α -synuclein isoforms 126 and 112 with cloning sites for insertion into peGFP-N1. Arrows indicate the primer binding sites and orange boxes represent the untranslated regions.

Name	Primer Sequence
SNCA126 2/4 Junction	GGTGTTCCTCTATGTAGTGGCT
2/4 Junction Adaptor 1	GTGTGGCAGAAGCAGCAGGAAAGACAAAAGAGGGTGTTCCTCTATGTAG
2/4 Junction Adaptor 2	GAGGGAGTTGTGGCTGCTGCTGAGAAAACCAAACAGGGTGTGGCAGAAGCAGCAG
2/4 Junction Adaptor 3	ATGGATGTATTCATGAAAGGACTTTCAAAGGCCAAGGAGGGAGTTGTGGCTGCT
5'Sall site	CTCT-GTCGAC-ATGGATGTATTCATGAAA
SNCA112 4/6 Junction	GTCTTGATAACCCTTCCTTGC
3' SacII site	CTCT-CCGCGG-TTAGGCTTCAGGTTCGTAGTC
4/6 Junction Adaptor 1	TTAGGCTTCAGGTTCGTAGTCTTGATAACCCTTCCTT
SNCA Exon 2	GGAATTCATTAGCCATGGATGT
SNCA Exon 6	GGCTTCAGGTTCGTAGTCTTG

Table 6: Primers used during amplification of the α -synuclein 126 and 112 isoforms

The α -synuclein isoforms were sub-cloned into pGEM-T-easy and sequenced to check for mutations in the CDS and restriction sites. Any mutations were corrected by site mutagenesis (see section 2.1). The isoforms were digested from the pGEM-T-easy constructs and ligated into peGFP-N1 vector via the XhoI and SacII restriction sites. Due to incorrect annotation of the plasmid SalI sites were cloned onto the CDS of the α -synuclein isoforms but SalI ends are compatible with XhoI ends.

2.12 Real Time-PCR

2.12.1 SYBR® Green

To analyse the expression levels of the α -synuclein 5'UTR isoforms individual primer pairs were designed and used in a SYBR® Green system. An Additional pair of primers was designed to amplify a region within the α -synuclein ORF which allowed detection of total α -synuclein, and a separate pair for amplification of GAPDH. The primer pairs used are shown in Table 7.

Gene/Isoform	Fwd primer	Rev primer
α -synuclein 201	CCATTCGACGACAGTGTG	GTCTTTCCTGCTGCTTCTG
α -synuclein 202	CATTCGACGACAGGTTAGC	GAAAAGGAGCGCACAGGA
α -synuclein 003	CAGCTGTCTTCCAGCTCTGA	CCACACCCTGTTTGGTTTTTC
Total α -synuclein	GGAATTCATTAGCCATGGATGT	CCACACCCTGTTTGGTTTTTC
GAPDH	AATCCCATCACCATCTTCCAG	AAATGAGCCCCAGCCTTC

Table 7: SYBR® Green primers used for amplification of the α -synuclein 5'untranslated region (UTR) isoforms by real-time-PCR

RNA was extracted from cells and used to create a cDNA copy as described previously. Negative RT reactions for one sample within each treatment group were routinely included. cDNA was diluted 1 in 5 in nuclease-free H₂O and a small volume from each sample (e.g. 2 μ l) was used to make a stock mixture. This stock cDNA solution was serially diluted 2-fold a total of four times to create standards. These were then used to confirm the efficiencies of the primer pairs. Acceptable efficiencies were 100% $\pm$ 15%.

Individual reactions were set up as follows: 10 μ l SYBR® FAST ABI Prism® 2X qPCR Master Mix (KAPA Biosystems), 0.4 μ l (10 μ M) forward primer, 0.4 μ l (10 μ M) reverse primer, 1 μ l cDNA (untreated and MPP⁺-treated samples) or 2 μ l (brain samples) and H₂O to 20 μ l. Samples were tested in biological triplicates and technical duplicates. The largest possible volumes were always pipetted.

Once reactions were plated in 96-well MicroAmp Fast Optical 0.1ml qPCR plates (Applied Biosystems) the plates were briefly vortexed to mix and centrifuged to collect at the bottom of the wells. The run method on the ABI StepOnePlus RT-PCR machine was 3 minutes at 95°C, followed by 40 cycles of 3 seconds at 95°C and 20 seconds at 60°C.

2.12.2 TaqMan Assays

To analyse the expression levels of the alternatively spliced α -synuclein isoforms TaqMan assays for isoforms 112, 126 and total α -synuclein were developed using Integrated DNA Technologies (IDT) online RT-PCR Custom Assay Design software. The primer sets and probe sequences are shown in table 8. These arrived lyophilised and were reconstituted using TE buffer to a 4x concentration, as per the manufacturer's instructions.

Gene/Isoform	Forward primer	Reverse primer	Probe
α -synuclein 112	GGGAGCAGGGAG CATTG	GGCTTCAGGTTTCG TAGTCTTG	56-FAM/CAA AAA GGA /ZEN/CCA GTT GGG CAA GGA AG/3IABkFQ
α -synuclein 126	CAGAAGCAGCAG GAAAGACA	CCTCCAACATTTG TCACTTGC	56-FAM/TCA GCC ACT /ZEN/ACATAG AGA ACA CCCTCT T/3IABkFQ
Total α - synuclein	GGTGTAAGGAA TTCATTAGCCATG	ACCCTCTTTTGTC TTTCCTGC	56-FAM/CAC ACC CTG /ZEN/TTT GGTTTT CTC AGC A/3IABkFQ

Table 8: Real Time-PCR primer and probe sequences ordered as TaqMan Assays from IDT

An additional assay for GAPDH was ordered from Applied Biosystems at a 20x concentration (Hs99999905_m1).

2.12.2.1 cDNA synthesis for TaqMan assays

Initially, cDNA synthesised using Superscript II was used but the assay efficiencies, as tested for SYBR[®] Green using a cDNA stock, were low. At this point we switched to use a High capacity cDNA reverse transcription kit (Invitrogen) in combination with Ambion (non-DEPC-treated) H₂O. For cDNA synthesis an RT master mix was prepared containing 2 μ l 10x RT buffer, 0.8 μ l 25x dNTPs (100Mm), 2 μ l RT random primers, 1 μ l Multiscribe reverse transcriptase and 4.2 μ l H₂O. Final RT reactions contained 10 μ l of above master

mix plus up to 10µl RNA (volume adjusted accordingly depending on the amount of RNA). Every effort was made to ensure each sample received the same amount of input RNA in the RT reaction. For untreated and MPP⁺-treated BE(2)M17 reactions 700ng was added. As there was less RNA, brain sample reactions had 200ng of RNA per RT reaction.

2.12.2.2 TaqMan assay reaction set up

Final reactions contained 10µl of TaqMan® Gene Expression Master Mix (Applied Biosystems), 1µl of 20x TaqMan assay (GAPDH) or 5µl of 4x TaqMan Assay (custom α -synuclein IDT), cDNA and H₂O to a total volume of 20µl. 1µl cDNA per reaction was added for the standards, untreated and MPP⁺-treated samples. 2µl of cDNA per reaction for the brain samples were added as there was less input RNA for these samples.

Once reactions were plated in 96-well MicroAmp Fast Optical 0.1ml qPCR plates (Applied Biosystems) the plates were briefly vortexed to mix and centrifuged to collect at the bottom of the wells. The run method on the ABI StepOnePlus RT-PCR machine was 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

2.13 Amplification of TF ORFs

The ORFs of transcription factors PitX3, FoxA2, Lmx1a, Nurr1, Mash1 and Hif1 α were amplified from a number of plasmids kindly given to us by different sources; Tet-o-fuw-hNurr1, pOTB7-hMash1 and pBluescript-hLmx1a were donated by Dr Vania Broccoli (Stem cells and Neurogenesis Unit, Milan), pHGCX-EBNA-ratFoxA2 and pHGCX-EBNA-hPitX3 were donated by Professor Richard Wade-Martins (University of Oxford) and LZRS-pBMN-IRES-EGFP-hHIF1a triple mutant (P402A, P564G, N803A) was donated by Professor Johannes Schoedel (University of Oxford). Primers at the beginning and end of the ORF were designed for each TF and are shown in table 9.

Transcription Factor	Forward Primer	Reverse Primer	Expected Product size (bp)
PitX3	GAGTTCGGCCTGC TCAGC	TCATACGGGCCTTTCCAC	906
FoxA2	CTGGGAGCCGTGA AGATG	TTAGGACGAGTTCATAATAGGCC	1377
Mash1	GAAAGCTCTGCCA AGATGGA	TCAGAACCAGTTGGTGAAGTCG	730
Nurr1	CCTTGTGTTTCAGG CGCAG	TTAGAAAGGTAAAGTGTCCAGGAAA	1794
Lmx1a	CTGGACGGCCTAA AGATGG	TCAAGATGTGAAGTAAGAATTCTGC	1146
Hif1 α	GAGGGCGCCGGCG GCGCG	TCAGTTAACTTGATCCAAAGCTC	2478

Table 9: Sequences of the primers used to amplify the transcription factor open reading frames

Forward primers for each of the TFs were phosphorylated to aid the ligation of UTRs at a later stage. Phosphorylation reactions contained 3 μ l 100 μ M forward primer, 5 μ l T4-Polynucleotide Kinase (PNK-NEB) buffer (10x), 2.5 μ l ATP (1mM), 1 μ l PNK and H₂O to 50 μ l. Reactions were incubated at 37°C for 30 minutes and inactivated at 60°C for 25 minutes. 250 μ l H₂O added per reaction to create a final concentration of 1 μ M.

PCRs for TFs PitX3, FoxA2, Lmx1a, Nurr1, Mash1 were performed with reactions containing using 10 μ l 2x HiFi HotStart ReadyMix (KAPA Biosystems), 1 μ l cDNA, 4 μ l phosphorylated forward primer (1 μ M), 4 μ l reverse primer (1 μ M), and H₂O to 20 μ l. The PCR program was 95°C for 5 minutes, 30 cycles of 98°C for 5 seconds; 55°C 15 seconds and 72°C for 2 minutes followed by 72°C for 5 minutes. PCR products were visualised on a 1% agarose gel. The required bands were excised, extracted and the DNA purified using the Wizard® SV Gel and PCR Clean-Up extraction kit (Promega) following the manufacturer's protocol.

The PCR for Hif1 α was performed using Phusion DNA polymerase (as described in the general protocols) using a 10x GC rich buffer, and a 3 minute extension at 72°C with a total of 35 cycles.

All the resulting PCR products were incubated with 0.5µl Taq polymerase (NEB) (plus 1x buffer and 0.5µl dNTPs 10mM) at 68°C for 10 minutes to create A-overhangs, cloned into p-GEM-T-easy and send for sequencing to check the ORF of the TFs for mutations.

2.14 Ligation of UTRs to TF ORFs

For the TF ORFs to be used for *in vitro* transcription to make mRNA UTRs were added. This was based on the protocol by Warren et al. and required ‘splint’ oligomers to act as a bridge between the end of the coding sequence and the UTRs (Warren, Manos et al. 2010). These splints were designed for each TF and the sequences are shown in table 10.

Transcription Factor	5' splint	3'splint	Expected PCR products following ligation of UTRs (bp)
PitX3	GCTGAGCAGGCCGAAGCTCC ATGGTGGCTCTTATATTCT TCTT	CCCGCAGAAGGCAGCTC ATACGGGCCTTTCCAC	1138
FoxA2	CATCTTCACGGCTCCCAGC ATGGTGGCTCTTATATTCT TCTT	CCCGCAGAAGGCAGCTT AGGACGAGTTCATAATA GGCC	1609
Mash1	TCCATCTTGGCAGAGCTTTC CATGGTGGCTCTTATATTCT TTCTT	CCCGCAGAAGGCAGCTC AGAACCAGTTGGTGAAG TCG	962
Nurr1	CTGCGCCTGAACACAAGGC ATGGTGGCTCTTATATTCT TCTT	CCCGCAGAAGGCAGCTT AGAAAGGTAAAGTGTCC AGGAAA	2026
Lmx1a	CCATCTTTAGGCCGTCCAG CATGGTGGCTCTTATATTCT TTCTT	CCCGCAGAAGGCAGCTC AAGATGTGAAGTAAGAA TTCTGC	1378
Hif1α	CGCGCCGCCGGCGCCCTCC ATGGTGGCTCTTATATTCT TCTT	CCCGCAGAAGGCAGCTC AGTTAACTTGATCCAAA GCTC	2710

Table 10: Sequences of the transcription factor splint oligomers for ligation of untranslated regions (UTRs) and the product size following UTR ligation and PCR amplification

The 5' and 3' UTR sequences were common to all TFs and are shown below.

5'UTR- GCTCTAGAACTAGTGTGACCCCTCATTGGACCCTCGTACAGAAG
CTAATACGACTCACTATAGGGAAATAAGAGAGAAAAGAAGAGTA
AGAAGAAATATAAGAGCCACCATG

3'UTR- GCTGCCTTCTGCGGGGCTTGCCTTCTGGCCATGCCCTTCTTCTC
TCCCTTGACCTGTACCTCTTGGTCTTTGAATAAAGCCTGAGTAGGAA
GTGAGGGGTGACACTAGTTCTAGAGC

The ligation reactions contained ~20ng of ORF PCR reaction, 4µl 5'UTR (1µM), 4µl phosphorylated 3'UTR (1µM-as described for the forward primers), 2µl 5' splint (1µM), 2µl 3' splint (1µM) and H₂O to 20µl. Reactions were heated to 95°C for 4 minutes and then left to cool to room temperature for ~30 minutes.

The ligation products were amplified using 25µl 2x HiFi HotStart ReadyMix (KAPA Biosystems) with 15µl ligation reaction, 10µl primer (10µM) and H₂O to 50µL. The primer used is found in both the 5' and 3'UTR and the sequence is shown here.

Ligation amplification primer- GCTCTAGAACTAGTGTCGACCCCTCA

The conditions of the PCR were as described previously with the exception that the extension time at 72°C was increased to 3 minutes.

The sizes of the PCR products expected are shown in table 10. Products were visualised on a 1% agarose gel, excised and the DNA purified using the Wizard® SV Gel and PCR Clean-Up extraction kit (Promega) following the manufacturer's protocol.

The resulting PCR products were incubated with Taq polymerase (as described for the ORFs), cloned into p-GEM-T-easy and send for sequencing to check the ORF and restriction sites in the UTRs for mutations. Attempts to correct any mutations present were made using site-directed mutagenesis (see general protocols).

We were unable to ligate the UTRs to the PitX3 ORF, as it resulted in a loss of the ORF sequence. Several different methods were attempted, including direct PCR of the UTRs and slower cooling of the ligation reaction, but none of these were successful.

2.15 Initial experiment into substrate and medium change for iPSCs prior to modified mRNA transfection

2.15.1 Seeding iPSCs onto substrate

iPSCs were seeded on either Matrigel (BD Bioscience) or Poly-D-lysine and laminin (Invitrogen). For wells that required Poly-D-lysine (Sigma) and laminin 250µl of Poly-D-

lysine was added and the plate incubated at 37°C for 30 minutes. Wells were washed 3x with PBS and air-dried. 200µl laminin (thawed on ice) was added on top of the Poly-D-lysine and incubated at 37°C for approximately 1 hour 30 minutes. Matrigel was added to empty wells and also incubated at 37°C for approximately 1 hour 30 minutes. Matrigel and laminin solutions were removed and the laminin wells washed with PBS. Two wells of a 6 well plate of iPSCs were trypsinised using 1ml TrypLE (Invitrogen) and incubated at 37°C for 5 minutes. Cells were collected in a falcon, 18ml PBS added and spun at 400g for 5 minutes. The cell pellet was resuspended in 5ml mTeSR™1 (Stem Cell Technologies) supplemented with 10µl of 1mM ROCK inhibitor (Merck/Calbiochem). iPSCs were plated at 500,000 cells/cm² in mTeSR™1 medium and incubated overnight at 37°C.

2.15.2 Replacing the medium

The following day medium was changed to either fresh mTeSR™1, 100% neural induction medium (contained 300µl 2mM GlutaMAX (Invitrogen), 300µl N2 supplement (100x, R and D systems), 600µl 50mg/ml BSA, 300µl 10µM ROCK inhibitor, 30µl 10µM SB431542 (Tocris), 30µl 200ng/ml noggin (TM Biosource/Invitrogen), 300µl antimyotic/antibiotic (Invitrogen) and DMEM/12 (Invitrogen) to 50ml (filtered before use) or 50:50 mTeSR™1:neural induction medium. Cells were returned to 37°C for a further 24 hours. Light microscopy images were captured after incubation.

2.15.3 Transfection with modified GFP-mRNA

Half the medium per well was replaced with fresh medium that contained 2x 800ng/ml interferon inhibitor B18R (eBioscience). Cells were incubated for 4 hours at 37°C and transfected with 240ng modified GFP-mRNA (synthesised by Martina Hallegger) per well using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's protocol. Four hours post transfection the entire medium was replaced with either mTeSR™1, 100% neural induction medium or 50:50 mTeSR™1: neural induction medium, all containing 1x

B18R. Cells were incubated overnight at 37°C and transfection was repeated as described above, with the exception that the medium already contained B18r and so did not require changing 4 hours prior to transfection. Cells were incubated for a further 24 hours, imaged by light microscopy and fixed for immunofluorescence.

2.16 Immunofluorescence

Cells were fixed with 4% Paraformaldehyde (PFA) for 15 minutes at room temperature. Following washing PBS cells were permeabilised for 5 minutes in 0.2% Triton X-100 in PBS. Cells were blocked with 1% BSA in PBS for 1-2h. Cells were stained with primary antibody diluted in 1% BSA in PBS (concentrations shown in table 11) for 1 hour at room temperature. Species specific secondary fluorescent conjugate antibodies diluted in 1%BSA in PBS (shown in table 8) were added to the coverslips and incubated for 1 hour. Following washes with PBS DAPI (1:1500) was added and cells viewed under a fluorescent microscope.

Antibody	Concentration
Rabbit anti-TH (Sigma)	1:600
Mouse anti- β -III Tubulin (TUJI-Covance)	1:500
Rabbit anti-DAT Alpha Diagnostics	1:200
Mouse anti-NeuN (Chemicon)	1:50
Mouse anti-Nestin (Millipore)	1:200
Goat anti-mouse alexa-Fluor 488 (Invitrogen)	1:500
Goat anti-rabbit alexa-Fluor 594 (Invitrogen)	1:500

Table 11: Primary and secondary antibodies and the concentrations used for immunofluorescence

3 The role of α -synuclein non-coding regions

Recently the importance of non-coding regions within genes has been recognised and their role, especially in the control of gene expression, is now the focus. Analysis of α -synuclein transcripts annotated within the human genome indicates that there may be several different 5' and 3' UTRs expressed. In this chapter we explored this possibility and highlight the possible advantages of expressing such isoforms.

3.1 5'UTR isoforms of α -synuclein

Due to the removal of introns from pre-mRNA transcripts by the spliceosome (Cooper, Wan et al. 2009) exons and UTRs can also be differentially spliced, producing alternative isoforms. This allows many different mRNAs to be produced from a single gene. As UTRs are known to contain many elements that regulate translation, including protein-binding sites, secondary structures and miRNA binding-sites (Gebauer and Hentze 2004), inclusion or exclusion of these regions could have major impact on the level of protein produced.

As already mentioned, the levels of WT α -synuclein protein are extremely important and over-expression due to duplications and triplication of the α -synuclein loci result in aggressive early onset PD (Singleton, Farrer et al. 2003). Several different α -synuclein 5'UTRs are annotated within the human genome, which could potentially affect the levels of α -synuclein protein produced. With the exception of a predicted IRE (Friedlich, Tanzi et al. 2007) there has been little investigation into whether the α -synuclein 5'UTR contains regulatory elements or modulates protein expression.

In this chapter these isoforms have been cloned and their effect on protein translation has been explored via luciferase assays. As oxidative stress has been widely linked with PD we developed an oxidative stress assay which aimed to adequately stress the cells but not cause excessive cell death. Using this we have studied the α -synuclein 5'UTR isoforms in

cells treated with MPP⁺ to determine whether the expression levels change when compared to untreated cells. Finally, we investigated the levels of expression in cingulate gyrus samples taken from a control and dementia with LB disease patient.

3.2 3'UTR isoforms of α -synuclein

3'UTRs are known to play a role in the regulation of translation. The 3'UTRs of many mature mRNA transcripts can contain protein binding sites such as PABP which binds to the polyA tail and eIF4G and is thought to circularize the mRNA (Wells, Hillner et al. 1998). 3'UTRs are also the location of many miRNA binding sites. MicroRNAs are non-coding, short; ~22 nucleotides RNAs that display only partial complementarity to their target sequence. Following processing by Drosha (Lee 2003), transportation into the cytoplasm by Exportin-5 and further processing by Dicer the mature miRNA is loaded into RISC and guided to a small target region in the 3' UTR of target mRNA. The miRNA imperfectly binds to the mRNA, via a seed region, usually 6-8 nucleotides in length (Gottwein and Cullen 2008), leading to translation inhibition. The mechanism of this inhibition is not yet fully understood but it is widely accepted that mRNA degradation does not occur, unlike in siRNA mediated silencing.

The 3'UTR of α -synuclein transcript has been more widely studied than its 5' counterpart. Several papers confirm the presence of two miRNA binding sites; miR7 and miR153 within this region and have been shown to regulate α -synuclein expression (Junn, Lee et al. 2009; Doxakis 2010). The annotated α -synuclein transcripts in the human genome browser indicates that all isoforms have the same 3'UTR, however when compared to those annotated in Ensembl a disparity was observed. There appears to be a range of 3'UTR lengths, shown previously in chapter 1.4.4 in figure 11. Interestingly the miRNA sites all fall before the first polyA termination site. This raises the question, what is the function of

the 3'UTR beyond the first polyA termination site? Does it contain additional regulatory sequences?

About half of mammalian genes use alternative cleavage and polyadenylation to generate multiple mRNA isoforms differing in their 3' UTRs (Edwards-Gilbert, Veraldi et al. 1997; Beaulieu and Gautheret 2001; Zhang, Lee et al. 2005). There is some evidence that proliferating cells express shorter 3'UTRs (Sandberg, Neilson et al. 2008), therefore containing fewer regions for regulatory interactions and consequently subjected to lower levels of translational regulation. Indeed shortening of 3'UTRs and therefore removal of miRNA binding sites increased levels of translation (Sandberg, Neilson et al. 2008).

In this chapter we try to identify α -synuclein 3'UTR isoforms in our proliferating and differentiated BE(2)M17 cells and in iPSC-derived dopaminergic neurons, kindly donated from Elizabeth Hartfield in Richard Wade-Martins Laboratory, were also tested.

3.3 Aims

In light of the fact that there has been very little investigation into the 5'UTR of α -synuclein transcripts and no mention of alternative isoforms the initial aim is to determine whether any α -synuclein 5'UTR isoforms could be detected in our human neuroblastoma cell line (BE(2)M17) by PCR. Identified α -synuclein 5'UTR isoforms were cloned, in their entirety, into the dual luciferase reporter vector (PsiCHECK2.2), upstream of the Renilla luciferase ORF and used in a luciferase expression assay to determine whether the 5'UTR TSS has an effect on α -synuclein expression *in vitro*. The Luciferase assay was carried out in both BE(2)M17 and HEK-293 cells to determine whether any neuronal-specific mechanisms occurred.

Secondarily, to determine whether BE(2)M17 cells produce different α -synuclein isoforms when stressed, an oxidative stress protocol was developed using MPP⁺. MPP⁺ is a neurotoxin that inhibits the mitochondrial complex I, causing a loss of ATP production and an increase in oxidative stress within the cell. The protocol needs to stress the cells adequately to determine whether a change in the isoforms is present but not result in excessive cell death. Different concentrations of MPP⁺ were tested and the effect on cell viability monitored using an MTS assay. Once a robust oxidative stress assay had been developed, BE(2)M17 cells were treated and used to determine whether any additional 5'UTR isoforms of α -synuclein are expressed. These were also cloned into the luciferase reporter PsiCHECK2.2 and analysed alongside the others for their effects on protein translation.

Finally Real Time-PCR SYBR[®] Green primers for all the identified 5'UTR isoforms were designed and used to compare the expression levels of the isoforms in untreated and MPP⁺-treated BE(2)M17 cells.

3.4 Results

3.4.1 Detection of α -synuclein 5'UTR TSS isoforms in BE(2)M17 cells

Primers were designed to PCR amplify the specific 5'UTR isoforms that were distinctly different from each other; 201, 001, 003 and 008. There is evidence that a truncated 201 5'UTR is expressed so this was also amplified using junction primers. The PCR products obtained are shown in figure 18.

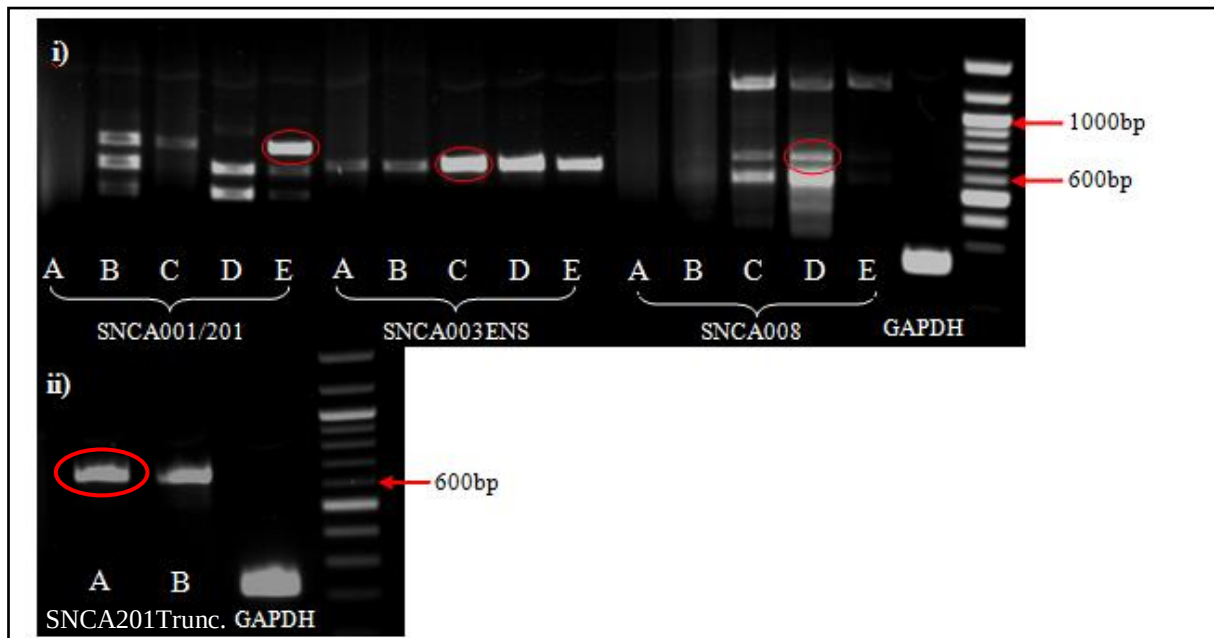


Figure 18: α -synuclein (SNCA) 5' untranslated region isoform amplification by PCR. i) PCR products; expected sizes; SNCA001=982, SNCA201=763, SNCA003=657bp, SNCA008=669bp where annealing temperatures were A=52.4°C, B=53.9°C, C=56.3°C, D=58°C and E=61.3°C. ii) Truncated SNCA201 PCR product (638bp) where A=56°C and B=59°C. Circled bands indicate the PCR product cut and purified.

Of the isoforms expected SNCA201 (full length and truncated) and SNCA003 were successfully identified in cDNA transcribed from RNA extracted from undifferentiated BE(2)M17 cells. Unfortunately the bands seen in the SNCA008 reactions were non-specific amplification and no band of the correct size was present for SNCA001.

All three 5'UTR isoforms of α -synuclein (201, 201-truncated and 003), circled in figure 23, were successfully cloned, confirmed by colony PCR or digestion and eventually sequenced to confirm the correct insert was present.

3.4.2 Cloning into psiCHECK 2.2 Dual Luciferase Vector

3.4.2.1 Creation/amplification of entire 5'UTR of SNCA isoforms

As we aim to investigate the effect of the different SNCA 5'UTRs on translation we needed to clone these regions into a luciferase reporter vector. As the primers used to identify and clone the different isoforms initially did not select the entire 5'UTR or create cloning restriction sites we needed to repeat the amplification with new primers. This was achieved directly from amplifying undifferentiated BE(2)M17 cDNA for the SNCA003 and truncated SNCA201 isoforms, creating NheI restriction sites at both ends (see figure 19ii and iii respectively).

However, the start of the SNCA201 5'UTR was too repetitive to allow successful amplification by PCR directly from cDNA. Therefore using the region cloned into pGEM-T-easy stepwise PCR reactions were run, we amplified using overlapping primers to increase the UTR until finally the entire region was created with NheI restriction sites at either end. The PCR products obtained after each stage are shown in figure 19.

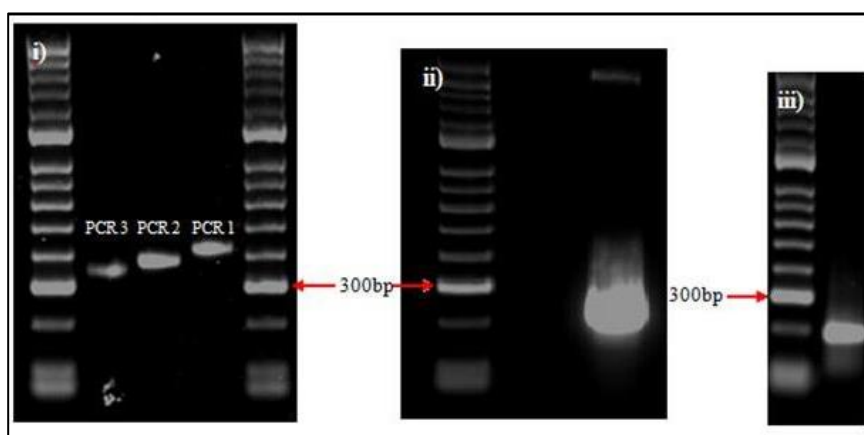


Figure 19: PCR amplification of entire α -synuclein 5' untranslated regions with cloning restriction sites. i) Stepwise PCR creating the entire 5'UTR of 201 with NheI restriction sites (PCR 1= 336bp, PCR 2= 371bp, PCR 3=409bp), ii) PCR amplifying003 5'UTR isoforms (217bp), iii) PCR product 201-truncated (191bp).

3.4.2.2 Digestion and ligation of α -synuclein 5'UTR isoforms into psiCHECK 2.2

As cloning the digested PCR products into psiCHECK 2.2 directly was inefficient, the inserts were first TA cloned into pGEM-T-easy. This also allowed us to sequence the inserts and check for mutations in the UTRs and restriction sites. Mini preps were digested with NheI to release the inserts (see figure 20) and ligated into psiCHECK 2.2.

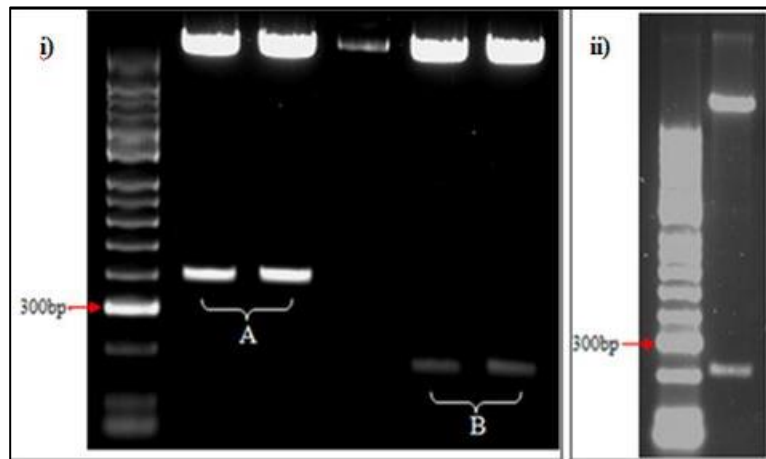


Figure 20: Digestion of α -synuclein 5' untranslated region (5'UTR) isoforms from pGEM-T vector. i) A= 201 5'UTR NheI digested from pGEM-T clone, B= 201-truncated 5'UTR NheI digested from pGEM-T clone, ii) 003 5'UTR NheI digested from pGEM-T clone.

Colonies were screened for the correct insert in the correct orientation by colony PCR using T7 forward and SNCA specific reverse primers. The colony PCR products of both positive and negative colonies are shown in figure 21.

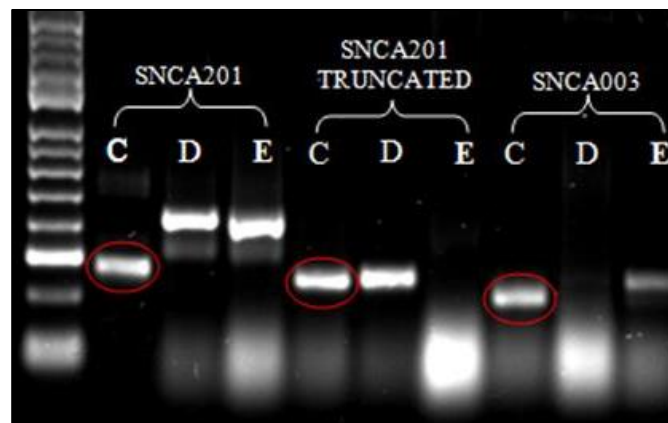


Figure 21: i) Colony PCR of psiCHECK- α -synuclein (SNCA)-5' untranslated region inserts; 201, 201 truncated and 003. Red circles indicate positive colonies.

Positive colonies were grown up in LB, the plasmid purified and sequenced. Once sequencing confirmed the 5'UTR isoforms were present and with no mutations the plasmids were grown up to midi preps and purified. The resulting constructs and the psiCHECK2.2 control vector are shown in appendix 1.

3.4.3 Effect of the different α -synuclein TSS isoforms in a luciferase reporter assay

Luciferase assays provide strong evidence as to whether a 5'UTR affects translation of a protein transcript. Numerous studies have used the dual vector psiCHECK2 (Promega). This vector contains two luciferase expression genes. The resulting proteins are detected by a luminometer reader. The Firefly luciferase gene is constitutively active and acts as a reference reading, while the Renilla luciferase gene contains a NheI cloning site at its 5' region, into which the 5'UTRs are cloned. The ratio of luminescence from these two proteins is measured and a change in this ratio implies that the levels of Renilla luciferase expression have been altered. If the ratio is sufficiently altered it provides good evidence the 5'UTR confers some regulation on protein translation. If a notable difference is seen in the change of Renilla luciferase protein levels between HEK293 and BE(2)M17 cells a tissue specific translational regulation mechanism can be deduced.

The α -synuclein constructs, shown in appendix 1, were transfected into BE(2)M17 and HEK293 cells. The results of the luciferase assay are shown in figure 22.

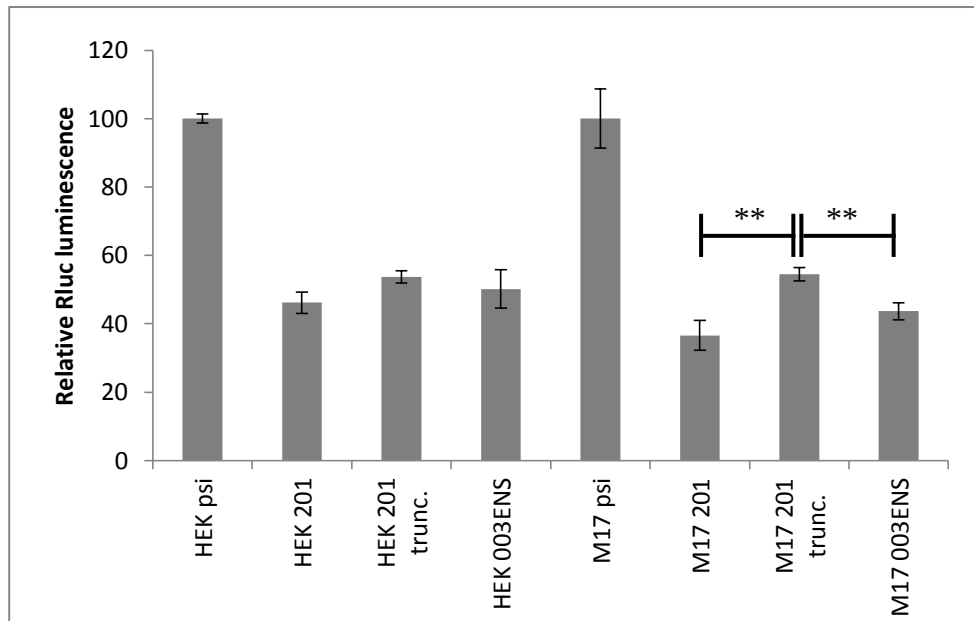


Figure 22: Luciferase data collected in HEK293 and BE(2)M17 cells when transfected with α -synuclein 5' untranslated region (5'UTR) constructs. Graph indicates that the α -synuclein 5'UTRs decrease RLuc expression relative to a psiCHECK control (no insert) in both HEK293 and BE(2)M17 cells. ** indicates $p < 0.001$ calculated using an ANOVA test. (BE(2)M17 samples compared using a Games-Howell post hoc test as samples have different variances, The HEK293 samples had equal variance so Bonferroni post hoc test was applied). N=3 per sample group. Error bars indicate the % standard error.

Figure 22 shows that the insertion of any of the α -synuclein 5'UTRs results in significantly decreased protein expression in both HEK293 and BE(2)M17 cells, when compared to empty PsiCHECK2.2. It is also evident that in BE(2)M17 cells, but not HEK293 cells, there is a statistical significance between the levels of protein expressed when under the control of the different 5'UTRs. α -synuclein 201 stimulated the least protein expression whilst its truncated counterpart, 201 truncated, resulted in the highest level of protein expression. Comparison of the two gave a p -value < 0.001 . The isoform 003 stimulated intermediate levels of protein expression and was significantly different when compared to 201 truncated with a p -value < 0.001 .

3.4.4 Development of an oxidative stress treatment for BE(2)M17 cells

Oxidative stress is thought to play a major role in the decline of dopamine neurons throughout the cause of PD and oxidative compounds have long been used to create animal models of PD. Oxidative stress responses include alterations in the expression of many

genes, one of which is α -synuclein. In order to assess whether oxidative stress induces the use of alternative transcription start sites and as a result produces alternative 5'UTRs, or if expression levels alter, an oxidative assay needed to be developed.

MPP⁺ is the active metabolite of MPTP, the compound first discovered to cause PD and regularly used to make animal models. MPP⁺ is taken up by DAT and transported into mitochondria where it acts as a complex I inhibitor, disrupting the cells production of energy. The concentrations used in previous literature vary, so we initially tested untreated, 50 μ M, 450 μ M and 2000 μ M MPP⁺ on our BE(2)M17 cells. Following 24 hour incubation the cell viability was assessed using an MTS assay. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. The results are shown in figure 23.

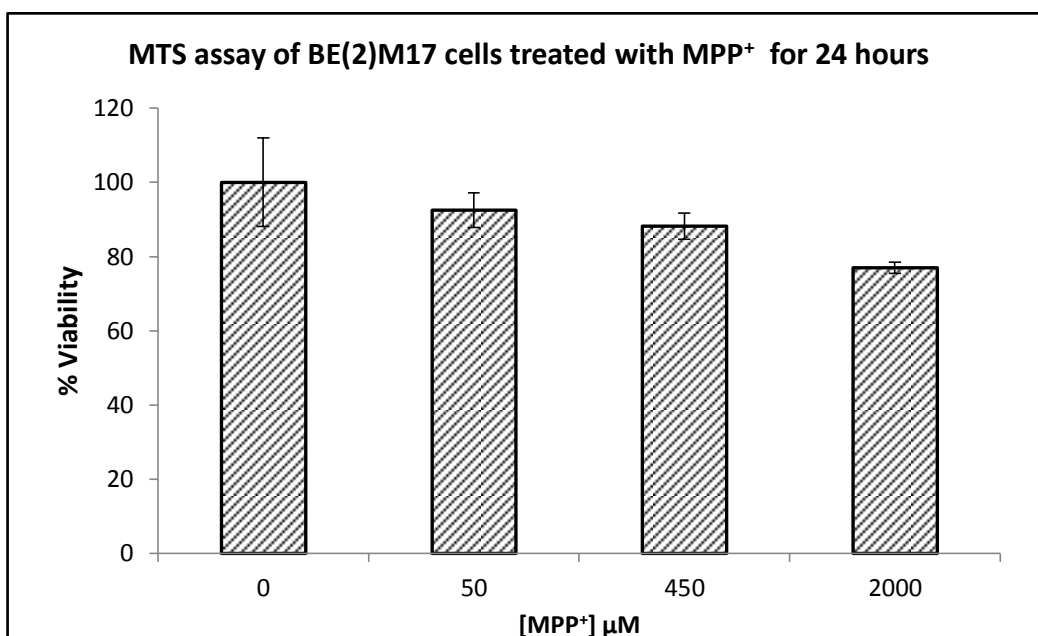


Figure 23: Percentage survival of BE(2)M17 cells following treatment with MPP⁺. Viability observed after 24hr incubation with varying concentrations of MPP⁺ as analysed using an MTS assay. Error bars plotted as % standard error. N=12 for controls and N=6 per concentration.

The results show that BE(2)M17 cells are quite resistant to oxidative stress and have a viability of 98, 88 and 77% when treated with 50μM, 450μM and 2mM MPP⁺ respectively, compared to untreated cells. Based on this, we decided to use 2mM for all future experiments.

To confirm that the cells were not undergoing apoptosis following treatment with MPP⁺ we stained them with Annexin V and Propidium Iodide (PI). Annexin V, a calcium and phospholipid binding protein, binds preferentially to phosphatidylserine, a negatively charged phospholipid located in the inner surface of the plasma membrane, that appears very early in apoptosis at the external cell surface. PI binds to DNA by intercalating between the bases but cannot permeate cells with an intact membrane. When cells are double-stained with Annexin V and PI, three different cell populations may be observed (i) live cells that do not stain either with Annexin V or with PI, (ii) necrotic or late apoptotic cells that stain with both Annexin V and PI, and (iii) early apoptotic cells that stain with

Annexin V only. Representative images collected for untreated and MPP⁺-treated cells are shown in figure 24.

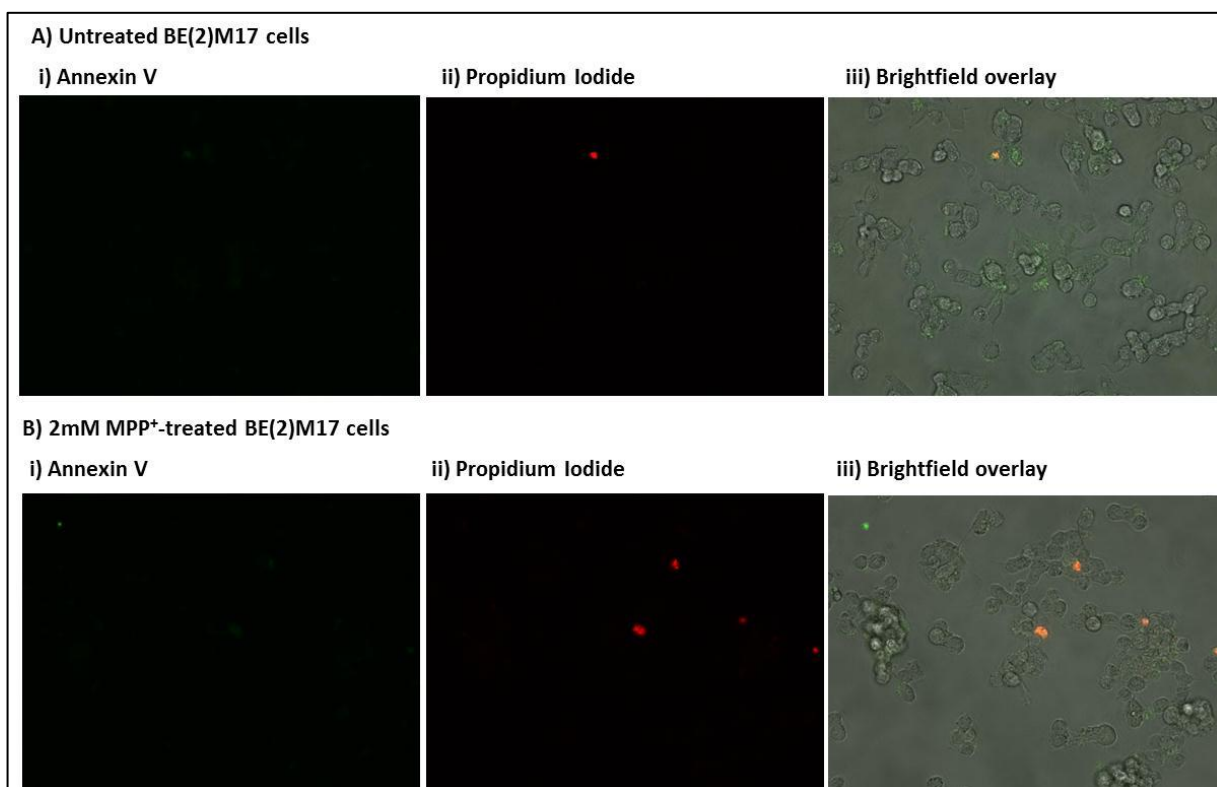


Figure 24: Analysis of A) untreated and B) 2mM MPP⁺-treated BE(2)M17 cells for early (Annexin V) and late (Propidium Iodide) apoptosis after 24 hours incubation.

The images shown in figure 24 show that there is little difference in either early (i) apoptosis markers between untreated and MPP⁺-treated BE(2)M17 cells. There does appear to be fractionally more PI labelled cells in the 2mM MPP⁺-treated wells as indicated in panels ii. But these “late apoptotic” cells do not account for the majority of the cells and therefore should not affect the analysis of transcription levels.

3.4.5 Detection of an additional α -synuclein 5'UTR isoform in oxidatively stressed BE(2)M17 cells

BE(2)M17 cells were treated with 2mM MPP⁺ and incubated for 24hrs. In order to remove any detached dead cells before RNA extraction and remove residual MPP⁺, cells were washed with PBS following removal of medium. Untreated cells were also washed to

remove the bias from this step. Following this the RNA was analysed for alternative α -synuclein 5'UTR isoforms. Exciting an additional isoform, 202, was detected, as shown in figure 25i. The additional isoform was amplified to create NheI cloning sites and ligated into PsiCHECK2.2, as described for the previous isoforms. The stages of amplification and cloning are shown in figure 25i-iv. The resulting construct is shown in appendix 1.

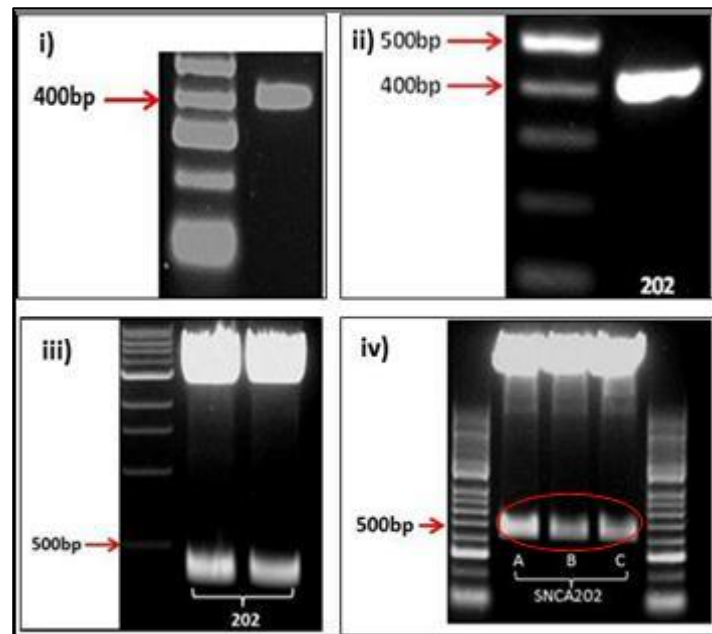


Figure 25: Amplification and cloning of α -synuclein 5' untranslated region (UTR) isoform 202. i) PCR amplification of α -synuclein isoform 202 from 2mM MPP⁺-stressed BE(2)M17 cells (391bp) ii) PCR product 202 (405bp) ii) PCR amplification of α -synuclein 5'UTR isoform 202 to create NheI cloning sites iii) SNCA202 NheI digested from pGEM-T-easy clones iv) NheI digestion of psiCHECK-202 5'UTR purified plasmids to confirm insert. Red circles indicate positive colonies.

3.4.6 Comparison of all the α -synuclein 5'UTR isoforms on protein expression in a luciferase assay

The luciferase assay was repeated, including the MPP⁺-induced 202 5'UTR construct. This time the assay was only carried out in BE(2)M17 cells as the previous study indicated no significant difference when transfected in HEK293 cells. The results are graphically represented in figure 26.

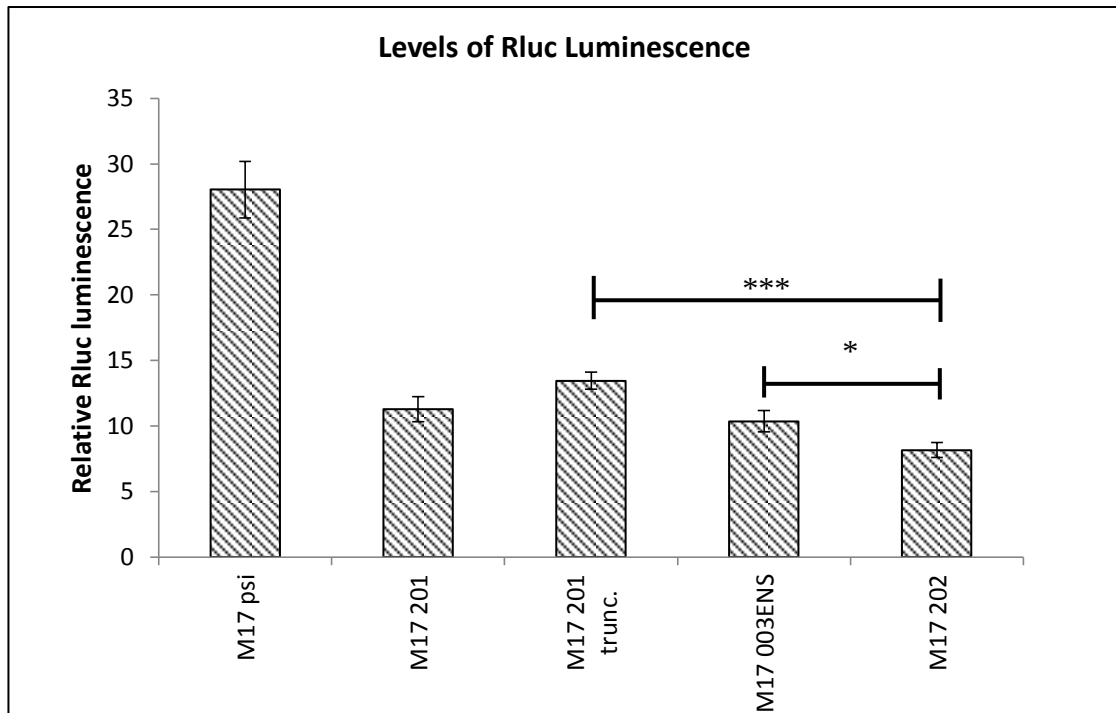


Figure 26: Luciferase data collected from BE(2)M17 cells when transfected with α -synuclein 5' untranslated region (5'UTR) PsiCHECK2.2 constructs. Graph indicates that the 5'UTRs decrease Rluc expression relative to a psiCHECK control (no insert) in BE(2)M17 cells. * indicates $p < 0.05$ *** indicates $p < 0.0001$ calculated using an ANOVA test. (BE(2)M17 samples compared using a Bonferroni post hoc test. N=6 for samples psi, 201, 201 truncated and 003 (N=3 in two separate experiments) N=3 for sample 202. Error bars indicate the standard error.

The results indicate that, as observed before, there is significant variation in the levels of Renilla luciferase protein expressed when preceded by the α -synuclein 5'UTR isoforms. Interestingly the 202 isoform, found in MPP⁺-treated BE(2)M17 cells, appears to simulate the lowest levels of protein expression and when compared to 201 truncated and 003 gave statistically significant p-values of >0.0001 and >0.05 respectively.

To assess whether MPP⁺ affected the luciferase assay, i.e. whether treatment would alter the levels of protein expression observed, BE(2)M17 cells were transfected with the piCHECK constructs and, after a 24 hr incubation, were treated with 2mM MPP⁺. After a further 24hrs the cells were lysed and the luciferase assay performed. The results are shown in figure 27.

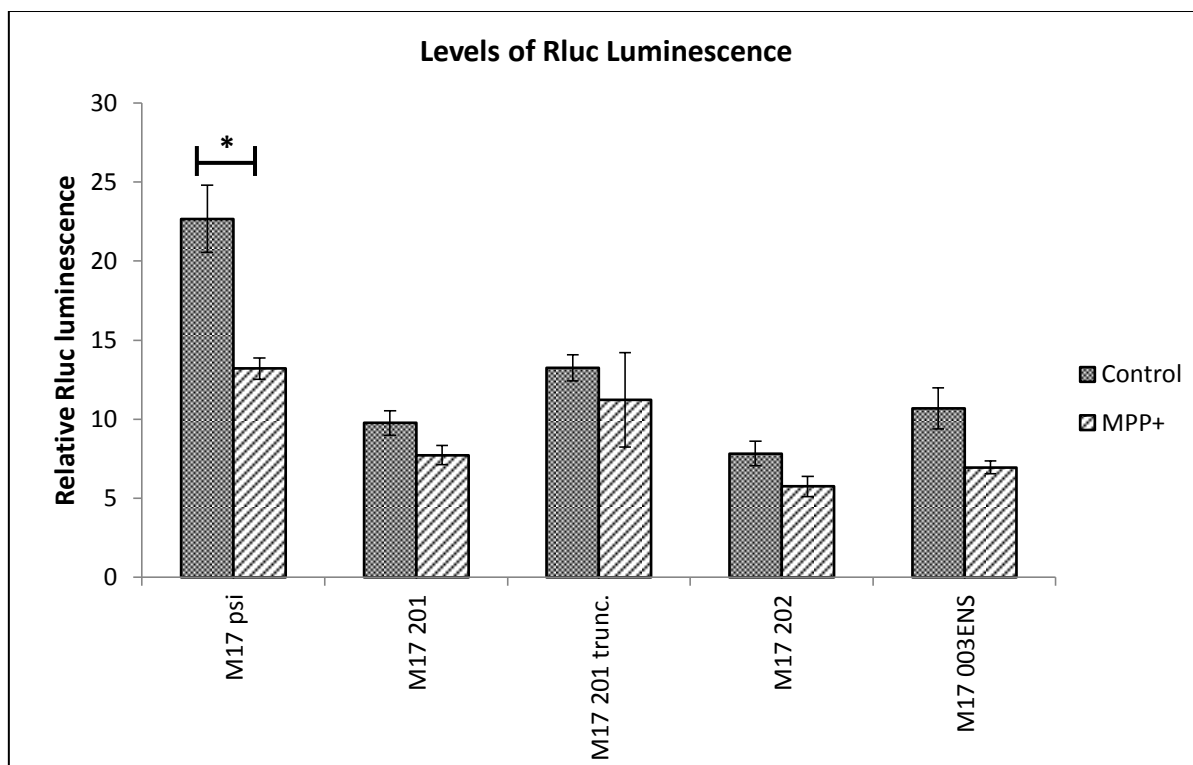


Figure 27: Luciferase data collected comparing Rluc expression of BE(2)M17 cells transfected with psiCHECK2.2 constructs containing α -synuclein 5' untranslated region (5'UTR) isoforms in either untreated or 2mM MPP⁺-treated conditions. (N=3 for all samples. Error bars represent standard error. Treated samples compared to untreated controls using a two-tailed TTest with unequal variances assumed) * indicates $p < 0.05$.

The results show that there is a significant decreased expression of Renilla luciferase protein in the empty PsiCHECK transfected cells treated with MPP⁺, with a reported P-value of 0.037. However, while there is a general decreasing trend observed in the α -synuclein 5'UTR isoform-PsiCHECK transfected cells (treated vs. untreated), the data collected is not significant.

To check that insertion of the α -synuclein 5'UTRs did not alter the splicing of the PsiCHECK intron, and therefore affect the luciferase readout, a PCR was performed using a forward primer in the SV-40 promoter and a reverse primer in the Renilla luciferase ORF. The vector map with the different components labelled is found in appendix 1. The results of this splicing test are shown in figure 28.

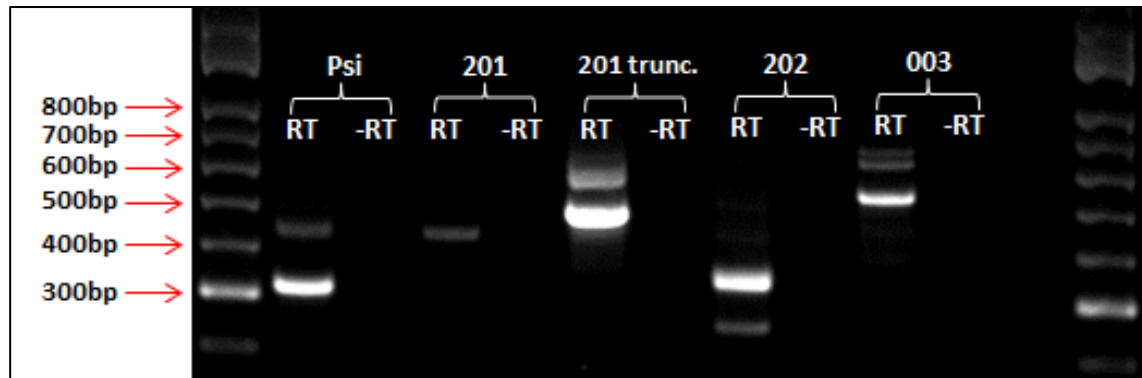


Figure 28: Agarose gel indicating the PCR products amplified from cells transfected with empty PsiCHECK2.2 and α -synuclein 5' untranslated region (UTR)-PsiCHECK2.2 constructs 201, 201 truncated, 202 and 003. Expected sizes of un-spliced/spliced per isoform are as follows; PsiCHECK only: 447/315bp, 201: 838/706bp, 201 truncated: 621/489bp, 202: 834/702bp, 003: 664/532bp. -RT; cDNA synthesis performed without reverse transcriptase to check for DNA contamination.

The results indicate that PsiCHECK2.2 only, PsiCHECK-201 truncated and PsiCHECK-003 appear to splice correctly producing the expected band sizes of 315, 489 and 532bp with only minimal un-spliced product detected in PsiCHECK2.2 only and PsiCHECK-003. Un-spliced products are the larger bands seen on the gel with sizes of 447, 664bp for samples PsiCHECK2.2 only and PsiCHECK-003 respectively.

Unfortunately, samples PsiCHECK-201 and PsiCHECK-202 did not amplify bands of the expected size, for either spliced or un-spliced product. This indicates that the addition of these α -synuclein 5'UTR isoforms 202 disrupts the splicing of the intron and/or introduces new splice sites. This may result in Renilla luciferase not being translated correctly and could explain the low levels of protein expression observed following transfection with these constructs.

To assess the splicing the PCR products from PsiCHECK-201 and PsiCHECK-202 reactions were ligated into p-GEM-T-easy and sequenced. The sequencing revealed that only 103bp of the 201 5'UTR are retained in the mature transcript with the other 135bp removed with the intron during splicing. With isoform 202, the entire 5'UTR isoform and

intron are spliced. This is interesting as despite the UTRs being partially or completely removed translation levels are still reduced.

3.4.7 Quantification of α -synuclein 5'UTR isoforms

3.4.7.1 Real Time PCR

Real Time-PCR is a method commonly used to compare expression levels in treated and untreated cells. SYBR[®] Green binds all double-stranded DNA molecules, emitting a fluorescent signal on binding, allowing the detection of all amplified DNA products within a reaction. As the number of cycles (Ct) increases so does the amount of bound SYBR[®] Green and therefore the fluorescent signal. By creating primer combinations specific for each α -synuclein 5'UTR isoform we could determine the different levels of expression based on the levels of fluorescent signal. There are several ways to analyse Real Time PCR data. First you can use the comparative method where you compare the Ct values obtained of your gene of interest (GOI) to that of a reference gene within the same sample. This normalises the data for varying amount of input cDNA. Then you compare your normalised values for your GOI from your control and your test samples (e.g. untreated and treated cells). If one assumes 100% efficiency (i.e. the PCR product doubles every cycle) and raise this to the power of your $-C_t$ value (2^{-C_t}) it will give you a fold change in expression levels.

However, it is unlikely that the primer efficiency will be 100% or that the different primer pairs will have the same efficiencies. To take this into account a Pfaffl analysis can be performed (Pfaffl 2001). This first requires the calculation of the primer efficiencies which is carried out using a standard curve. A mixture of cDNA is used to perform serial dilutions which are used as input for the PCR reactions. As the dilution increases, the number of cycles required to produce a detectible product should increase proportionally,

resulting in standard curve. The standard curve gives you an efficiency, as well as a correlation coefficient. You then perform the analysis as described above but using the efficiencies calculated for each primer pair; if the efficiency is 50% the number you raise to the power of $-C_t$ becomes 1.5, 75% becomes 1.75, etc. This is generally considered the more accurate method and is why it was used during the analysis of Real Time PCR data in this thesis.

3.4.7.2 Expression levels of α -synuclein 5'UTR isoforms in BE(2)M17 cells

We wanted to determine whether α -synuclein 5'UTR isoforms are differentially expressed when comparing untreated BE(2)M17s with 2mM MPP⁺-treated BE(2)M17 cells. It is possible that if, as the luciferase results indicate, the isoforms do stimulate different levels of protein expression the cells are trying to modulate the levels of α -synuclein protein production. In order to compare the levels of the different transcripts expressed Real-Time PCR SYBR[®] Green primers were developed for each isoform and one for total α -synuclein expression. The locations of the primer binding sites for each isoform are shown in figure 29.

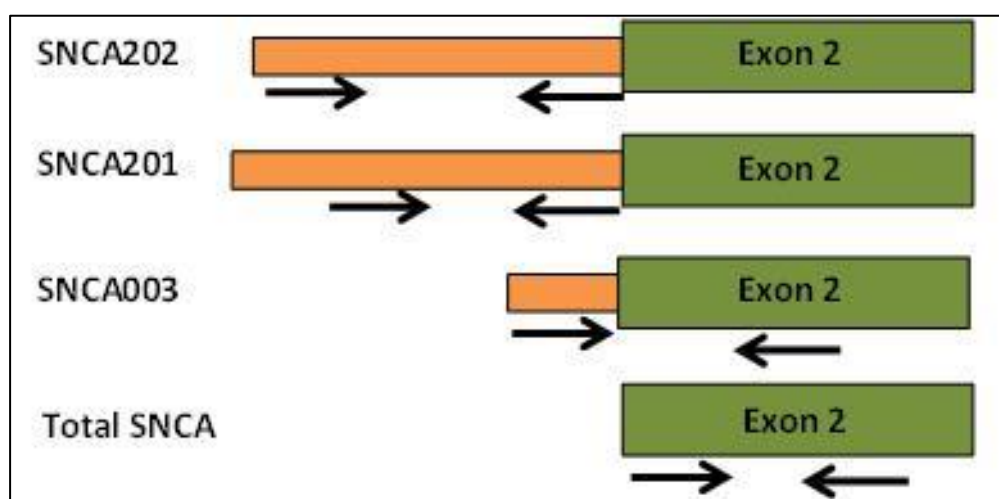


Figure 29: Real-Time PCR primer-binding locations for the different α -synuclein (SNCA) 5' untranslated region (5'UTR) isoforms 202, 201 003 and total SNCA. Forward and reverse primers are indicated by arrows facing right and left respectively. Exon 2 represents the start of the coding sequence. Orange boxes indicate 5'UTRs.

These primer sets were tested for their efficiencies using the standard curve method and used to analyse untreated and 2mM MPP⁺-treated BE(2)M17 cells. Expression levels were initially normalised to the house-keeping gene GAPDH the results interpreted using the Pfaffl method (Pfaffl 2001). The results collected are represented in figure 30A.

There was a statistically significant increase in the expression of α -synuclein 5'UTR isoforms 202 and 003 as well as the expression of total α -synuclein transcripts in 2mM MPP⁺-treated BE(2)M17 cells compared to the controls, with reported P-values less than 0.01. Isoforms 202 and 003 saw expression increase 3- and ~1.5-fold respectively in the treated cells. However, while there was also increase in the expression of α -synuclein isoform 201 transcripts it was not statistically significant. Total α -synuclein expression was also increased 1.5-fold and therefore the data was normalised to account for this. These results are shown in figure 30B.

Now that the total levels of α -synuclein have been normalised between treated and untreated samples the expression levels of 201 and 003 α -synuclein transcripts appear similar. Interestingly, the increase in expression of the α -synuclein 202 isoform is maintained when the total levels of α -synuclein are normalised between treated and untreated samples. This is a ~2-fold increase in expression in MPP⁺-treated cells which is statistically significant when compared to untreated cells, with a P-value >0.01.

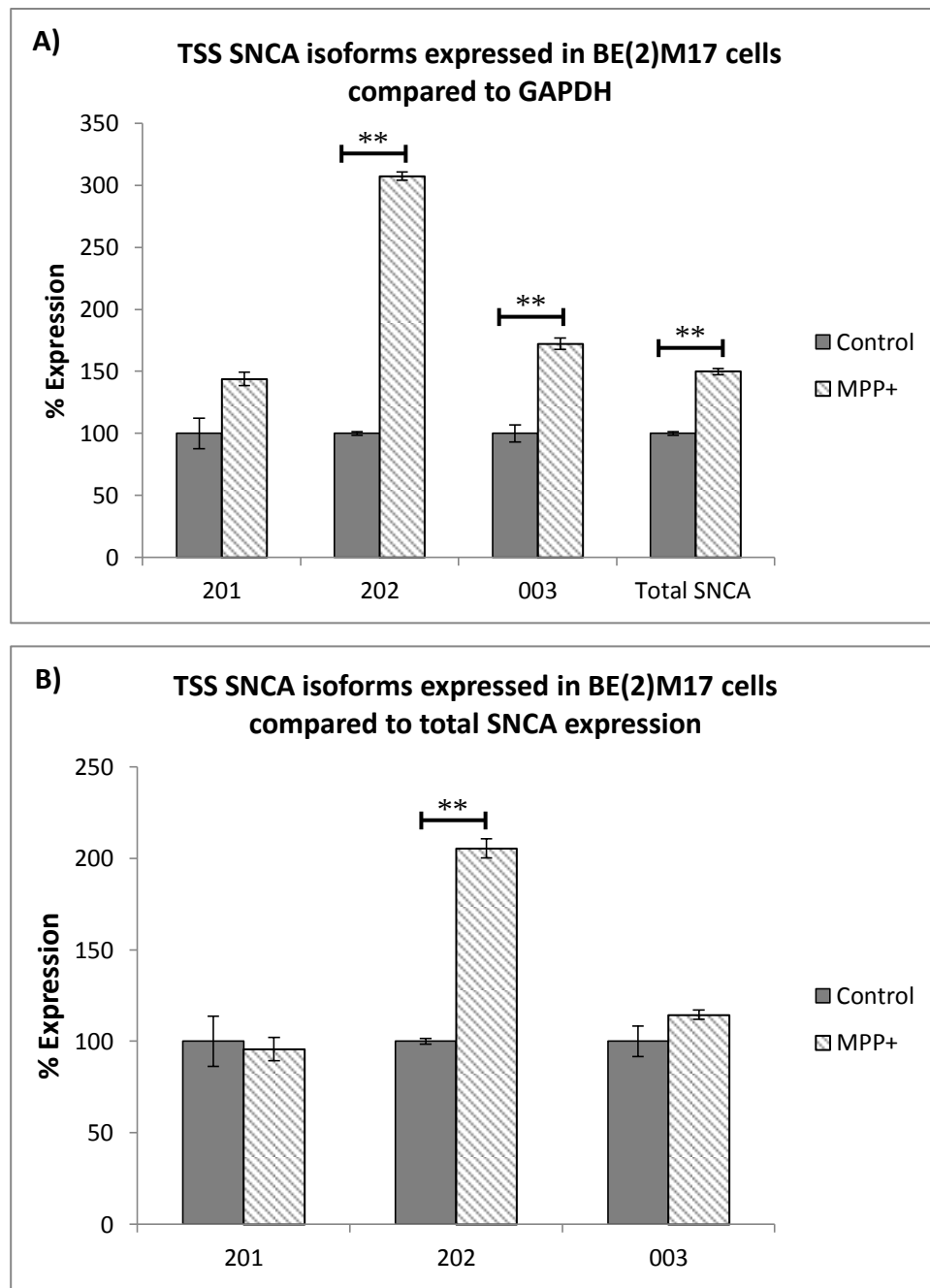


Figure 30: A) Percentage expression of α -synuclein untranslated region (5'UTR) isoforms in untreated and 2mM MPP⁺-treated BE(2)M17 cells normalised to GAPDH. B) Percentage expression of α -synuclein 5'UTR isoforms in untreated and 2mM MPP⁺-treated BE(2)M17 cells normalised to total α -synuclein expression. N=3 per sample per isoform for treated and untreated conditions. RT-PCR technical duplicates were also carried out. Statistical significance determined by a two-tailed TTEST with unequal variances assumed where **p<0.01.

3.4.7.3 Expression levels of α -synuclein 5'UTR isoforms in cingulate gyrus samples taken from a control and dementia with LB disease patient

We then decided to test a sample of cingulate gyrus from a control and DLB patient (provided by L.Parkkinen) for expression of α -synuclein 5'UTR isoforms. Due to the lack of biological replicates the samples were cut into three pieces and the RNA extracted individually. The samples were analysed using the same SYBR[®] green primers as for the BE(2)M17 cells. The results were normalised to GAPDH using the Pfaffl analysis method. The results are represented graphically in figure 31.

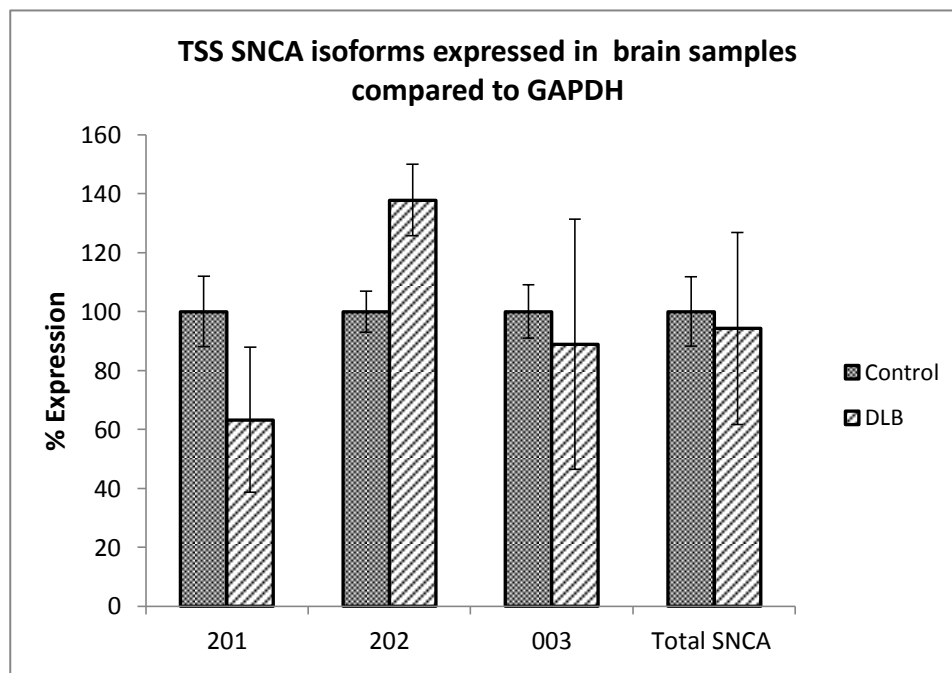


Figure 31: A) Percentage expression of α -synuclein 5' untranslated region (5'UTR) isoforms in cingulate gyrus taken from control and dementia with lewy body (DLB) patients normalised to GAPDH. 3 sections of cingulate gyrus per control and patient were used to extract RNA. RT-PCR technical duplicates were also carried out. Statistical significance determined by a two-tailed TTEST with unequal variances assumed.

The results indicate that based on the two samples tested there is no significant difference in the expression of α -synuclein 5'UTR isoforms in the cingulate gyrus between a control and DLB patient. There is visible increase in the levels of α -synuclein 202 but the p-value obtained was 0.14 and therefore not significant. Additionally there was no difference in the overall levels of α -synuclein expression.

3.4.8 Detection of α -synuclein 3'UTRs in BE(2)M17 cells

Undifferentiated and differentiated BE(2)M17 cells (differentiated according to a protocol from Richard Wade-Martin group) as well as iPSC-derived dopaminergic neurons (produced by the Richard Wade-Martins group) were analysed for the expression of different length 3'UTRs. The PCR products obtained are shown in figure 32.

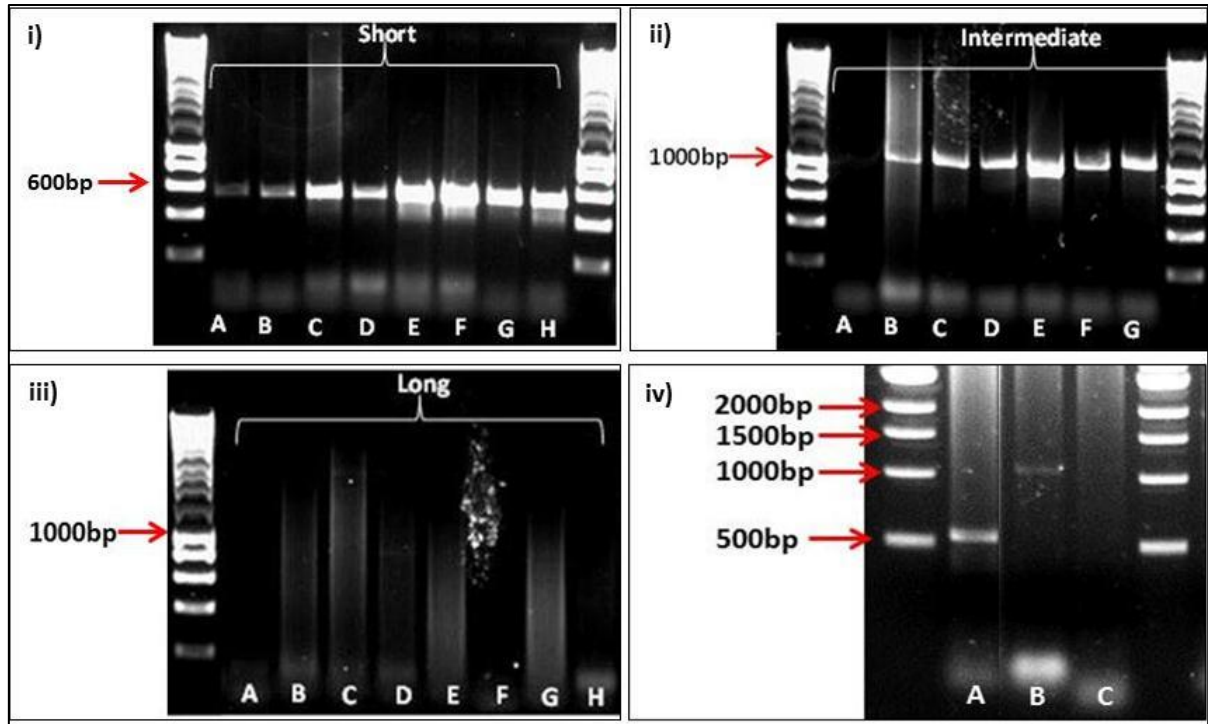


Figure 32: Amplification of the α -synuclein 3' untranslated region (3'UTR) using primers for the short (i) (548bp), intermediate (ii) (1074bp) and long (iii) (2327bp) UTR. Where A and B) undifferentiated BE(2)M17, C) iPSC-Rosetta JR36, D) iPSC-derived JR36 Neurons 1, E) iPSC-derived JR36 Neurons 3, F) iPSC-Rosetta MK24, G) iPSC-derived MK24 Neurons 1, H) iPSC-derived MK24 Neurons 2 iv) Differentiated BE(2)M17 where A) short (548bp), B) intermediate (1074bp), C) Long (2327bp).

We identified that the 1st PolyA site was not used for termination and detected the presence of the intermediate length 3'UTR (up to the 2nd PolyA site) in all of the cell types tested.

We were unable to detect any longer 3'UTRs.

3.5 Discussion

As discussed in the introduction of this thesis (section 1.3) translational regulation is governed by a host of regulatory elements, some of which are contained within the 5'UTR regions.

Prior to our work there was just a single paper on the 5'UTRs of α -synuclein; predicting the formation of an IRE, suggesting it might be regulated by cellular iron levels (Friedlich, Tanzi et al. 2007). In this chapter we identified several different 5'UTRs of α -synuclein, differing both in length and sequence, one appearing only when the cells were oxidatively stressed, and tested them for their ability to stimulate protein translation. Additionally the expression levels of the 5'UTRs within α -synuclein transcripts was determined in untreated and oxidatively stressed neuroblastoma cells using Real Time PCR. Finally, the expression levels of these 5'UTRs were compared in pieces of anterior cingulate gyrus from a control and a dementia with Lewy body patient. All cells and tissues analysed expressed the three α -synuclein 5'UTR isoforms but the levels of expression varied.

3.5.1 Detection of alternative α -synuclein 5'UTRs in BE(2)M17 cells

Investigation into the reported α -synuclein transcripts revealed that there were several different 5'UTRs expressed. These appeared to vary in length and sequence, as pictured in figure 33.

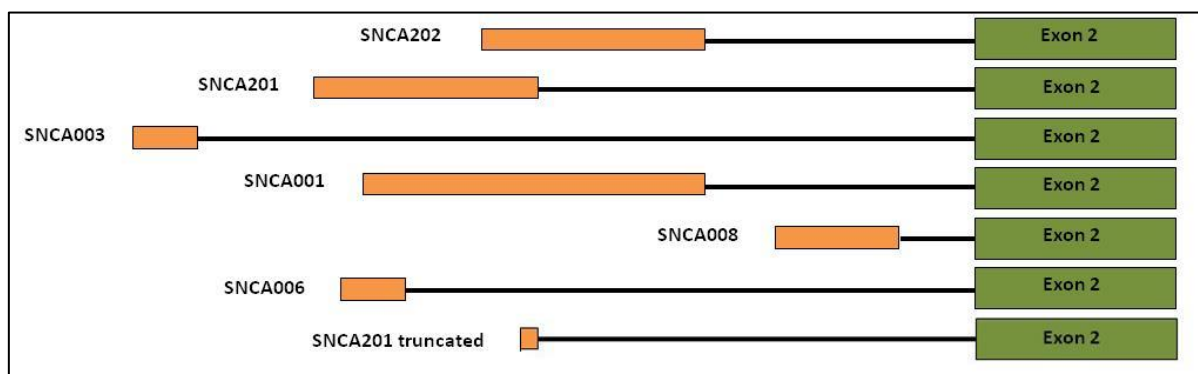


Figure 33: Schematic representation of the α -synuclein (SNCA) 5' untranslated region (5'UTR) transcripts annotated within the human genome. Orange boxes represent the 5' non-coding regions and Exon 2 indicates the start of the coding sequence.

Using cDNA obtained from BE(2)M17 cells two α -synuclein 5'UTR isoforms; 201 and 003, were successfully fully amplified. This indicated that there were alternative 5'UTR isoforms expressed and that more may be expressed under different conditions. We were also interested in the effect on translation when the 5'UTR was truncated. To assess this we designed junction primers so that only a small region of the 201 5'UTR was amplified.

3.5.2 Developing an oxidative stress assay

Since the discovery that MPTP induced parkinsonism (Lin and Beal 2006) oxidative compounds including the MPTP active metabolite MPP^+ , have been widely used to produce animal models of PD. Therefore it seemed reasonable to develop our oxidative stress assay based on this. As our cells are not co-cultured with glial cells and have no MAO- β they are unable to convert the MPTP into MPP^+ . For this reason and, due to lower safety concerns as MPP^+ cannot cross the blood brain barrier, we decided to add MPP^+ solution directly to our cells. There are several publications using MPP^+ to study the effects on α -synuclein (Kanda, Bishop et al. 2000) which used a range of concentrations. As the publications used 0.05–0.1 mM (Kanda, Bishop et al. 2000) and 2-3mM (Kalivendi, Yedlapudi et al. 2010) we decided to test 50 μM , 450 μM and 2mM solutions of MPP^+ . BE(2)M17 cells appeared quite resistant to oxidative stress, showing a viability of 77% when incubated with the highest concentration of MPP^+ (2mM) for 24 hours. Based on this, we decided to use a concentration of 2mM MPP^+ in all future experiments.

3.5.3 Detection of an additional α -synuclein 5'UTR isoform from oxidatively stressed BE(2)M17 cells

Excitingly, an additional α -synuclein 5'UTR isoform; 202 was identified in BE(2)M17 cells treated with 2mM MPP^+ . This result suggested that the 5'UTRs of α -synuclein may be differentially expressed, depending on environmental triggers and posed the question why would this be advantageous to the cell? Could it be a mechanism to try and protect

itself from oxidative damage? To begin to answer this question we looked at the effect of inserting the different 5'UTRs in front of the Renilla luciferase ORF in a dual luciferase vector.

3.5.4 The effect of different α -synuclein 5'UTRs on protein expression in a luciferase assay

As already mentioned, the levels of WT α -synuclein protein are extremely important and over-expression due to duplications and triplication of the α -synuclein loci result in aggressive early onset PD (Singleton, Farrer et al. 2003).

Luciferase assay results indicate that all the 5'UTRs reduce Renilla: Firefly luciferase luminescence ratio and therefore decrease Renilla luciferase protein expression, in both HEK293 and BE(2)M17 cells, when compared to empty PsiCHECK2.2 vector. However a significant difference between the protein expression stimulated by the different 5'UTRs was only seen in BE(2)M17 cells. Based on this it is tempting to speculate that the translational regulation observed is being influenced by factors only present in neuronal cells.

Interestingly α -synuclein isoform 202, the isoform found in MPP⁺-treated cells, stimulated the lowest levels of protein expression in the luciferase assay. As high α -synuclein levels have been shown to decrease cell survival when presented with oxidative insult (Kanda, Bishop et al. 2000; Song, Shults et al. 2004) it suggests that the cells might be trying to protect themselves from damage by initiating transcription giving rise to a UTR that does not lead to high levels of protein production.

It also appears that treatment with MPP⁺ altered the luminescence ratio significantly when transfected with empty PsiCHECK2.2. While there was a general decrease in

luminescence seen between treated and untreated cells it was not statically significant. This could indicate that MPP⁺ treatment results in an overall decrease in translation, possibly by proteolytic cleavage or phosphorylation of the EIFs (Jackson, Hellen et al. 2010). This is very interesting as the same significant decrease was not seen in the constructs containing the α -synuclein 5'UTRs. Therefore it could be postulated that the UTR's are contain regulatory elements that allow translation to occur with lower levels of EIFs. There is evidence that the expression of some stress response genes, including HSP, utilise IRES when the cell is in distress in order to ensure that translation of these proteins continues even when conventional cap-dependent translation is down-regulated (Spriggs, Bushell et al. 2010). It may be that α -synuclein is one of these necessary proteins and has been linked to chaperoning activity (Chandra, Gallardo et al. 2005). It is also worth mentioning that as the luciferase genes within the PsiCHECK2.2 vector are under the control of two different promoters (a Herpes Simplex Virus (HSV) promoter and a SV-40 promoter) it may be that the different promoters are affected by the MPP⁺-treatment unequally. This could account for the observed decrease in expression levels.

Unfortunately, luciferase assays have several caveats which cannot be ignored when interpreting the data. PsiCHECK2.2 contains an intron, between the SV-40 promoter and the Renilla luciferase ORF, which contains splice sites at either end. Generally splicing allows more efficient export and therefore translation of transcripts. The NheI site used to clone the α -synuclein 5'UTRs is located between the end of the intron and the start of the ORF and an insert there could potentially interfere with the intron splicing. If this was the case with our 5'UTRs then the decreased luminescence and assumed drop in protein expressed may be due to intron retention and not regulatory mechanisms contained in the UTRs. To investigate this, PCRs were performed amplifying the region from the HSV

promoter through to the Renilla luciferase ORF. The products were analysed against the expected bands for both intron splicing and retention. While empty PsiCHECK2.2, and PsiCHECK2.2-201 truncated and PsiCHECK-003 produced predominantly spliced products with low levels of larger un-spliced products PsiCHECK2.2-201 and -202 produced products which were the incorrect size for both intron splicing and retention. As these products were smaller than expected, it is likely that the UTRs contain additional splice sites and that sections of the UTRs were removed along with the intron. This could explain the altered translation efficiency.

The sequencing results show that the second half of the 201 5'UTR is spliced along with the intron. This indicates that this isoform contains an additional splice site. In the case of the 202 isoform it appears that the entire 5'UTR is removed in addition to the intron. In both cases this sequencing is interesting: even with a shorter or no 5'UTR translation levels are decreased. This could indicate that the remaining region in 201 contains a strong regulatory element or that the introduction of additional splice sites in some way impacts on translation of the downstream ORF.

3.5.5 Real Time-PCR results show that α -synuclein-202 expression is up-regulated in cells treated with MPP⁺

3.5.5.1 α -synuclein 5'UTR isoform expression in MPP⁺-treated cells

Based on the Pfaffl analysis, MPP⁺-treated BE(2)M17 cells express a significantly higher level of 5'UTR isoforms -202 and -003, when normalised to the house keeping gene GAPDH, compared to untreated cells. Treated cells also express an increase in the total amount of α -synuclein when normalised to GAPDH. This overall increase means that we are able to draw any conclusions on the expression of individual isoforms as it is possible that all the isoforms are increased proportionally. Therefore, to try and tease apart the

results further we normalised to the total levels of α -synuclein between treated and untreated cells. Interesting there was still a 2-fold increase in the expression of α -synuclein 202 transcripts in MPP⁺-treated cells after this normalisation, while isoforms 201 and 003 were similar in both conditions.

This indicates that expression of α -synuclein isoform 202 is specifically switched on in BE(2)M17 cells during oxidative stress. We can speculate that the cells have developed mechanisms to cope with toxic insult. One such mechanism could be to try and restore α -synuclein homeostasis by decreasing levels of translation through the expression of this 202 5'UTR. It may be this regulation of homeostasis that is impaired in PD and leads to the accumulation and aggregation of α -synuclein in LBs.

3.5.5.2 α -synuclein 5'UTR isoform expression in a brain sample from a patient with Dementia with Lewy Bodies

Synucleinopathies are a group of neurodegenerative disorders characterised by the presence of α -synuclein protein aggregates (fibrils) in specific neurons and glia (Martí, Tolosa et al. 2003). They include PD, DLB and multiple system atrophy (MSA) (Martí, Tolosa et al. 2003). The prevalence of dementia in PD has been estimated at approximately 30% and there is controversy over whether DLB should be considered a separate disease as PD and DLB cannot be pathologically separated but are two different clinical syndromes (Martí, Tolosa et al. 2003). As the factor common to all these diseases is the presence of α -synuclein aggregates there may be a central mechanism or particular isoform that predisposes aggregation.

The cingulate gyrus is located in the cortex, just above the corpus callosum. It forms part of the limbic system and is involved in emotion, learning and memory. It is an area often analysed for α -synuclein LBs in DLB patients and is thought to be affected in the late

stages PD (stages 5-6) (Baba, Nakajo et al. ; Braak, Tredici et al. 2003), contributing to impaired cognition (Dubois and Pillon 1996).

Our Real Time PCR data does not indicate any differences in the expression of α -synuclein 5'UTR isoforms between the DLB patient and our control. However, we only analysed samples from one patient and one control and variation in the samples was large. It is possible that changes in 5'UTR expression are subtle and simply require a larger sample group.

However our results also show no increase in total α -synuclein expression. As DLB is characterised by the presence of α -synuclein-rich LBs (Martí, Tolosa et al. 2003) this is unexpected. This could be explained by storage and sample preparation. There was no information available as to how the patients died or how their tissues were stored. Patients who suffered respiratory arrest and therefore reduced oxygen levels in the brain prior to death often yield RNA of a poor quality due to the degradation that occurs. Likewise, poor treatment of brain samples (i.e. repeated freeze-thaw cycles) also results in poor quality RNA. Any one, or more likely all of these factors, will have impacted on the results obtained and therefore these results should not be discarded but used as preliminary experiments in which to test further samples.

It should also be noted that PD and DLB are generally considered two different diseases with the cingulate gyrus only being affected in the very late stages of PD. Additionally the cingulate gyrus does not contain dopaminergic neurons and thus further experiments with three different PD patients, especially if the SNpc was analysed, could yield different results.

3.5.6 Potential function of α -synuclein 5'UTR isoforms

While there is little literature regarding the 5'UTRs of α -synuclein we can compare to other genes which are known to express different UTRs, such as brain-derived neurotrophic factor (BDNF). BDNF has 34 identified mRNA transcripts, with different 5'UTRs, which arise via the alternative splicing of 11 exons upstream of protein coding exon 9 (Pruunsild, Kazantseva et al. 2007). Investigations into these different 5'UTRs indicate that some of them promote cell survival and protect against cytotoxicity (Baj and Tongiorgi 2009). Interestingly a later publication also shows that the 5'UTRs may play a role in the localisation of the transcripts, determining whether they remain near the cell body or are transported to distal compartments (Baj, Leone et al. 2011). It is possible that α -synuclein transcripts could contain similar traits, especially as the 202 5'UTR isoform is up-regulated in oxidatively stressed cells, and function to improve cell viability. In the future it would be interesting to silence 202 transcripts and compare cell survival when treated with MPP^+ . Additionally, as α -synuclein has been linked to vesicle release, targeting of its transcripts to synapses is quite possible and may be achieved via sequences in different 5'UTRs.

As the translation of α -synuclein transcripts has already been shown to be regulated by iron levels (Febbraro, Giorgi et al. 2012) and one 5'UTR isoform is predicted to contain an IRE element (Friedlich, Tanzi et al. 2007) more research is needed to determine which isoforms are subject to this regulation and in which conditions are these isoforms specifically expressed.

3.5.7 No α -synuclein 3'UTR isoforms were identified in undifferentiated or differentiated BE(2)M17 cells or iPSC-derived neurons

The results indicate that only the intermediate 3'UTR, up to the 2nd polyA site, is found in proliferating undifferentiated BE(2)M17 cells. This is consistent with current literature (Sandberg, Neilson et al. 2008) as longer UTRs allow down-regulation of translation via mechanisms such as miRNA binding (as described in the previous translation regulation section). Based on this terminally differentiated cells should express longer 3'UTRs and therefore be subjected to increased translational regulation (Sandberg, Neilson et al. 2008). However, our results do not show this; both differentiated BE(2)M17 and iPSC-derived dopaminergic neurons appear to express the same intermediate 3'UTR as proliferating immature BE(2)M17 cells.

However, it is interesting that the first PolyA site does not result in termination. This indicates that despite the fact that both identified miRNA-binding sites are before the first PolyA site, it is advantageous to translate a longer UTR. Further investigation is needed to determine whether there are additional regulatory elements between the two PolyA sites and their role in translational regulation.

4 Alternative splicing of α -synuclein

4.1 Introduction

4.1.1 Alternatively spliced α -synuclein

The α -synuclein transcript comprises of 7 exons, 5 of which are protein coding. α -synuclein is one of the many genes known to undergo alternative splicing, a process by which exons in a pre-mRNA transcript are differentially spliced by the spliceosome during intron removal (Cooper, Wan et al. 2009). The advantage of alternative splicing is that many different mRNAs can be produced from a single gene, differing either in coding regions or UTRs. This allows the regulation of altered proteins either in different cell types, developmental stage or environmental changes.

Within the literature three well defined isoforms of the α -synuclein transcripts and protein are described; 140, 126 and 112. α -synuclein 140 is the full length transcript while -126 and -112 are missing exons 3 and 5 respectively (Beyer 2006). Most recently a fourth alternatively spliced transcript was identified: α -synuclein 98 which is missing both exons 3 and 5 (Beyer, Domingo-Sábat et al. 2008) but to our knowledge has not been confirmed by other research groups and are not annotated in any EST-databases. These alternatively spliced isoforms are shown in chapter 1.4 in figure 8.

The roles of α -synuclein still remain unclear but it has been linked to regulation of dopamine synthesis (Perez, Waymire et al. 2002; Peng, Tehranian et al. 2005; Gao, Li et al. 2007; Yu, Zuo et al. 2004; Tehranian, Montoya et al. 2006; Baptista, O'Farrell et al. 2003), synaptic vesicle trafficking (Nemani, Lu et al. 2010; Yavich, Tanila et al. 2004; Venda, Cragg et al. 2010; Larsen, Schmitz et al. 2006), with the capacity to act as a

chaperone (Chandra, Gallardo et al. 2005; Souza, Giasson et al. 2000; Ostrerova, Petrucelli et al. 1999), and regulate the up-take of dopamine via DAT (Fountaine, Venda et al. 2008). Unfortunately the effect of alternatively spliced α -synuclein protein isoforms on the known roles of α -synuclein within neurons has not been well characterised. Investigations into the function and localisation of these isoforms have been hindered by a lack of molecular tools able to distinguish between them with the required accuracy and specificity. To our knowledge there are no isoform-specific antibodies commercially available for use in either immunofluorescence or western blotting making quantification of protein levels and localisation studies difficult.

In this chapter the three major alternatively spliced isoforms of α -synuclein have been amplified from oxidatively stressed cells and their expression levels quantified via Real Time PCR. In order to help develop isoform-specific antibodies the isoforms were cloned into a peGFP reporter vector and transfected so that cell lysates could be used to test novel antibodies (a kind gift to Olaf Ansorge's group from Katrin Beyer).

4.2 Aims

With the exception of one research group, the literature available appears to agree that isoforms 140, 126 and 112 can be detected in a variety of different cell lines and in PD brains (Kalivendi, Yedlapudi et al. 2010). Therefore our primary aim was to develop Real Time PCR assays for analysis of alternative splicing in undifferentiated and oxidatively stressed BE(2)M17 cells and post mortem tissues. To this end we used the published method of MPP⁺ treatment to cause oxidative stress in the BE(2)M17 cells. The levels of isoforms expressed have also been analysed in cingulate gyrus samples taken from a control and dementia with LB disease patient.

Finally, we cloned the CDS of the major splice isoforms into a peGFP plasmid and used them to test novel antibodies synthesised to be specific for the alternatively spliced isoforms 112 and 126. These were a gift from Katrin Beyer and will be tested on isoform-transfected cell lysates by western blotting. The development of isoform specific antibodies would provide an invaluable tool for analysing the alternatively spliced α -synuclein isoforms, enabling studies such as localisation, prevalence in PD patient brains and quantification of protein expression.

4.3 Results

4.3.1 Detection in BE(2)M17 cells

BE(2)M17 cells were treated with 2mM MPP⁺. Using the RNA extracted from the treated and untreated cells we tested for the presence alternatively spliced α -synuclein isoforms; 140, 126, 112 and 98 by PCR. The results from this PCR are shown in figure 34.

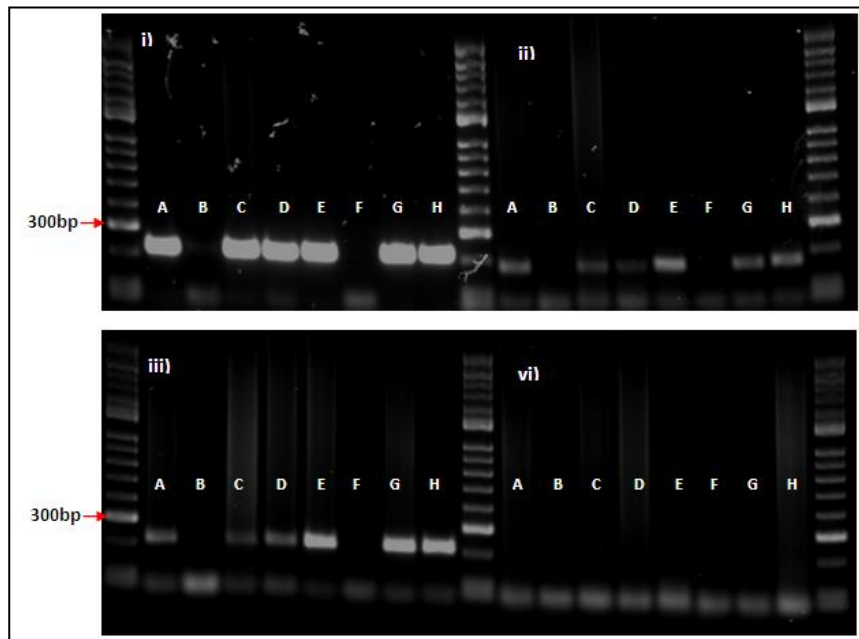


Figure 34: Amplification of α -synuclein alternatively spliced isoforms from OptiMEM controls and MPP⁺-treated cells. i) α -synuclein-140, ii) α -synuclein-126, iii) α -synuclein-112 and iv) α -synuclein-98. Where A-D= OptiMEM controls (B=negative RT), E-H=2mM MPP⁺ (F=negative RT).

It is evident that both the controls and 2mM MPP⁺-treated cells express SNCA140, 126 and 112. However it appears that the 2mM-treated cells express a higher level of α -synuclein-112, when compared to the controls. It also appears that there is a slight increase in expression of α -synuclein-126 isoform in 2mM MPP⁺-treated cells compared to the controls. The shortest α -synuclein isoform (α -synuclein-98) was not detected in any samples.

4.3.2 Quantification of α -synuclein alternatively spliced isoforms in BE(2)M17 cells

As RT-PCRs cannot be accurately quantified we performed Real Time PCR on untreated and 2mM MPP⁺-treated BE(2)M17 cells using custom made TaqMan assays. Like SYBR[®] Green, TaqMan assays contain a pair of primers that bind to the target transcript allowing its amplification but they also contain a labelled probe which also binds to the target transcript, over an exon boundary to avoid detection any contaminating genomic DNA. As the polymerase moves along the target sequence the probe is displaced, resulting in a signal. The amount of signal is proportional to the amount of amplification, which is dependent on the concentration of transcript present. The TaqMan assays were analysed for their efficiencies using the same method as for the SYBR[®] Green primers (see chapter 4). Results were analysed using the Pfaffl method, taking these efficiencies into account.

The location of the primers and probe binding sites for each alternatively spliced isoform and total α -synuclein transcripts are shown in figure 35. The probe for the alternatively spliced isoforms 126 and 112 are located over the boundaries for exons 2/4 and 4/6 respectively so that only amplification of that specific splice isoform results in signal generation.

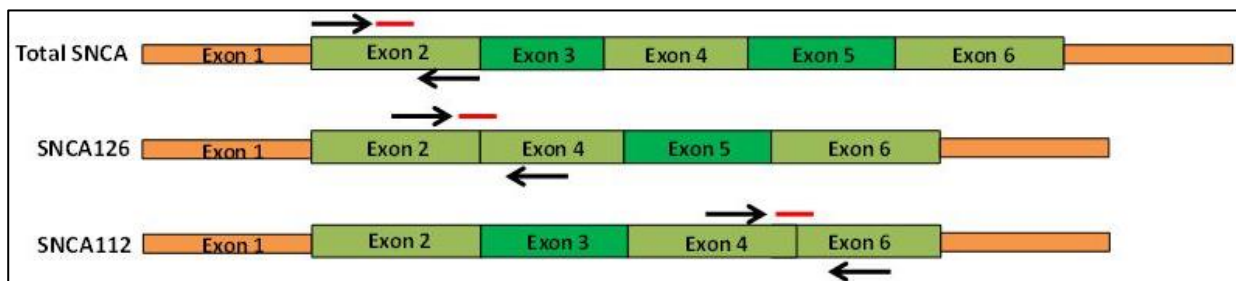


Figure 35: Location of the primer (black arrows) and probe (red lines) binding sites to the alternatively spliced α -synuclein (SNCA) isoforms 126 and 112 and total SNCA. Orange boxes represent untranslated regions.

These assays were tested for their efficiencies using the standard curve method. Expression levels were initially normalised to the house-keeping gene GAPDH the results interpreted using the Pfaffl method. The results collected are represented in figure 36A.

There was a statistically significant increase in the expression of α -synuclein splice isoform 112 as well as the expression of total α -synuclein transcripts in 2mM MPP⁺-treated BE(2)M17 cells compared to the controls, with reported P-values <0.01 and 0.05 respectively. Isoform 112 showed an expression increase of ~1.5-fold in the treated cells. However, while there was also increase in the expression of α -synuclein isoform 126 transcripts, it was not statistically significant. As total α -synuclein transcript expression was also increased over 2-fold and we decided to also normalise the data to account for this overall increase. These results are shown in figure 36B.

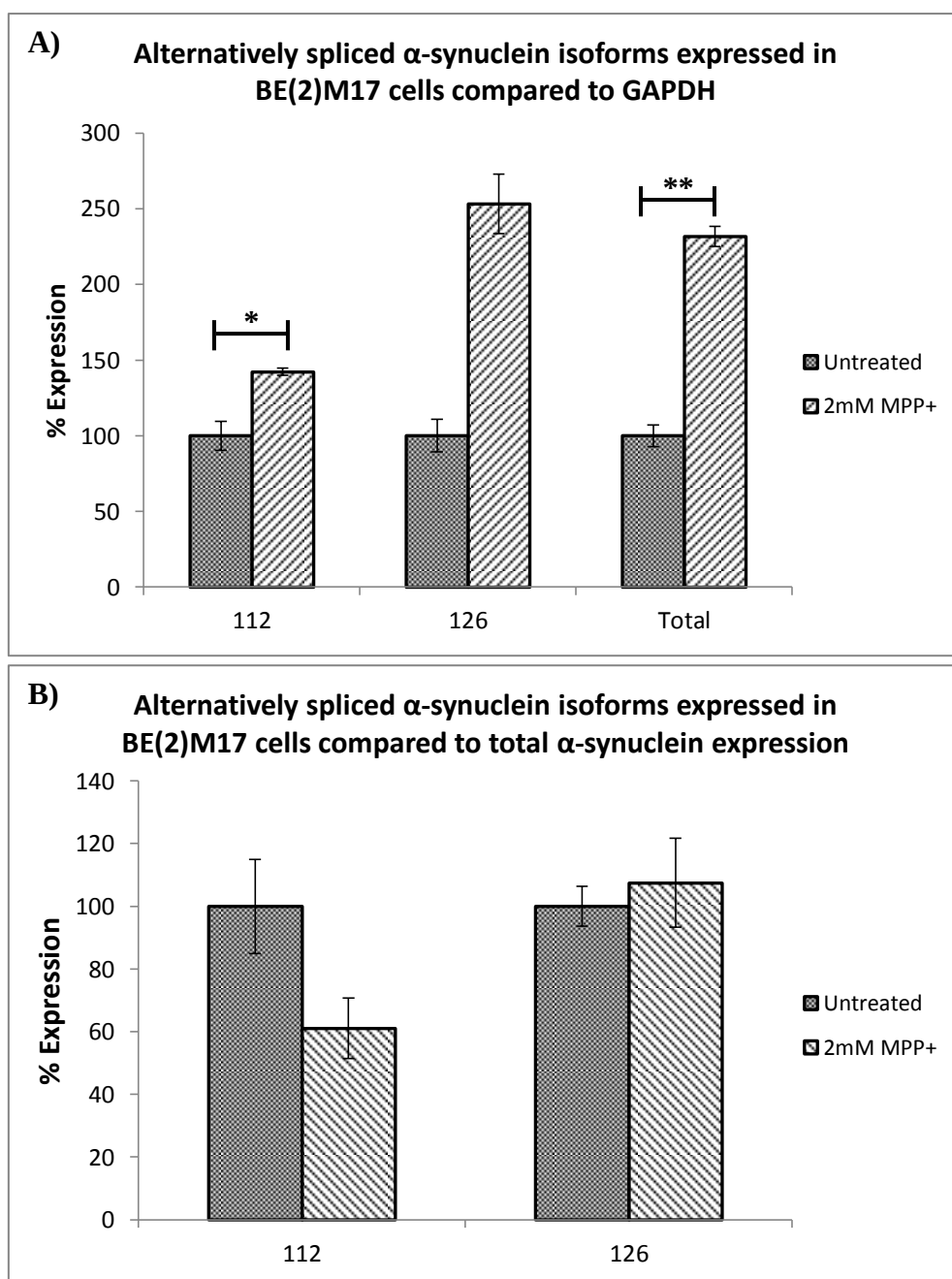


Figure 36: A) Percentage expression of α -synuclein alternatively spliced isoforms in untreated and 2mM MPP⁺-treated BE(2)M17 cells normalised to GAPDH. B) Percentage expression of alternatively spliced α -synuclein isoforms in untreated and 2mM MPP⁺-treated BE(2)M17 cells normalised to total α -synuclein expression. N=3 per sample per isoform for treated and untreated conditions. RT-PCR technical duplicates were also carried out. Statistical significance determined by a two-tailed TTEST with unequal variances assumed where **p<0.01 *p<0.05.

Normalisation of total levels of α -synuclein between treated and untreated samples results in similar expression levels of 112 and 126 α -synuclein transcripts, with insignificant P-values recorded.

4.3.3 Quantification of α -synuclein alternatively spliced isoforms in cingulate gyrus samples taken from a control and dementia with LB disease patient

Using the same Taqman assays described for the BE(2)M17 cells expression levels of three alternatively spliced α -synuclein isoforms were determined by Real Time PCR. Expression levels were initially normalised to the house-keeping gene GAPDH the results analysed using the Pfaffl method. The results collected are represented in figure 37.A.

There was a statistically significant increase in the expression of α -synuclein splice isoform 112 as well as the expression of total α -synuclein transcripts in cingulate gyrus taken from a DLB patient compared to the control, with reported P-values <0.05. There was also an increase in isoform 126 expression but this was not statistically significant. Isoform 112 showed an expression increase ~2.5-fold in DLB brain tissue and total α -synuclein transcript expression was also increased over 3-fold. Based on this we decided to also normalise the data to account for this overall increase. These results are shown in figure 37.B.

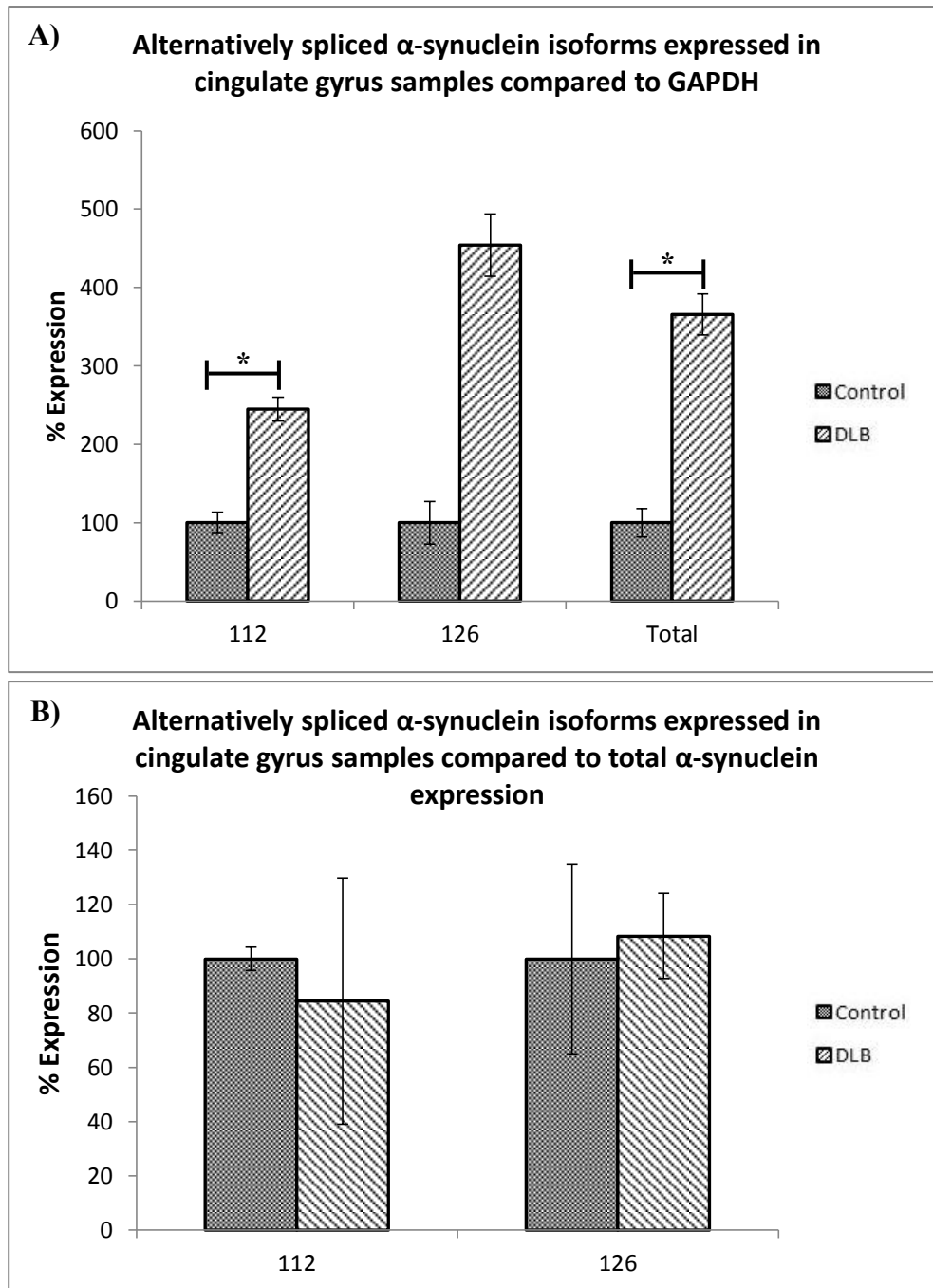


Figure 37: A) Percentage expression of α -synuclein alternatively isoforms in cingulate gyrus taken from control and dementia with lewy body (DLB) patients normalised to GAPDH. B) Percentage expression of alternatively spliced α -synuclein isoforms cingulate gyrus taken from control and dementia with lewy body (DLB) patients normalised to total α -synuclein expression. 3 sections of cingulate gyrus per control and patient were used to extract RNA. RT-PCR technical duplicates were also carried out. Statistical significance determined by a two-tailed TTEST with unequal variances assumed where * $p < 0.05$.

Now that the total levels of α -synuclein have been normalised between DLB and control brain samples the expression levels of 112 and 126 α -synuclein transcripts appear similar, with insignificant p-values recorded.

4.3.4 Cloning of alternatively spliced α -synuclein

The junction primers used to amplify the different isoforms from BE(2)M17 cells initially (shown in figure 36) and either an exon 2 or exon 6 primer were initially used to clone the majority of the coding sequence. Linking primers were then used to reconstruct the entire CDS with SalI and SacII restriction sites. The various PCR products are shown in figure 38.

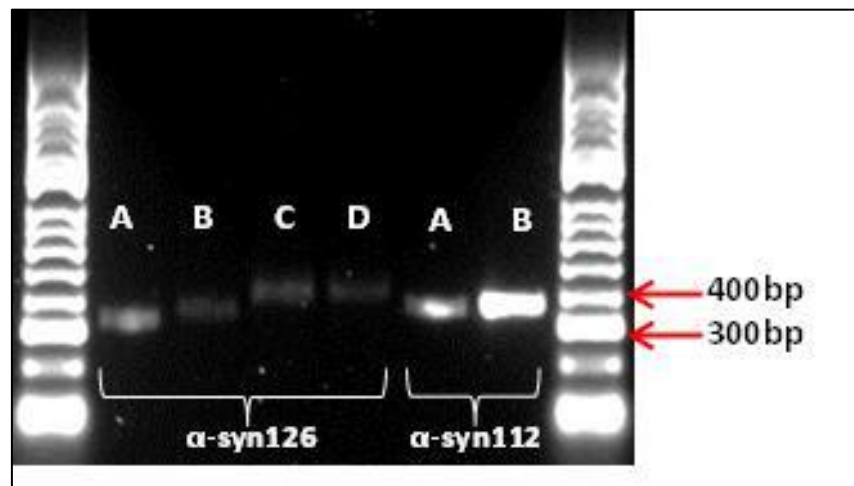


Figure 38: The stepwise PCR reaction products for amplifying the entire coding region of the α -synuclein isoforms 126 (reactions A-D) and 112 (reactions A-B).

The isoform coding sequences were ligated into pGEM-T-easy and sequenced to confirm the CDS was correct. Any mutations were corrected using site-directed mutagenesis. The α -synuclein isoforms were digested from pGEM-T-easy with SalI/SacII and ligated into pEGFP-N1, following the removal of α -synuclein 140 with XhoI and SacII (XhoI and SalI produce compatible overhangs). The digestions of the pGEM-T-easy and pEGFP-N1 constructs are shown in figure 39.

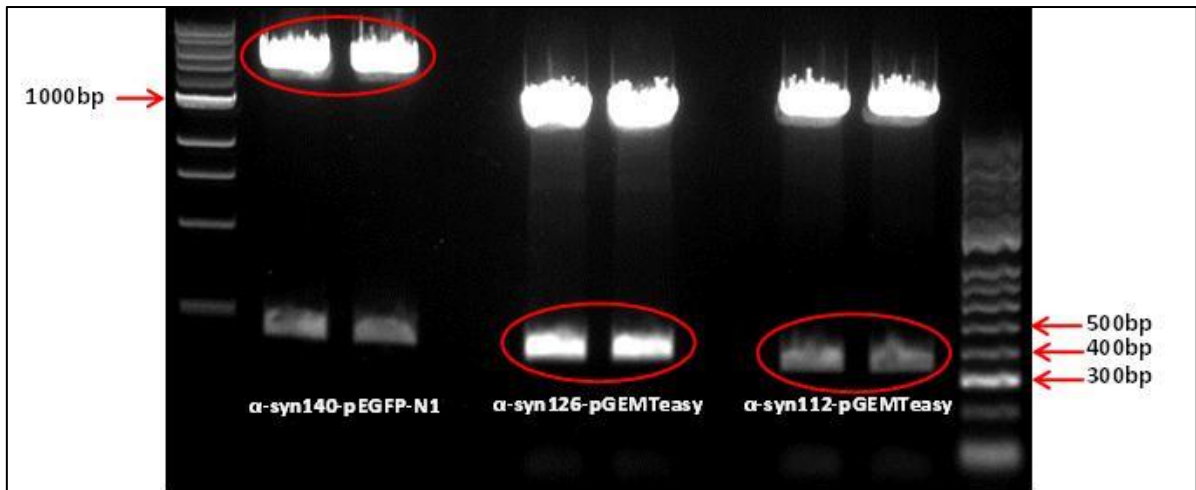


Figure 39: Digestion of the α -synuclein 140-pEGFP-N1 and α -synuclein 126/112-pGEMT-easy vectors with XhoI/SacII and Sall/SacII restriction enzymes respectively. Expected products; N1=4697bp, 126=387bp and 112=345bp.

The peGFP- α -synuclein isoform vectors were transfected into BE(2)M17 cells using Lipofectamine 2000. The GFP-tag allowed the detection of transfected cells by microscopy and similar transfection efficiencies for each construct were observed, see figure 40. Following imaging the cells were lysed, the protein extracted and analysed for total concentration.

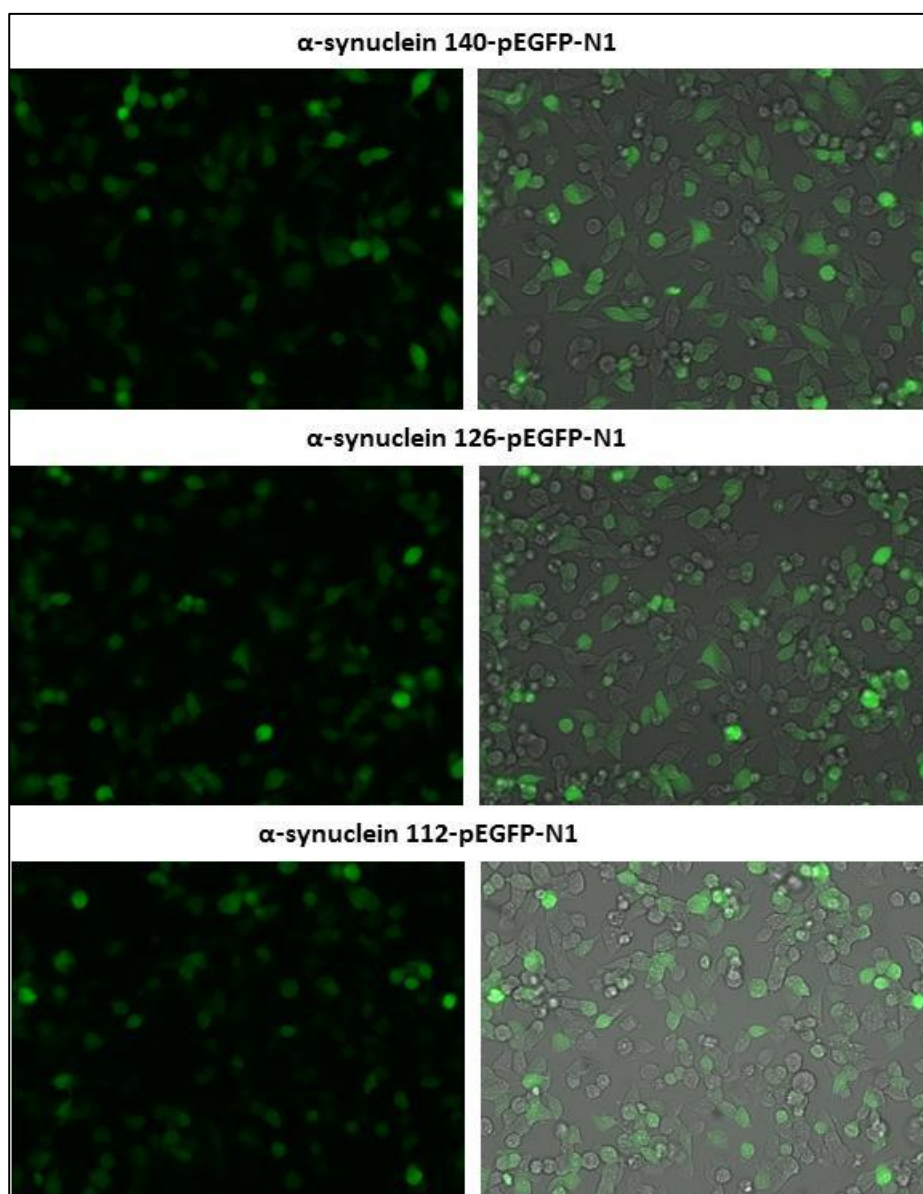


Figure 40: Representative images captured of BE(2)M17 cells transfected with the α -synuclein splice isoform-pEGFP-N1 plasmid constructs; 140, 126 and 112.

4.3.5 Testing the specificity of α -synuclein isoform specific antibodies

The protein lysates extracted from the α -synuclein transfection experiment were run on 4-12% Bis-Tris gels and blotted. The BD Biosciences anti- α -synuclein antibody, raised against amino acids 15-123 in rats, recognizes human α -synuclein 91-99aa (Perrin, Payton et al. 2003) and detects all three isoforms and is shown in figure 41i. The isoform specific antibodies were also tested against all three transfection lysates, 126 and 112 antibody membranes are shown in figures 41ii and 41iii respectively.

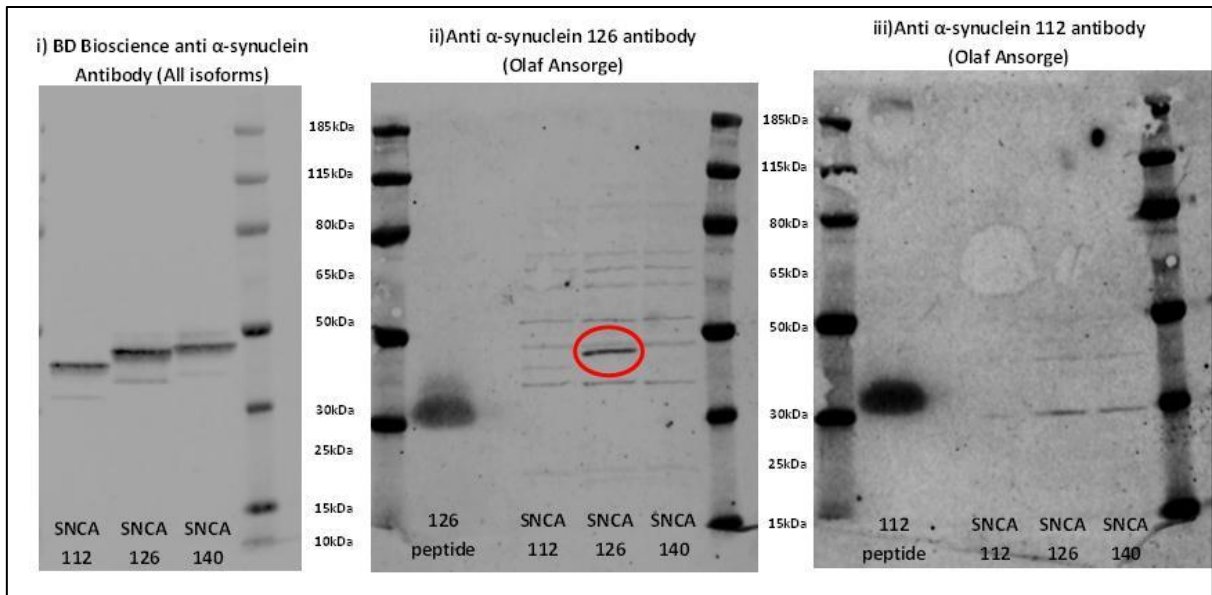


Figure 41; Western blot membranes containing BE(2)M17-transfected with α -synuclein splice isoform constructs cell lysates stained with i) BD Bioscience anti α -synuclein, ii) anti α -synuclein 126 and iii) anti 112 α -synuclein (both from Olaf Ansorge's laboratory). GFP-tagged α -synuclein has an expected molecular weight of 40-48kDa. Primary antibodies used at 1:1000. The 112 antibody was also tested at 1:500 but the membrane was similar to previous results and has been omitted.

As expected the BD Bioscience antibody detected all three GFP-tagged α -synuclein isoforms, with 140 the largest and 112 the smallest as the exon splicing results in smaller proteins. The 126 antibody appears to detect primarily the 126 α -synuclein isoform (circled in red on figure 41ii) with some faint non-specific background bands visible in all the transfected lysates. At a concentration of 1:1000 and 1:500 the 112 antibody did not detect any of the α -synuclein isoforms. As both membranes were similar only the one stained using 1:1000 primary antibody has been included.

4.4 Discussion

4.4.1 Detection of three alternatively spliced α -synuclein transcripts in BE(2)M17 cells

Of the four identified α -synuclein splice isoforms 140, 126 and 112 were detected in untreated and 2mM MPP⁺-treated BE(2)M17 cells using RT-PCR. The development of the MPP⁺ assay was fully discussed in chapter 8. These preliminary results indicate that while both treated and untreated cells expressed the isoforms differences in their expression levels could be detected. While RT-PCR is not an accurate measure of transcript expression, levels of 140 and 126 levels appeared similar between treated and untreated cells. However, the RT-PCR result shows an increase in the expression of α -synuclein-112 in cells treated with 2mM MPP⁺. This is consistent with current literature (Kalivendi, Yedlapudi et al. 2010).

4.4.2 Quantification of α -synuclein alternatively spliced isoforms reveals no isoform-specific increase in transcript expression in oxidatively stressed BE(2)M17 cells

Based on Pfaffl analysis 2mM MPP⁺-treated BE(2)M17 cells express increased levels of α -synuclein-112 transcript compared to untreated controls when normalised to the house-keeping gene GAPDH. This is consistent with some of the literature which showed a similar increase in MPP⁺-treated SHSY-5Y cells (a different human neuroblastoma cell line) (Kalivendi, Yedlapudi et al. 2010). There was also increased expression of α -synuclein-126 transcripts but this was not statistically significant.

It is interesting to speculate why skipping of exon 5 increases in MPP⁺-treated cells. Based on the literature, lack of exon 5 would potentially result in a shortening of the C-terminus and aggregation is expected to be increased (Beyer, Lao et al. 2004; Beyer 2006; Beyer, Domingo-Sábat et al. 2008). Interestingly the sites for phosphorylation, ubiquitination,

nitration and truncation are located in exons 3 and 5 (Beyer 2006; Oueslati, Fournier et al. 2010), so alternative splicing may limit the level of post transcriptional modifications and therefore physiological function (McLean, Hallett et al. 2012). The mRNA transcripts encoding these alternatively spliced isoforms have already been shown to be differentially expressed in human α -synucleinopathies including PD and DLB (Neystat, Lynch et al. 1999; Beyer, Humbert et al. 2006).

In the small number of studies performed α -synuclein 112 was shown to inhibit proteasome function, specifically the trypsin-like proteasome activity of 20S (Kalivendi, Yedlapudi et al. 2010). This inhibition would reduce the cellular capacity for degrading potentially harmful proteins such as α -synuclein, leading to the accumulation of larger toxic aggregates. Therefore it is likely that the increase in 112 transcript expression is not a cellular response to try and protect itself from damage but a direct result of the oxidative stress induced by MPP⁺ treatment. Therefore it is reasonable to propose that dopaminergic neurons, which are at increased risk of being exposed to ROS, would express higher levels of α -synuclein 112 as a result. Therefore, if the mechanism of isoform splice switching could be identified it could be a potential target for therapeutic intervention in PD, providing the majority of dopaminergic neurons in the SNpc remained.

However, expression of total α -synuclein transcripts were over 2-fold higher in MPP⁺-treated cells compared to untreated cells. Based on this we decided to also normalise the data to account for this overall increase. We believe that normalising to total α -synuclein is necessary to confirm whether any isoform transcript increases observed reflect a specific increase or just up-regulation of all isoforms. Now that the total levels of α -synuclein have been normalised between treated and untreated samples the expression levels of 112 and 126 α -synuclein transcripts appear similar, indicating that all the isoforms were up-

regulated in a non-specific manner. This contradicts the data previously shown in SHSY-5Y cells (Kalivendi, Yedlapudi et al. 2010) but we believe that this is due to the fact that the authors did not normalise for total levels of α -synuclein transcripts. It is also equally possible that the increase in 112 isoform expression is responsible for the overall increase in α -synuclein levels.

It also contradicts the initial PCR result where we observed a large increase in expression of 112 while expression of isoforms 140 and 126 appeared similar. However, like Kalivendi et al., we did not include a primer pair to amplify all α -synuclein transcripts and this could account for the discrepancy. One could argue that there was no increase in the more predominant full-length 140 expression so there would not have been an increase of total α -synuclein but it is possible that as there was a strong signal on the agarose gel the reaction was saturated, i.e. there were not enough reagents left regardless of the cDNA levels present.

4.4.3 Quantification of α -synuclein alternatively spliced isoforms in cingulate gyrus samples show no isoform-specific up-regulation of transcript expression in a DLB patient compared to a control

The current literature contains mixed reports carried out in different post-mortem brain regions. Some groups show up-regulation of specific isoforms; 140 and 126 isoforms in the SNpc of PD patients compared to controls (McLean, Hallett et al. 2012), and others observing a down-regulation of 112 and 140 in the SNpc of PD patients (Neystat, Lynch et al. 1999). In DLB patients there was a significant reported decrease in the levels of 126 expressed and an increase in expression of α -synuclein 112 transcripts (Beyer, Humbert et al. 2006). However the methodologies within these publications vary significantly and could account for the discrepancies (McLean, Hallett et al. 2012).

Our results indicate an increase in the expression of α -synuclein 112 transcripts when normalised to GAPDH in the cingulate gyrus of a DLB patient compared to that of a control. However, total α -synuclein transcript expression was also increased over 3-fold, as you would expect in a synucleinopathy, and so the data must be normalised to account for this. Normalisation of the total levels of α -synuclein between DLB and control brains results in similar expression levels of 112 and 126 α -synuclein transcripts. We believe that this normalisation may account for the conflicting results as many of the publications mentioned fail to take this into account (Neystat, Lynch et al. 1999; Beyer, Humbert et al. 2006; McLean, Hallett et al. 2012).

There was a discrepancy in the levels of total α -synuclein between the DLB patient and the control. When analysed using SYBR[®] Green primers (as described in chapter 9) there was no increase in total α -synuclein transcript expression compared to the control. However, when the same samples were analysed using TaqMan assays there was over 3-fold increase. This could be due to the sensitivity of the assay, as SYBR[®] Green binds to all double-stranded DNA while TaqMan assays only detect DNA where the probe is bound. Additionally it could be due to the cDNA synthesis: for the SYBR[®] Green assays we used SuperScript II (Invitrogen) with Oligo(dT) while for the TaqMan assays we used High capacity cDNA reverse transcription kit (Invitrogen) with the supplied random primers. If the RNA had been degraded the random primers are able to 'bridge' the degradation better than the Oligo(dT)s, resulting in more usable template RNA.

4.4.4 Novel α -synuclein antibodies detect -126 isoform with good specificity but there is no detection of -112

The novel anti- α -synuclein-126 antibody appears to detect primarily the 126 α -synuclein isoform with some faint non-specific background bands visible in all the transfected lysates. This level of background 'noise' is acceptable for use in western blotting as you

can clearly discriminate bands based on their size in comparison to a protein ladder. However, using this protein for immunofluorescence would be more difficult.

At a concentration of 1:1000 and 1:500 the novel anti- α -synuclein-112 antibody did not detect any of the α -synuclein isoforms.

The development of such antibodies is vital if the alternatively spliced isoforms of α -synuclein are to be fully characterised in terms of their localisation and function. Although we made good progress in confirming the specificity of the novel α -synuclein-126 antibody, the background staining of non- α synuclein proteins is not ideal for applications such as immunofluorescence. Clearly, further antibody development is required in the future.

5 Modelling PD

5.1 Introduction

As described in section 1.5 of the introduction relevant models of human neurons, both of normal and diseased physiology, are difficult to develop. While there has been significant progress with reprogramming utilising viruses these protocols are unlikely to yield anything clinically useful. Therefore the focus has shifted to developing methodologies that use different molecular tools.

In this chapter we start to a protocol using modified mRNAs to differentiate iPSCs into dopaminergic neurons. We create the vectors needed to produce the modified mRNAs encoding several dopaminergic TFs and start optimising iPSC substrate and medium conditions.

5.2 Aims

We aim to develop a method of differentiating of iPSCs into dopaminergic neurons. This technology provides the possibility of developing PD patient dopaminergic neurons allowing a closer analysis of the pathways involved in PD pathophysiology. There have been many protocols detailing which combination of growth factors are required to produce the highest yield of dopaminergic neurons but these are often time-consuming, expensive and ultimately non-reproducible in different hands. These protocols also produce a mixed population of neurons and immature neuronal precursors. To try and overcome some of these issues we decided to investigate differentiation of iPSCs into dopaminergic neurons using modified mRNAs. Warren et al. had previously shown a simple differentiation of their iPSCs into myogenic fibres using modified mRNA encoding MyoD (Warren, Manos et al. 2010). We want to replicate this using modified mRNAs encoding TFs involved in dopaminergic neuronal development. First we must select and clone the ORF of the TFs. UTRs will then needed to be ligated to the ORF before they can be used for *in vitro* transcription to create the modified mRNAs. Additionally we need to start optimising the conditions for seeding and transfecting iPSCs; as those required to maintain iPSCs and to differentiate them into dopaminergic neurons are significantly different.

5.3 Results

5.3.1 Selection and amplification of TFs

Based on the recent literature we chose a combination of TFs involved in dopaminergic development. Mash1, Nurr1 and Lmx1a over-expression had previously been shown sufficient to convert mouse and human fibroblasts directly into mature functional dopaminergic neurons (Caiazzo, Dell'Anno et al. 2011). While PitX3 and Nurr1 are required for maintenance of post-mitotic dopaminergic neurons (van den Munckhof, Luk et al. 2003; Wallen and Perlmann 2003). There is also evidence that FoxA2 and Lmx1a and Nurr1 and PitX3 work in a synergistic manner (Martinat, Bacci et al. 2006; Lee, Bae et al. 2010).

As low O₂ conditions are reported to increase the efficiency of differentiation (Caiazzo, Dell'Anno et al. 2011) we will try and recapitulate this by creating a modified mRNA encoding mutated Hypoxia-inducible factor 1 α (Hif1 α).

5.3.2 Cloning of the TF ORFs

The ORFs of hPitX3, hLmx1a, hNurr1, hMash1, rat FoxA2 and hHif1 α -triple mutant were amplified by PCR, gel purified and cloned into pGEM-T-easy. Gel images of the amplified TFs are shown in figure 42.

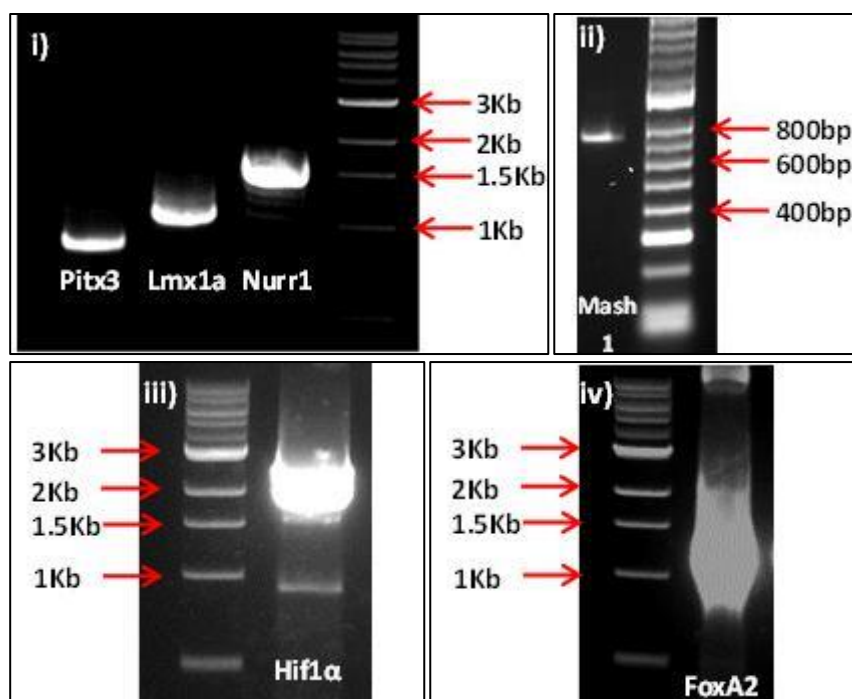


Figure 42: PCR amplification of the open reading frames for transcription factors PitX3 (906bp), Lmx1a (1146bp) and Nurr1 (1794bp) (i), Mash1 (730bp) (ii), Hif1 α (2478bp) (iii) and FoxA2 (1377bp) (iv). PitX3; Pituitary homeobox 3, Lmx1a; LIM homeobox transcription factor 1 alpha, Nurr1; Nuclear receptor related 1 protein, Mash1; Achaete-scute homolog 1, Hif1 α ; Hypoxia-inducible factor 1 α and FoxA2; Forkhead box protein A2 .

The constructs were sent for sequencing to confirm that there were no mutations affecting the amino acid sequences.

5.3.3 Ligation of the UTRs to the TF ORFs

To carry out *in vitro* transcription to obtain modified mRNAs a 5'UTR containing a strong Kozak translational initiation signal and an alpha-globin 3' UTR terminating with an oligo(dT) sequence for addition of a polyA tail were required (Warren, Manos et al. 2010). The 5' and 3'UTRs were ligated using 'splint oligos' which was complementary to both the end of the ORF and beginning of the UTRs. This is shown schematically in figure 43.



Figure 43: Ligation of splint oligos and untranslated regions (UTRs) to transcription factor open reading frames (ORF).

Once sequencing confirmed the present of the mutation-free ORFs the UTRs were ligated and the ligation product amplified by PCR. The PCR products were gel purified and run on a gel to confirm the correct size, shown in figure 44.

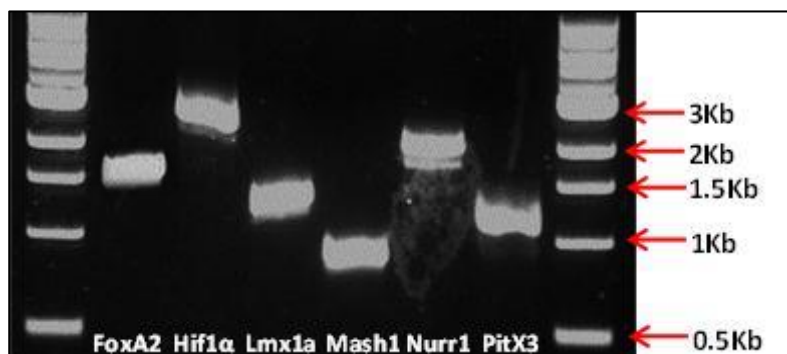


Figure 44: PCR amplification of transcription factor open reading frames with untranslated regions; FoxA2 (1609bp), Hif1 α (2710bp), Lmx1a (1378bp), Mash1 (962bp), Nurr1 (2026bp) and PitX3 (1138bp). PitX3; Pituitary homeobox 3, Lmx1a; LIM homeobox transcription factor 1 alpha, Nurr1; Nuclear receptor related 1 protein , Mash1; Achaete-scute homolog 1, Hif1 α ; Hypoxia-inducible factor 1 α and FoxA2; Forkhead box protein A2 .

The TF ORFs complete with UTRs were cloned into pGEM-T-easy and sequenced to confirm that there were no mutation in either the ORF or the UTRs that might affect translation. Ligation of UTRs and amplification of the ligated product was achieved for TFs Mash1, Lmx1a, Nurr1, Hif1 α and FoxA2. However sequencing of FoxA2+UTRs revealed 3 point mutations within the ORF that impacted on the protein sequence. One of these was corrected using site-directed mutagenesis but we were unable to correct the remaining two despite repeating it several times, using new Pfu enzyme, and reordering primers. Unfortunately we were also unable to ligate the UTRs to the ORF of PitX3. The PCR product obtained using the method for the other TFs resulted in presence of the UTRs but no ORF. To overcome this problem we tried direct PCR of the PitX3 ORF using the splint sequences and then ligating the UTRs but had no success. We also tried a slower cooling time during the ligation step.

5.3.4 Seeding iPSCs onto different substrates

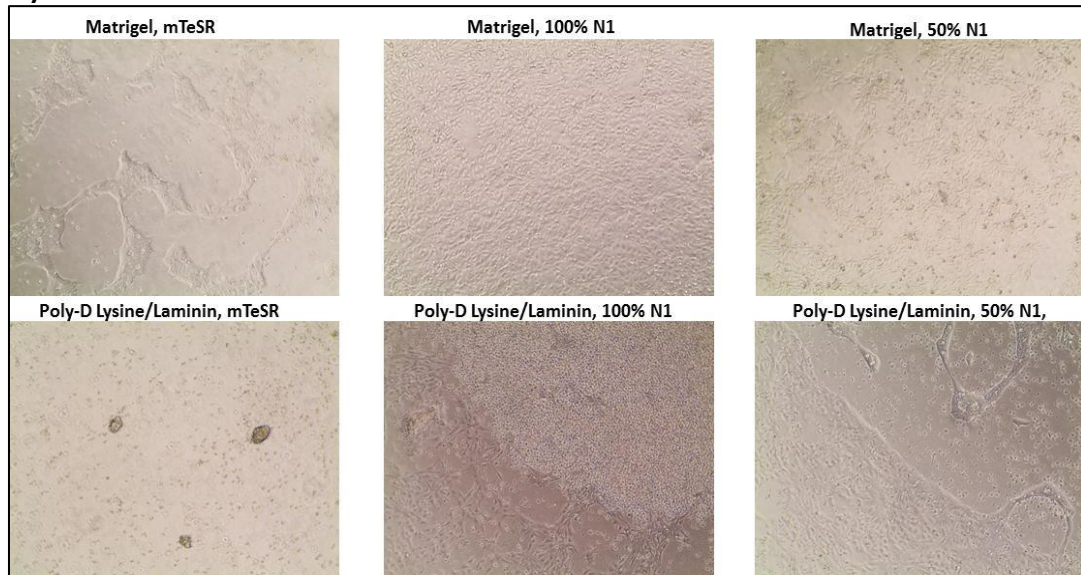
iPSCs were seeded either onto Matrigel or Poly-D-Lysine/laminin and incubated in mTeSRTM1 medium overnight at 37°C. The following day the medium was changed to either fresh mTeSRTM1, neural induction medium (N1) or a 50:50 mixture of the two. After 24 hours the cells were imaged by light microscopy. Representative images are shown in figure 45.A. The images captured indicate that the iPSCs grow in the normal ‘honeycomb’ formation on Matrigel and in mTeSRTM1 medium (figure 45A, top left panel), which is their normal proliferation condition. By day 1 the cells in 50:50 medium on Matrigel and in mTeSRTM1 medium on Poly-D-Lysine/Laminin form a monolayer but are looking rounded and starting to aggregate (figure 45A top right and bottom right respectively). Interestingly, the wells containing Poly-D-lysine/Laminin (figure 45A bottom row) appear to contain two populations with an inner and outer ring but both have lost the morphology of iPSC cells. Cells seeded onto Poly-D-Lysine/Laminin in mTeSRTM1 medium were already dead and were discarded.

Following two daily transfections with modified mRNA encoding GFP the iPSCs were imaged again by light microscopy. These images are shown in figure 45.B). Light microscopy images show that those transfected on Matrigel in mTeSRTM1 medium appear more as a monolayer than their untransfected counterparts (figure 45B left panels of top and middle rows respectively). iPSCs on Matrigel in 50:50 mTeSRTM1:N1 medium appear to stay in a monolayer with areas of dense cells regions visible in both transfected and untransfected wells (figure 45B middle panels of top and middle rows). The cells on Poly-D-Lysine/Laminin in N1 medium (figure 45B left panel of middle row and middle panel of the bottom row) showed a decrease in number and some rounding of cells was visible but the majority appeared alive. However, those cells plated on Poly-D-Lysine/Laminin in

50:50 medium still displayed the two cell populations observed in the initial images but in both transfected and untransfected well in the centre there are apoptotic-like inductions (panels left and right on the bottom row in figure 45B).

The transfected iPSCs were then fixed and stained using anti-Nestin and anti- β III tubulin immunofluorescent antibodies. Representative figures are shown in figures 46 and 47 respectively.

A)



B)

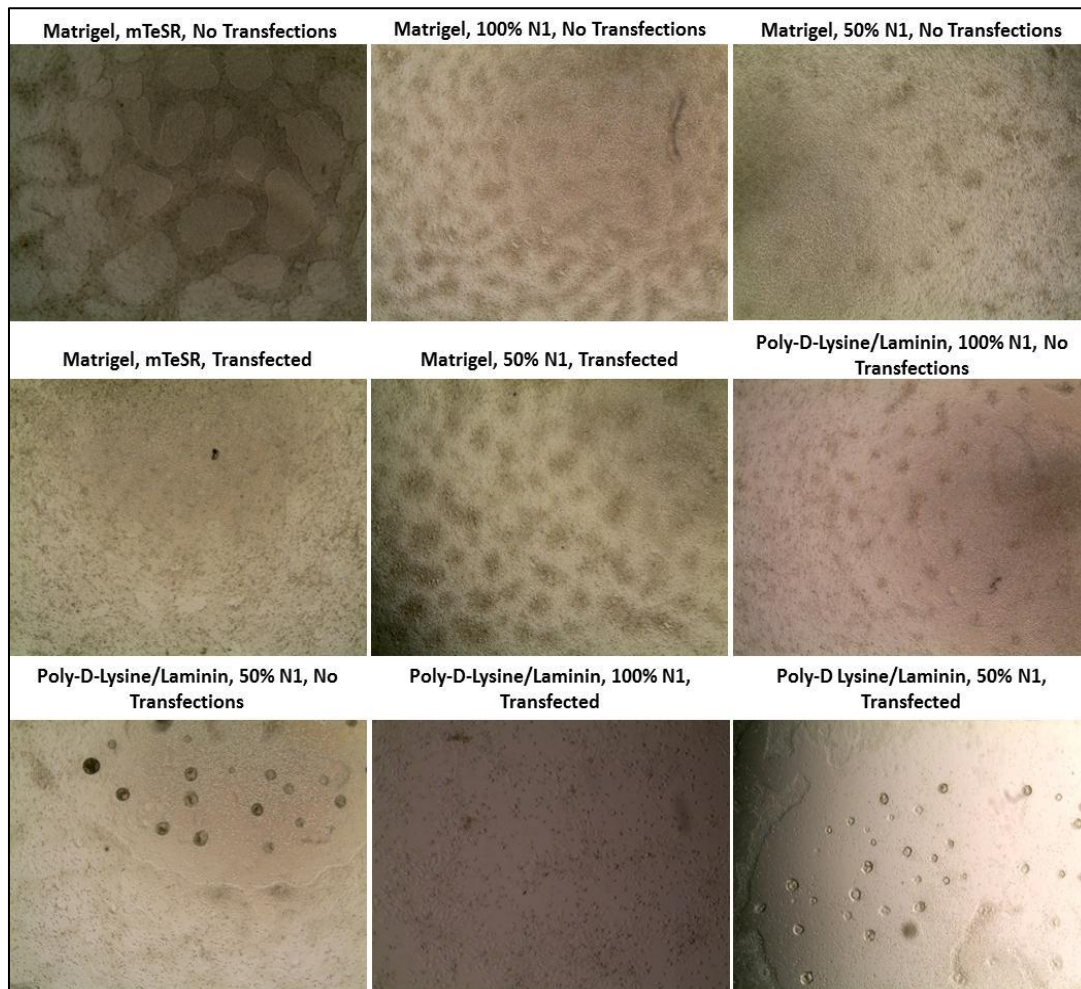


Figure 45: A) Induced pluripotent stem cells (iPSCs) seeded onto two different substrates after 24 hours in mTeSR™1, neural induction medium (N1) or a 50:50 mixture. B) iPSCs on different substrates in various media after two daily transfection with GFP mRNA and untransfected cells.

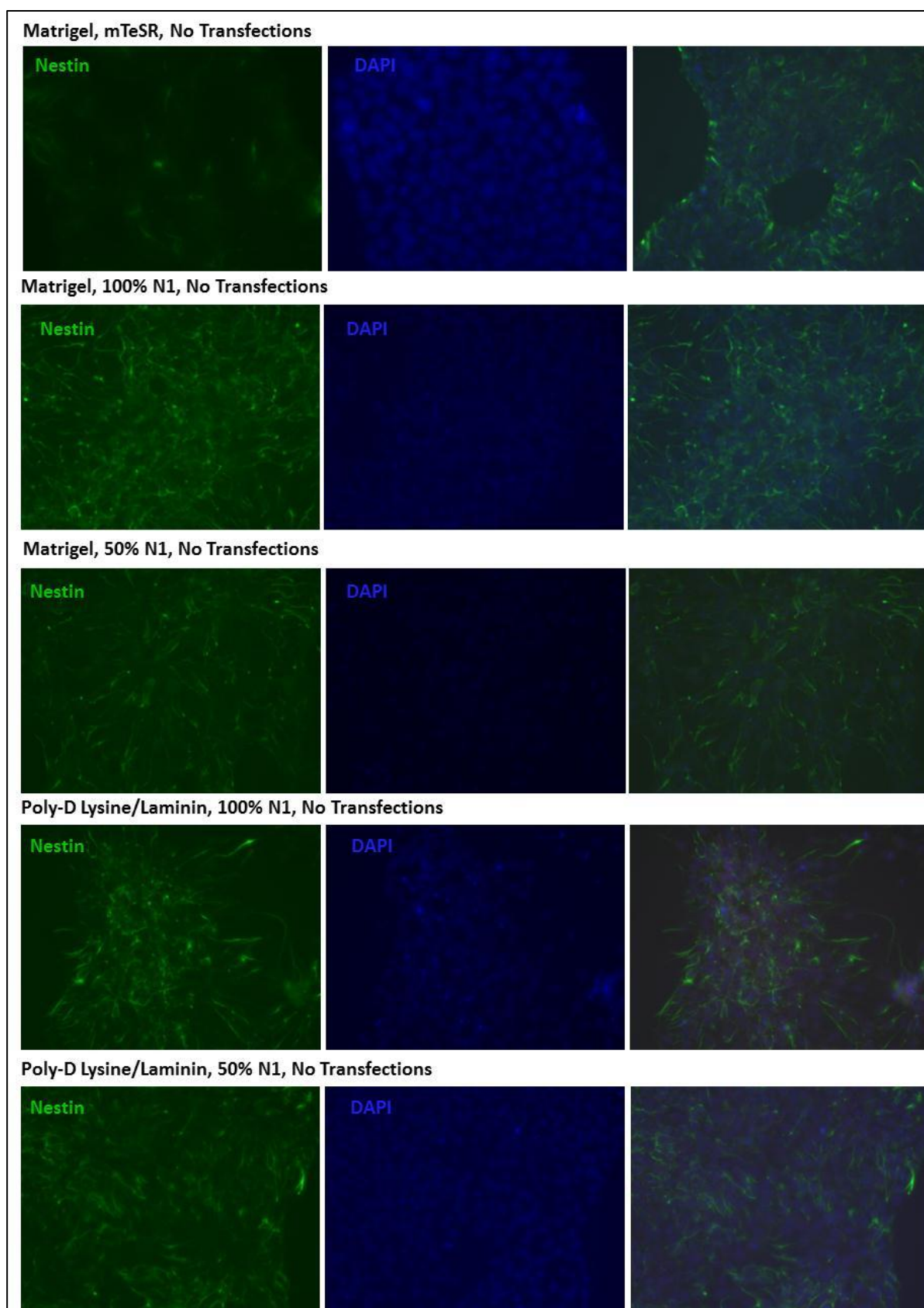


Figure 46: Staining of induced pluripotent stem cells (iPSCs) seeded onto Matrigel or Poly-D-Lysine/Laminin in mTeSR™1, neural induction medium (N1) or a 50:50 mixture with anti-Nestin and DAPI and an overlay of the two.

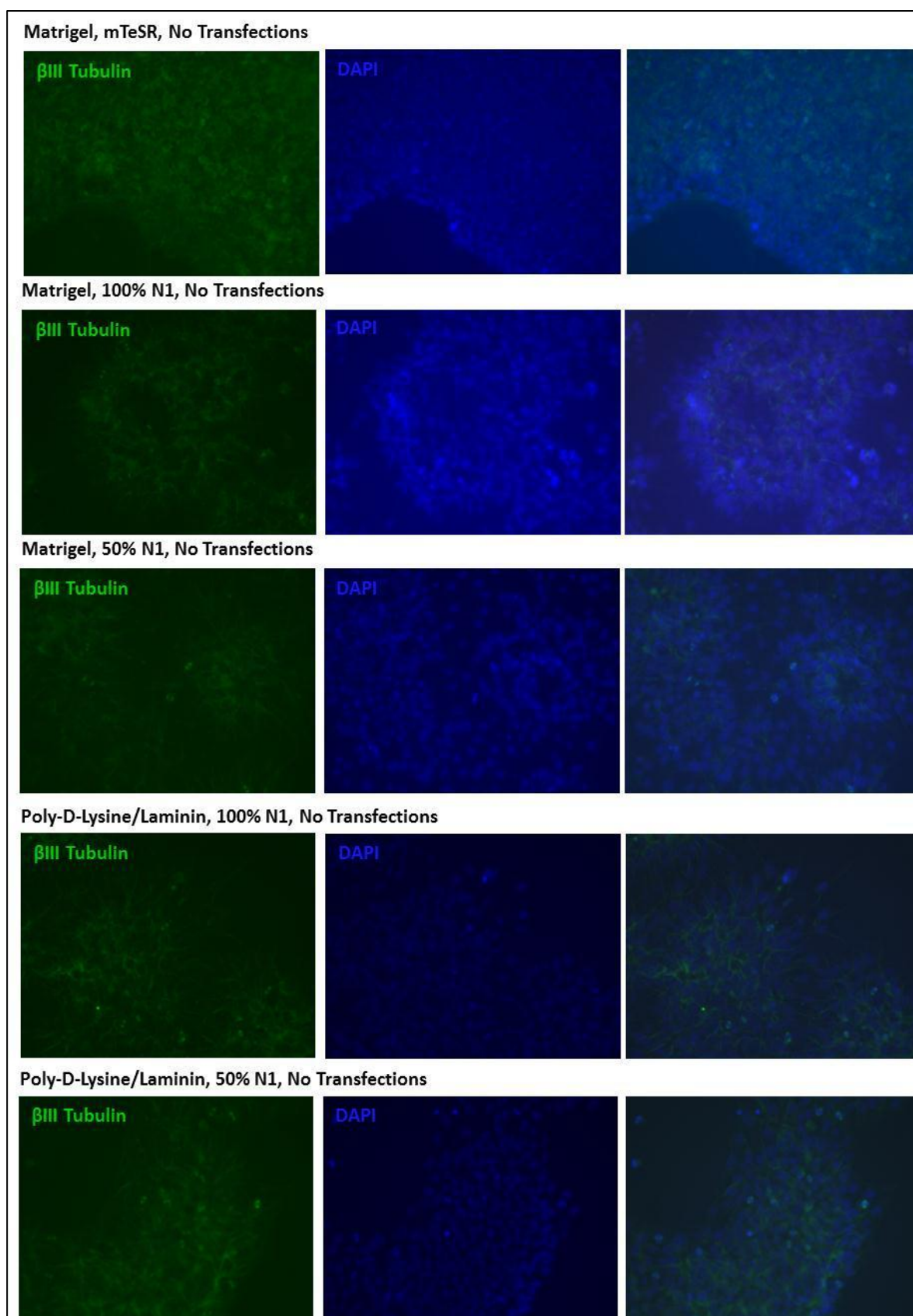


Figure 47: Staining of induced pluripotent stem cells (iPSCs) seeded onto Matrigel or Poly-D-Lysine/Laminin in mTeSR™1, neural induction medium (N1) or a 50:50 mixture with anti-βIII tubulin and DAPI and an overlay of the two.

The staining indicates that all cells stain positive for Nestin and β III tubulin regardless of the substrate and medium, although iPSCs on Matrigel in mTeSR™1 medium express the lowest levels. However the varying morphology of the cells is highlighted in the immunofluorescent images (shown in figures 46 and 47). Cells on Matrigel in mTeSR™1 medium do not show any neurite-like out growths (top rows in figures 46 and 47) while those on Matrigel in 50:50 medium (third rows in figures 46 and 47) show some small projections but appear fewer in number. Interestingly, cells plated onto Poly-D-Lysine/Laminin in 100% N1 medium exhibit neural rosette-like structures (groups of cells with longer neurite-like outward projections) that are Nestin positive (fourth rows in figures 46 and 47). Again cells plated in 50:50 medium on Poly-D-Lysine/Laminin substrate appear fewer in number with some small projections (fifth rows in figures 46 and 47).

5.4 Discussion

It is always difficult to try and develop good models of human diseases, especially when the cells affected are as inaccessible as dopaminergic neurons. As animal models of PD do not usually develop the same symptoms or pathophysiology seen in PD patients the focus is shifting to developing models in a dish.

A promising novel method involving no viral vectors was published by Warren et al., in 2010 and is achieved via the transfection of modified mRNAs encoding the Yamanaka factors (Warren, Manos et al. 2010). The authors then went on to differentiate their obtained iPSCs into myogenic fibres using modified mRNA encoding the TF MyoD. We have taken this principle and want to utilise it to produce dopaminergic neurons. In this chapter we have selected TFs involved in dopamine neuronal development and amplified their ORFs. We then ligated the UTRs required for *in vitro* transcription. Additionally we have carried out an initial experiment to determine the best substrate/medium combination to use for iPSC transfections.

5.4.1 Selection of TFs

Based on current literature we decided to use the TFs Mash1, FoxA2, Lmx1a, Nurr1, PitX3 and Hif1 α . Because of the quick turnover of Hif1 α transfection of a non-mutated transcript would not be effective. However a transcript with mutations in the three residues vital for hydroxylation enables the expression of Hif1a which is resistant to degradation by inhibiting its interaction with pVHL and preventing its targeting to the proteasome complex and leading to constitutive expression of hypoxic genes (Fu, Wang et al. 2011).

5.4.2 Ligation of UTRs to the TF ORFs

Ligation of UTRs and amplification of the ligated product was achieved for TFs Mash1, Lmx1a, Nurr1, Hif1 α and FoxA2. However sequencing of FoxA2+UTRs revealed three point mutations within the ORF that impacted on the protein sequence. One of these was corrected using site-directed mutagenesis but we were unable to correct the remaining two despite repeating it several times, using new PFu enzyme, and reordering primers. Unfortunately we were also unable to ligate the UTRs to the ORF of PitX3. The PCR product obtained using the method for the other TFs resulted in presence of the UTRs but no ORF, leading us to believe that the UTRs were self-priming.

With the exception of PitX3 and FoxA2 the TFs are ready to be polyT-tailed and used in *in vitro* transcription.

5.4.3 iPSCs can be seeded directly onto Poly-D-Lysine/Laminin in neural induction medium and survive two daily transfections

iPSCs are usually cultured on Matrigel in mTeSRTM1 medium to maintain their pluripotency. For differentiation into neurons, neuronal precursors are usually plated on Poly-D-lysine/Laminin. As we aim to differentiate the iPSCs directly without an intermediate stage we needed to determine whether iPSCs could survive a direct change of medium and substrate and then cope with daily transfections. To assess this we plated iPSCs on either Matrigel or Poly-D-Lysine and then changed their medium to mTeSRTM1, neural induction medium (used in the initial stages of dopaminergic neuronal differentiation) or a 50:50 mixture of the two. We then transfected the cells on two consecutive days with mRNA encoding GFP using RNAiMAX, keeping some untransfected controls.

Cells seeded onto Poly-D-lysine/Laminin in mTeSR™1 medium died within 24 hours. This is probably due to ‘mixed signals’ as mTeSR™1 medium is designed to ensure that the iPSCs remain pluripotent, while Poly-D-Lysine/Laminin stimulates neuronal differentiation. It is likely that any cells that started to differentiate did not receive the factors required to survive, which are normally contained in the neuronal induction media.

The iPSCs, with the exception of those on Poly-D-Lysine/Laminin in mTeSR™1 medium, survived two daily transfections with modified mRNA encoding GFP. Again, if the ring structure results from evaporation of Laminin these cells are probably not receiving the signals required to maintain differentiation and as they are only in half the amount of N1 medium compared to those in 100% N1 medium, are more likely to die.

Unfortunately we observed no GFP signal from any of the transfected cells. Following analysis of the GFP mRNA used (Martina Hallegger), it was apparent that the mRNA was degraded and therefore could not be translated in transfected cells. However, it is the use of the RNAiMAX during transfection that is usually the most toxic aspect and so the results of this experiment still indicate that iPSCs can survive (to some degree) two daily transfection after be seeded onto ‘non-iPSC’ substrate in neuronal media. Further investigation involving longer transfection regimes must be carried out before a cohesive differentiation protocol can be produced.

5.4.4 Immunofluorescence indicates that iPSCs on Poly-D-Lysine/Laminin substrate in either 100% neural induction medium or 50:50 with mTeSR™1 different neuronal morphology

Staining the untransfected wells for two early neuronal markers, Nestin and β III-Tubulin showed that while all the cells stained positive for both to some degree the most intense staining was seen in cells on Poly-D-Lysine/Laminin in either 100% or 50:50 N1 medium. These cells also exhibited neurite-like projections, the longest of which were seen in cells on Poly-D-Lysine/Laminin in 100% N1 medium. This indicates that these cells have already started to differentiate without transfection of mRNAs encoding dopamine-specific TFs.

In summary, the iPSCs appeared to survive best when directly plated on Poly-D-Lysine/Laminin in N1 medium and even developed promising neuronal morphology when imaged by immunofluorescence.

As we now know iPSCs can survive on neuronal substrate and in neuronal medium the next stage is to clone the remaining TFs without mutations present and use the constructs in *in vitro* transcription to produce modified mRNA. We can then start to determine whether iPSCs can survive a higher number of daily transfections and which order and combination of TFs provide the best yield of dopaminergic neurons. Hopefully, when fully developed, this protocol will produce mature and functional dopaminergic neurons that can be PD-patient specific with a high efficiency and specificity.

6 Discussion

In this thesis we have focused on three different areas of research; the 5'UTR and alternatively spliced α -synuclein transcripts and the development of a new protocol to differentiate iPSCs into dopaminergic neurons.

First we introduced the concept of alternative 5'UTRs, a widely known molecular mechanism for other genes including BDNF where differential splicing of the upstream non-coding region leads to decreased toxicity and altered protein localisation (Baj and Tongiorgi 2009; Baj, Leone et al. 2011), in the α -synuclein gene. Before our investigations there was a single publication predicting a secondary structure but no molecular study to confirm this. Not only did we confirm the presence of α -synuclein transcripts with different 5'UTRs but determined that the expression of at least one isoform is up regulated when our model cells (BE(2)M17) are exposed to the neurotoxin MPP⁺. This posed the question: what effect does this altered 5'UTR transcript have on the cells when they are oxidatively stressed?

To answer this question we determined the levels of protein translation stimulated by the different 5'UTRs via luciferase assays. These were found to stimulate different levels of protein expression in our neuronal cell line but not HEK293 cells, the lowest of which was stimulated by α -synuclein 202; the isoform identified in MPP⁺-treated cells. This indicates that the 5'UTRs of α -synuclein may contain a regulatory element that is acted on by neuronal-specific factors. The only way to determine whether this is occurring would be to repeat the luciferase experiments but co-transfect silencing RNA for individual transcription factors. When knocked down the transcription factor would alter the

luciferase result and we could then determine via further silencing experiments the neuronal specific mechanism.

We then wanted to try and compare the expression levels of the different α -synuclein 5'UTR transcripts semi-quantitatively so we investigated them using Real Time PCR. The results obtained indicate that expression of α -synuclein isoform 202 is up-regulated ~2 fold in oxidatively stressed cells even when normalised for total α -synuclein level. It is possible, that as excessive α -synuclein protein has been shown to be pathological (Kanda, Bishop et al. 2000; Song, Shults et al. 2004), the cells are trying to reduce the amount of α -synuclein protein produced by transcribing a UTR that contains regulatory elements resulting in decreased protein expression. While this is interesting we acknowledge that a dopaminergic neuroblastoma cell line directly stressed with a neurotoxin is not an accurate representation of the cells affected in PD patients. To try and determine whether we could see a similar differential expression we tested a sample of cingulate gyrus from a patient with DLB and a control.

While preliminary experiments analysing the expression levels of these 5'UTR isoforms in cingulate gyrus samples from a DLB patient showed no significant change in expression when compared to a control, this should not be discouraging. It may be that the expression changes, if present, are more subtle and require a larger number of patient and control samples. Similarly, the cingulate gyrus does not contain any dopaminergic neurons and DLB is not the same disease as PD. It would be interesting to look at expression levels in the SNpc of PD patients compared to age-matched controls. As the technology is developing at a significant rate it would also be appropriate to use laser capture microdissection to select individual dopaminergic neurons from the SNpc, ideally of early-onset PD patient brains and age-matched controls, and extract RNA to repeat the Real

Time PCR (Pietersen, Lim et al. 2011). In addition to comparing PD patient dopaminergic neurons of the SNpc to those of controls it would be extremely interesting to compare dopaminergic neurons from the SNpc and VTA of the same patients to see whether the alternative 5'UTRs could be involved in the survival of the VTA neurons in PD progression.

The alternative splicing of α -synuclein transcripts has also been explored. In agreement with other publications we found the isoform 112 expression increased in oxidatively stressed cells. However, following normalisation for total α -synuclein levels, the significance was obliterated. Therefore it is reasonable to presume that expression of α -synuclein 112 transcripts only increase due to an increase in total α -synuclein. We believe that previous publications have not accounted for this and therefore the results report an over-estimation of isoform expression. Likewise, it is also possible that the large increase in 112 expression accounts for the increased level of total α -synuclein. As with the 5'UTR isoforms it would be prudent to repeat the Real Time PCR using RNA extracted from single dopaminergic cells from PD patients captured using laser microdissection.

The function of these alternatively spliced α -synuclein proteins is yet to be determined. As point mutations appear to drastically alter protein function (Chandra, Gallardo et al. 2005; Yavich, Oksman et al. 2005; Tehranian, Montoya et al. 2006), one can only imagine what effect the removal of entire exons has. The characterisation of the spliced proteins is hindered because there are no molecular tools available to distinguish between them. Within this thesis we also test the specificity of novel antibodies, designed to specificity detect the 112 and 126 isoforms of α -synuclein. Excitingly the 126 antibody does detect the 126 α -synuclein with other background staining but not the other α -synuclein splice

variants. Unfortunately the 112 antibody did not detect any α -synuclein isoforms. While this novel antibody appears to be the first antibody specific for the 126 α -synuclein protein the level of background staining means that it has limited use as an immunofluorescent stain and thus will not help elucidate the localisation of the isoforms within the neurons. With further optimisation of western blotting, possibly by changing the concentration of antibody used or increasing the blocking solution concentration, it is possible that this antibody will help quantify the levels of alternatively spliced α -synuclein protein, an area that is currently lacking in the literature.

Finally we have developed the majority of vectors required to begin a novel method of differentiating iPSCs into dopaminergic neurons. Based on a publication using modified mRNAs to reprogram fibroblasts into iPSCs (Warren, Manos et al. 2010), we have cloned ORFs for TFs involved in dopamine neuron development; Mash1, FoxA2, Lmx1a, Nurr1, and PitX3. Additionally, the ORF of a mutated Hif1 α has been cloned to enable us to stimulate hypoxic conditions during the differentiation. The UTRs required for *in vitro* transcription were successfully ligated onto all but one of the TF ORFs. Unfortunately we were not able to ligate the UTRs onto the PitX3 ORF or correct two point mutations within the FoxA2 ORF. We did also determine that iPSCs could be seeded onto Poly-D-Lysine/Laminin substrate in neural induction medium and survive two transfections. Additionally, those iPSCs seeded in these conditions were observed to have neurite-like projections after just four days. This is very encouraging and indicates that with transfection of dopaminergic-specific TF mRNAs we will be able to produce dopaminergic neurons, hopefully with improved efficiency and specificity.

In order to continue with protocol development the two remaining TFs need to be cloned and ligated to the UTRs without any mutations. Once these have been attained the vectors can be used for in vitro transcription to produce mRNA transcripts. Following this investigation into the iPSC transfection regime can begin, determining variables such as the amount of RNA transfected, frequency of transfections and the length of exposure needed for the iPSCs to express neuronal markers. This would be assessed using immunofluorescent staining and PCR at various different time points.

Hopefully the data collected within this thesis will provide the foundations for future experiments investigating α -synuclein, especially the role of its non-coding regions in translation regulation, and help to improve our understanding of its role in PD. Additionally, it has provided the foundations for a novel method of iPSC differentiation into dopaminergic neurons which does not require the use of viruses for reprogramming and aims to increase the speed and specificity at which dopaminergic neurons are produced. Ultimately this should provide better in vitro models of PD and allow the development of PD therapies.

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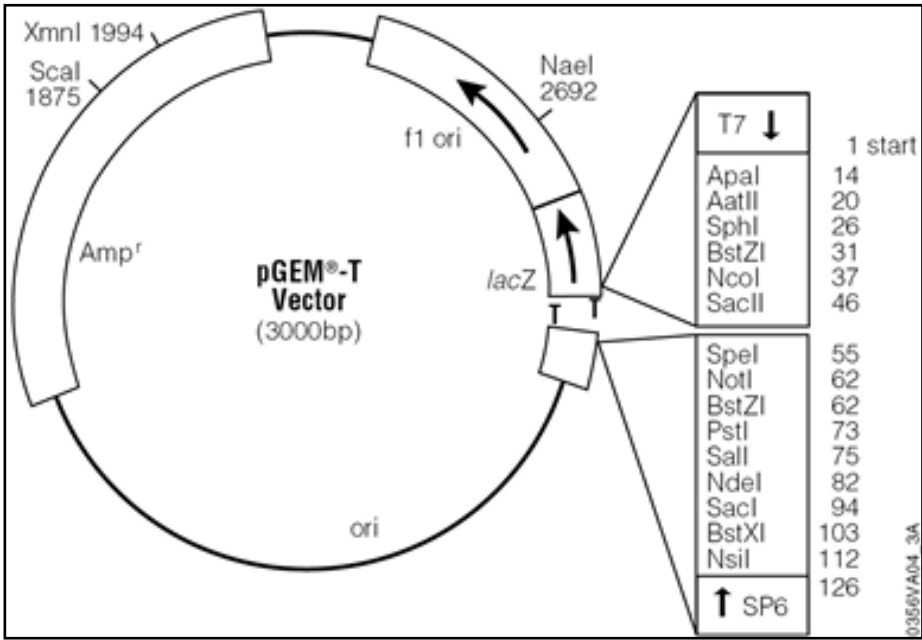
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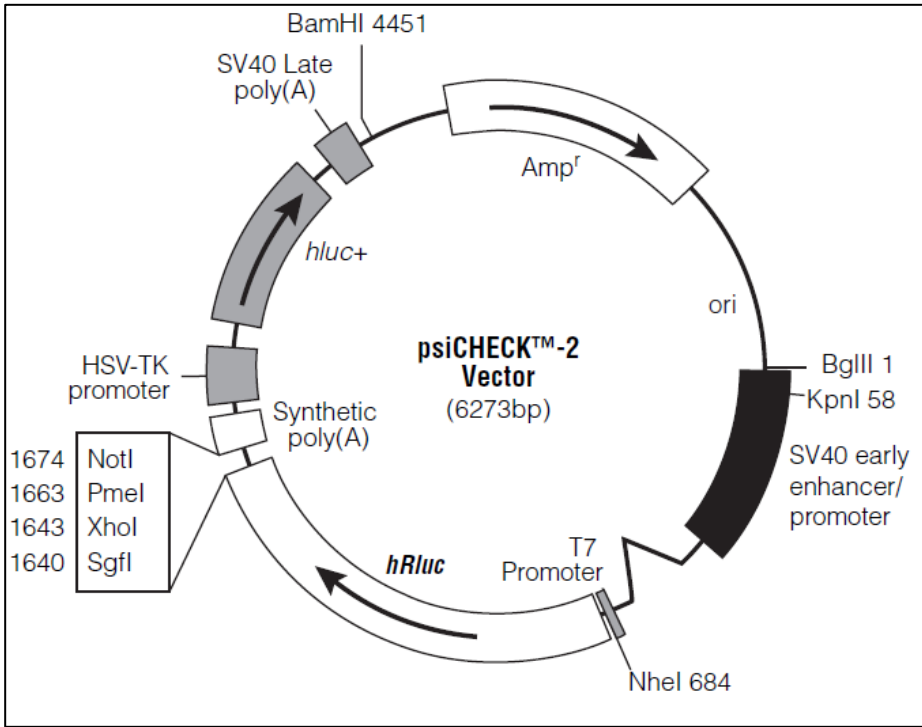
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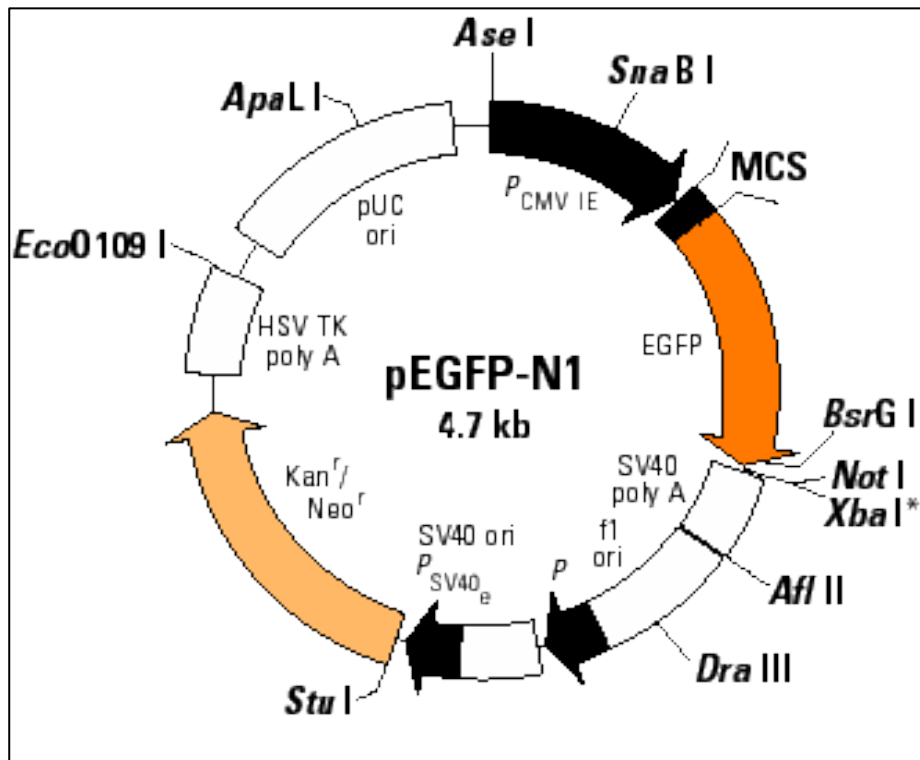
Appendix 1- Plasmid Maps



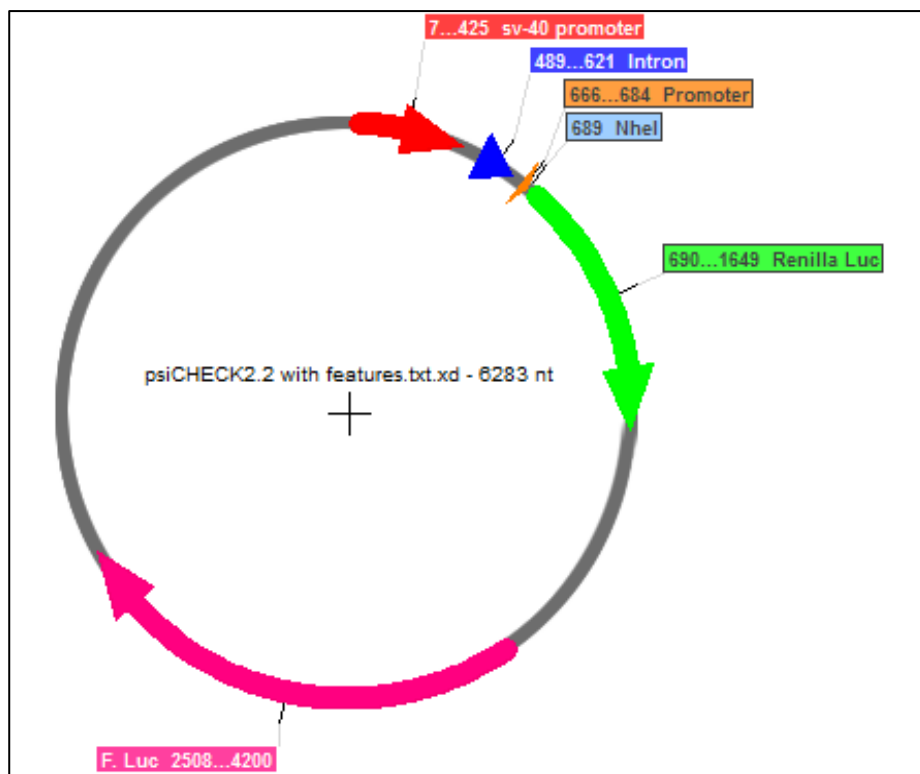
Plasmid map of pGEM-T-easy vector (Promega)



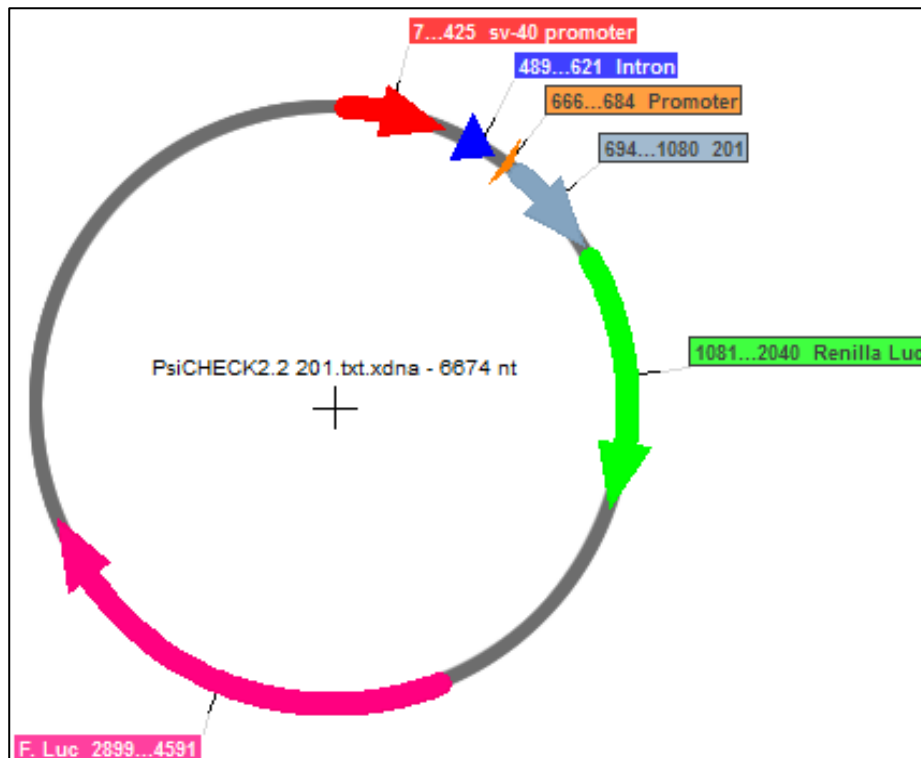
Map of PsiCHECK2 (Promega) indicating the Simian virus 40 (SV-40) promoter, NheI cloning site, Renilla luciferase ORF, HSV promoter and Firefly luciferase ORF.



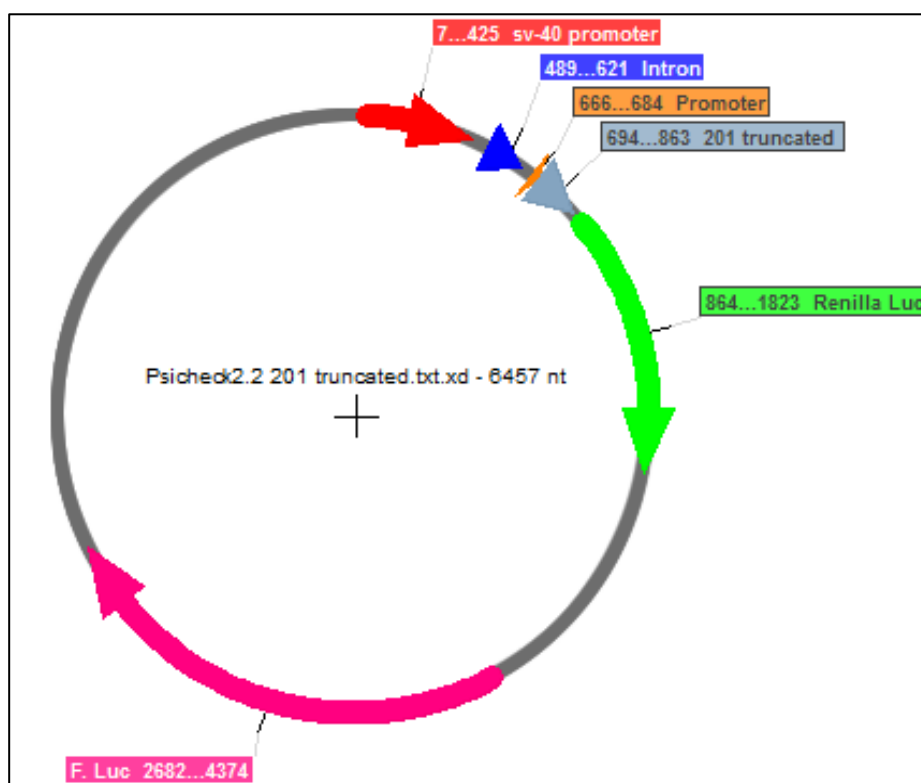
Plasmid map of pEGFP-N1



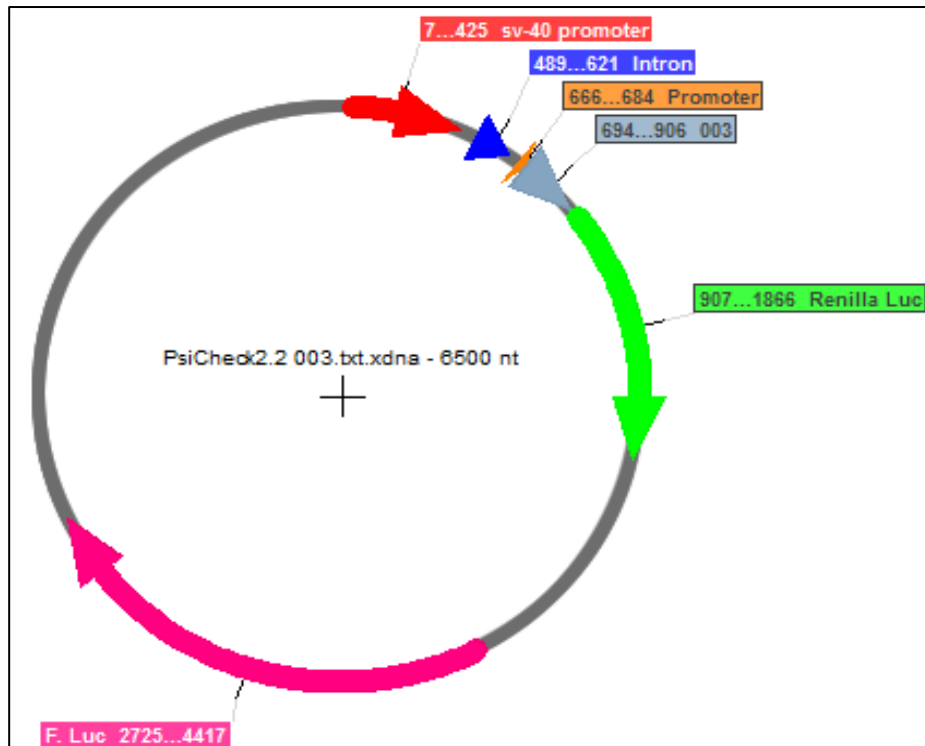
Plasmid map of PsiCHECK2.2 (modified by our lab from the original PsiCHECK2 vector-Promega) with the SV-40 promoter, intron, T7 promoter, NheI cloning site, Renilla luciferase coding sequence and firefly luciferase coding sequence labelled.



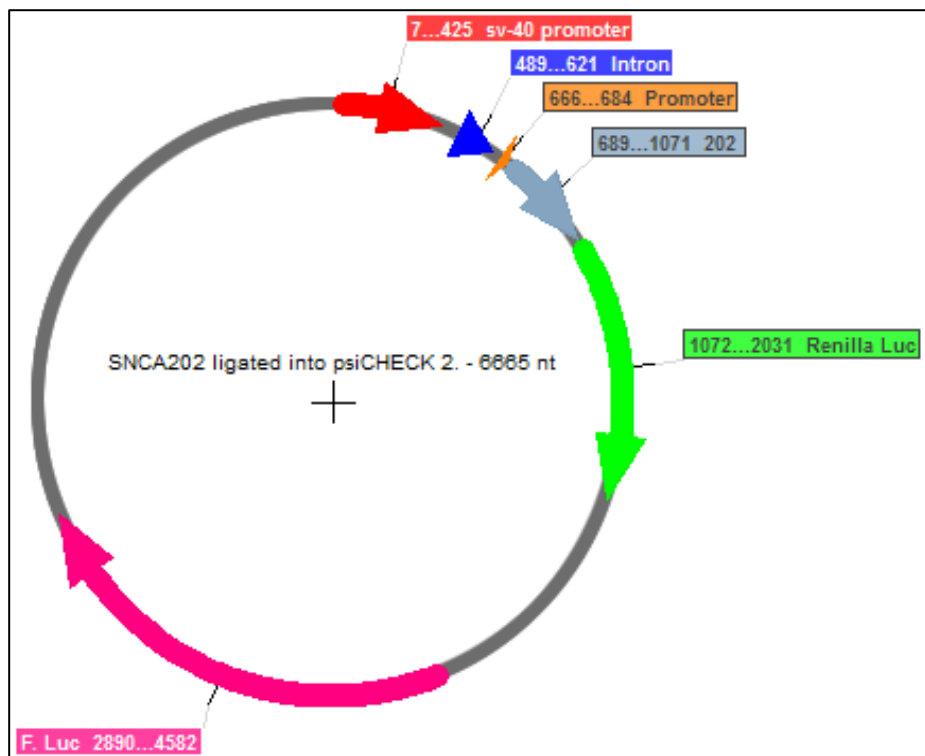
Plasmid map of PsiCHECK2.2- α -synuclein-201 vector with the SV-40 promoter, intron, T7 promoter, α -synuclein 5' untranslated region isoform insert, Renilla luciferase coding sequence and firefly luciferase coding sequence labelled.



Plasmid map of PsiCHECK2.2- α -synuclein-201 truncated vector with the SV-40 promoter, intron, T7 promoter, α -synuclein 5' untranslated region isoform insert, Renilla luciferase coding sequence and firefly luciferase coding sequence labelled.



Plasmid map of PsiCHECK2.2- α -synuclein-003 vector with the SV-40 promoter, intron, T7 promoter, α -synuclein 5' untranslated region isoform insert, Renilla luciferase coding sequence and firefly luciferase coding sequence labelled.



Plasmid map of PsiCHECK2.2- α -synuclein-202 vector with the SV-40 promoter, intron, T7 promoter, α -synuclein 5' untranslated region isoform insert, Renilla luciferase coding sequence and firefly luciferase coding sequence labelled.