**Regulation of alpha-synuclein (α SNCA) gene and protein expression by
hypoxia**

By

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A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

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ABSTRACT

Parkinson's disease (PD) is characterized by the death of neurons in the midbrain and by the formation of Lewy Bodies (LBs). Neurodegenerative diseases have been shown to be more prevalent in patients that have also suffered from ischemic cardiovascular diseases. This study links hypoxia-associated ischemic cardiovascular diseases such as stroke with the development of PD. We hypothesized that hypoxia would promote α -synuclein gene (*SNCA*) and protein (α SNCA) expression in a hypoxia-inducible factor-dependent manner. *SNCA* gene expression and α SNCA protein expression were examined in human embryonic kidney (HEK293T) and human neuroblastoma (SH-SY5Y) cells treated with short- and long-term hypoxia (< 21% O₂). α SNCA protein expression increased significantly under hypoxia, as well as modified and aggregated forms of the protein. *SNCA* gene expression did not show significant alterations during hypoxia. Thus, hypoxia plays a role in α SNCA protein accumulation without altering the level of expression of its gene. Further cell viability assays and siRNA knockdown targeting α SNCA were performed and results showed a significant change in cell viability under the short-term siRNA knockdown combined with hypoxia. The knockdown results were measured and analyzed at the monomer level of α SNCA without significant changes at the tetramer and hexamer forms of aggregation.

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3. TABLE OF CONTENTS

1.	ABSTRACT.....	iii
2.	ACKNOWLEDGEMENTS.....	iv
3.	TABLE OF CONTENTS.....	v
4.	LIST OF FIGURES.....	ix
5.	LIST OF ABBREVIATIONS.....	xii
6.	INTRODUCTION.....	1
6.1.	Dementia of neurodegenerative disorders.....	1
6.2.	Parkinson's Disease (PD).....	3
6.3.	α -Synuclein Gene (<i>SNCA</i>) and Protein (α SNCA).....	19
6.4.	Oxidative Stress in PD and Hypoxia.....	30
6.5.	Environmental Stressors: Stroke and Hypoxia.....	37
6.6.	HIF-1 Transcription Factors.....	43
6.7.	Hypothesis and Objectives.....	52
7.	MATERIALS AND METHODS.....	54
7.1.	Promoter Search for Hypoxia-Inducible α -Synuclein Cis-acting Elements.....	54
7.2.	Chemicals and Cell Culture.....	54
7.3.	Hypoxia Treatments and Chemicals Hypoxic Mimetics.....	58
7.4.	Cell Harvest, Whole Cell Lysis and Isolation of Soluble α SNCA.....	58

7.5.	Urea Extraction of Insoluble α SNCA.....	59
7.6.	Protein Determination of Soluble and Insoluble α SNCA.....	60
7.7.	Western Blot Analysis.....	60
7.8.	RNA Isolation and Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).....	61
7.9.	Design of siRNA Molecules.....	63
7.10.	Transient Transfection of HEK293T cells.....	64
7.11.	MTT Assay Cell Viability Under siRNA-SNCA and Hypoxic Conditions.....	65
7.12.	Statistical Data Analysis.....	66
8.	RESULTS.....	67
8.1.	Analysis of Hypoxia-Inducible α -Synuclein Cis-acting Elements.....	67
8.2.	Changes of α SNCA Soluble Protein Levels in HEK293T Cells under Hypoxic Conditions.....	67
8.3.	Changes of Insoluble α SNCA Protein in HEK293T Cells Under Hypoxic Conditions.....	71
8.4.	Changes of Insoluble α SNCA Protein in PC12-AC in Comparison to Soluble Levels Under Hypoxic Conditions.....	71
8.5.	Changes of Soluble α SNCA Protein in SH-SY5Y Cells Under Hypoxic Conditions.....	77
8.6.	Protein Expression of Insoluble α SNCA in SH-SY5Y Cells Under Hypoxic Conditions.....	77
8.7.	Optimization For Total RNA Amount by Real-Time RT-PCR.....	78

8.8.	Gene Expression of <i>SNCA</i> in HEK293T, PC12-AC, and SH-SY5Y Cells Under Long-Term Hypoxic Versus Normoxic Conditions.....	83
8.9.	Gene Expression of <i>SNCA</i> in HEK293T Cells Under Hypoxic Conditions by Real-TimeRT-PCR.....	84
8.10.	Gene Expression of <i>SNCA</i> in SH-SY5Y Cells Under Hypoxic Conditions by Real-Time RT-PCR.....	92
8.11.	Knockdown By siRNA Molecules Targeting <i>SNCA</i>	92
8.12.	Optimization of Amount of siRNA Molecules for Maximal <i>SNCA</i> Knockdown by Western Blot Analysis.....	96
8.13.	Optimization of Transfection Time for siRNA Knockdown by Western Blot Analysis.....	99
8.14.	Transfection Efficiency of siRNA Molecules by Fluorescence Microscopy.....	102
8.15.	Changes of Soluble Monomer α SNCA Protein Expression in HEK293T Cells Under siRNA Knockdown and Hypoxic Conditions.....	102
8.16.	Changes of Soluble Tetramer α SNCA Protein Expression in HEK293T Cells Under siRNA Knockdown and Hypoxic Conditions.....	103
8.17.	Cell Viability of HEK293T Cells Under siRNA Knockdown and Hypoxia by MTT Assay.....	110
8.18.	Gene Expression Analysis of <i>SNCA</i> in HEK293T Cells Under siRNA Knockdown and Hypoxic Conditions by Real –Time RT-PCR.....	113
9.	DISCUSSION.....	117
9.1.	Putative HRE Sites Search.....	118

9.2.	Protein Expression of α SNCA in HEK293T Cells Under Hypoxic Conditions.....	119
9.3.	Protein Expression of α SNCA in Neuronal-Like Cells Under Hypoxic Conditions.....	123
9.4.	Gene Expression of <i>SNCA</i> in HEK293T , PC12-AC, SH-SY5Y Cells Under Hypoxic Conditions.....	128
9.5.	Knockdown of α SNCA by siRNA Molecules in HEK293T Cells.....	129
9.6.	Gene and Protein Expression of α SNCA in HEK293T Cells Under siRNA Knockdown and Hypoxia.....	133
9.7.	Cell Viability of HEK293T Cells under siRNA knockdown / Hypoxic Treatments.....	136
10.	REFERENCES.....	140

4. LIST OF FIGURES

Figure 1: A schematic representation of the neuropathology of PD and the accumulation of LBs in SNpc region.....	6
Figure 2: Proposed mechanisms of neurodegeneration in PD.....	9
Figure 3: Schematic representation of MPTP metabolism as a toxic model in studying PD pathology.....	14
Figure 4: The MPP ⁺ intracellular pathway in the dopaminergic neurons.....	16
Figure 5: The potential function of α SNCA in formation of synaptic vesicles for dopamine storage in presynaptic terminals.....	21
Figure 6: The motif structure and functional regions of α SNCA.....	23
Figure 7: The pathological pathway involved in the aggregation of α SNCA and the formation of LBs.....	28
Figure 8: The role of oxidative stress in the pathogenesis of PD.....	31
Figure 9: The imbalance between the production and the elimination of ROS leading to the pathogenesis of PD.....	34
Figure 10: The adaptation process of mammalian cells in response to hypoxia (O ₂ <21%).....	39
Figure 11: The schematic representation of the HIF α and HIF β subunits and their important functional domains.....	45
Figure 12: Schematic representation of HIF-1 α stabilization versus degradation pathway.....	47
Figure 13: The three cell lines models were used to study α -synuclein gene and protein expression under normoxic and hypoxic conditions.....	55

Figure 14: Western blot analysis of α SNCA soluble and insoluble protein expression in HEK293T cells under hypoxic conditions.....	69
Figure 15: Western blot analysis of insoluble α SNCA protein expression in HEK293T cells under hypoxic conditions.....	73
Figure 16: Western blot analysis of soluble versus insoluble α SNCA protein expression in PC12-AC cells under hypoxic conditions.....	75
Figure 17: Western blot analysis of soluble α SNCA protein expression in SH-SY5Y cells under hypoxic conditions.....	79
Figure 18: Western blot analysis of insoluble α SNCA protein expression in SH-SY5Y cells under hypoxic conditions.....	81
Figure 19: Real-time RT-PCR analysis of <i>SNCA</i> gene expression using different amounts of total RNA isolated from HEK293T cells under normoxic conditions.....	85
Figure 20: Real-time RT-PCR analysis of <i>SNCA</i> gene expression at 100 ng of total RNA from HEK293T, PC12-AC, and SH-SY5Y cells during hypoxic and normoxic conditions.....	87
Figure 21: Real-time RT-PCR analysis of <i>SNCA</i> gene expression in HEK293T cells under hypoxic conditions.....	89
Figure 22: Real-time RT-PCR analysis of <i>SNCA</i> gene expression in SH-SY5Y cells under hypoxic conditions.....	93
Figure 23: Western blot analysis of α SNCA knockdowns by (0.25 to 1 μ g) siRNA molecules in HEK293T cells under normoxic conditions.....	97
Figure 24: Western blot analysis of α SNCA knockdown by 1 μ g siRNA molecules in HEK293T cells under different durations of normoxic conditions.....	100

Figure 25: Localization of fluorescent small interference RNA (siRNA) molecules in the cytoplasm region of HEK293T cells.....	104
Figure 26: Western blot analysis of soluble monomeric form of α SNCA protein expression in HEK293T cells under siRNA knockdown and hypoxia conditions.....	106
Figure 27: Western blot analysis of the soluble tetrameric form of α SNCA protein expression in HEK293T cells under siRNA knockdown and hypoxia treatments.....	108
Figure 28: Cell viability of HEK293T cells under α SNCA knockdown by siRNA molecules and hypoxia exposure as measured by MTT.....	111
Figure 29: Real-time RT-PCR analysis of <i>SNCA</i> gene expression in HKE923T cells transfected with 2 μ g siRNA molecules under hypoxic conditions.....	114
Figure 30: Induction of oxidative stress and stabilization of HIF under hypoxic conditions.....	124
Figure 31: Overview of the several ways to induce RNAi.....	131

5. LIST OF ABBREVIATIONS

AD: Alzheimer's Disease

ARNT: Aryl Hydrocarbon Receptor Nuclear Translocator

ALS: Amyotrophic Lateral Sclerosis

α SNCA: Alpha-Synuclein

CBZ: Proteasome Inhibitor (Cbz-Leu-Leu-Leu-al)

DA : Dopamine

DA-Q : Dopamine-Quinone

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DTT: Dithiothreitol

FCS: Fetal Calf Serum

FBS: Fetal Bovine Serum

HEK293T: Human Embryonic Kidney with SV40 T antigen

HD: Huntington's Disease

HIF: Hypoxia Inducible Factor

HIF-1 α : Hypoxia Inducible Factor-1 Alpha

HIF-1 β : Hypoxia Inducible Factor-1 Beta

HRE: Hypoxia Response Element

HRP: Horse Radish Peroxidase

HS: Horse Serum

LBs: Lewy Bodies

LRRK2: Leucine-Rich Repeat Kinase 2

MAO-B: Monoamine Oxidase B

MPDP⁺: 1-Methyl-4-Phenyl-2,3-Dihydropyridinium

MPP⁺: 1-Methyl-4-Phenylpyridinium

MPTP: 1-Methyl -4-Phenyl-1,2,3,6-Tetrahydropyridine

NGF: Nerve Growth Factor

ODDD: Oxygen Dependent Degradation Domain

6-OHDA: 6-Hydroxydopamine

PAS: Per/ARNT/SIM

PBS: Phosphate Buffered Saline

PC12-AC: Adherent Pheochromocytoma Rat Cells

PD: Parkinson's Disease

PHD: Prolyl Hydroxylase Domain

PMSF: Phenylmethanesulfonyl fluoride

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

SH-SY5Y: Human Neuroblastoma Cells

SNpc: Substantia Nigra Pars Compacta

TH: Tyrosine Hydroxylase

TNF: Tumor Necrosis Factor

UCHL-1: Ubiquitin C-Terminal Hydroxylase L 1

vHL: von Hippel Lindau protein

VMAT2: Vesicular Monoamine Transporter-2

6. INTRODUCTION

6.1. Dementia of neurodegenerative disorders

Dementia has a major impact on the health and welfare of the Canadian populace. Recent data suggests that on a worldwide aspect, dementia is a disabling disorder characterized by a general degeneration in brain function and it is more common with aging (Agüero-Torres et al., 1998; De Ronchi et al, 2005; Hill et al., 1996; Kawas and Brookmeyer, 2001; Fratiglioni et al, 2008; Salmon and Bondi, 2009). The types of dementia are dependent on the cause and regional factors leading to a decline in the cognitive functions (Janvin et al., 2006; Kröger et al., 2008; Turner et al., 2002). There are several common neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS) (Beal, 1995; Ross and Poirier, 2004). Treatment of human neurodegenerative disorders is one of the challenging aspects in the medical field due to the complexity of the disorders and the limited number of solutions that are normally aimed at the symptoms level.

The brain contains between 10 and 100 billion nerve cells differing in shape, size, and function, depending on the chemicals they produce (Burke and Barnes, 2006). Human neurological disorders are characterized mainly by neuronal death in specific areas of the brain (Mattson, 2000; Mattson, 2006). These affected areas might be related to cognitive behaviour, memory, language acquisition, and motor functions that will cause impairment in voluntary movements (Janvin et al., 2006; Maccioni et al., 2001; Mattson, 2006). Therefore, dementia can lead to a severe loss of intellectual abilities. The

specific regions of dementia can be divided in two types: cortical and subcortical dementias depending on the affected brain structures (Salmon and Filoteo, 2007; Salmon and Bondi, 2009; Argno-Lasprilla et al., 2006; Janvin et al., 2006; Turner et al., 2002). The cortical-type dementias target abnormalities in the hippocampus, cortex association areas and medial temporal lobes with a gradual decline in learning abilities, language and perception (Argno-Lasprilla et al., 2006). The subcortical-type dementias, on the other hand, target the basal ganglia, thalamus and brainstem structures (Argno-Lasprilla et al., 2006). The deficits in these areas result in declines in motivations, movements, mood and attention (Argno-Lasprilla et al., 2006; Turner et al., 2002).

The cortical dementias include AD and vascular dementia (stroke) (Lopez-Neblina et al., 2005; Maccioni et al., 2001; Mattson, 2000). AD is the most common cause for cortical dementia. It is characterized mainly by the death of hippocampal and cortical neurons (Carter and Lippa, 2001; Maccioni et al., 2001). The hippocampus is a collection of structures which is located in the cerebral hemisphere of the brain (Burke and Barnes, 2006). The hippocampus plays a critical role in memory and thus people with dementia or disorder in this region often lack the ability to learn anything new. The second most common cause of cortical dementia is vascular dementia, also known as multi-infarct dementia (Carmichael, 2006; White et al., 2000). In humans, stroke is correlated with a limited degree of recovery in the cognitive operations in the peri-infarct cortex and connected cortical areas (Carmichael, 2006; Popa-Wangner et al., 2007; Lopez-Niblina et al., 2005). Stroke may result from blocking the blood supply due to occlusion in blood vessels; known as ischemic stroke (Lipton, 1999).

The subcortical dementias include PD and HD (Argno-Lasprilla et al., 2006, Janvin et al., 2006; Turner et al., 2002). PD is pathologically characterized by the death of midbrain neurons which produce the neurotransmitter dopamine (Sawle, 2004; Thomas and Beal, 2007; Zaccai et al., 2005). These midbrain neurons are specifically located in the substantia nigra pars compacta (SNpc). The progression of neuronal death eventually leads to “parkinsonism” which is characterized by motor dysfunction, such as tremor, rigidity and slowness of voluntary movements (Janvin et al., 2006; Turner et al., 2002; Przedborski, 2005). HD is an autosomal dominant neurodegenerative disease characterized by the expansion of trinucleotide (CAG) repeat in the Huntington gene leading to the production of an insoluble polyglutamine region in the protein and aggregation of insoluble protein in the basal ganglia, striatum and cortex region (Duyao et al., 1993; Walker, 2007).

6.2. Parkinson’s Disease (PD)

PD is classified as the second most common neurodegenerative disorder after AD (Przedborski, 2005). The age onset of PD is usually 55 years; moreover, the incidence rate of PD increases from 20/100,000 at age 55 to 120/100,000 at the age 70 (Przedborski, 2005). Statistics show a yearly increase of more than 50,000 cases of affected individuals in United States (Przedborski, 2005). About 80% of PD patients suffer from motor disabilities characterized by a decrease in voluntary and normal unconscious movements, an increase in rigidity of passive movements in patient's limbs, postural instability and freezing (Björklund and Lindvall 2000). PD affects movement, mood, behaviour, thinking and sensation (Sawle, 2004). However, the symptoms may

vary amongst individuals and thus their medical treatments will differ depending on the severity and level of progression. The early symptoms of PD include rigidity (resistance to movement) of limbs, fingers and muscle stiffness (Eriksen et al., 2005; Moore et al., 2005, Sawle, 2004). These symptoms are likely to affect one side of the body first and then slowly progress to affect the other side. Furthermore, PD produces a number of other symptoms including depression, anxiety, lack of motivation and mood swings (Ferreri et al., 2006; Sawle, 2004). Other major clinical symptoms occur when the disease is expressed fully, including bradykinesia (slow movement), tremor (maximal at rest and decreased with movement) and impaired postural reflexes (poor balance) (Sawle, 2004).

PD is a neuropathological condition characterized by gradual loss of dopamine-producing neurons in the SNpc of the midbrain and other monoaminergic neurons in the brain stem (Ross and Poirier, 2004). The pathological hallmark of PD is the presence of intraneuronal inclusions called Lewy Bodies (LBs) (Sawle, 2004; Thomas and Beal, 2007). LBs are spherical cytoplasmic protein aggregates, as illustrated in **Figure 1**, which contain a variety of proteins such as aggregated forms of α -synuclein, parkin, ubiquitin, a synuclein interactor referred to synphilin-1, proteasome proteins and neurofilaments (Ross and Poirier, 2004; William and Przedborski, 2003). LBs are found to be densest in the SNpc but they can be also found in the cerebral and cortical neurons and neurites known as Lewy neurites (Ross and Poirier, 2004). Electron microscopy has found that LBs are 15 μ m in diameter and contain a dense hyaline core surrounded by a ring of clear, halo radiating 8-10 nm fibrils (William and Przedborski, 2004). The role of LBs in PD has been controversial; some groups suggest that they are neuroprotective by sequestering abnormal misfolded proteins away from cellular elements while others

suggest that they are cytotoxic by building up toxic misfolded protein and eventually initiating programmed cell death (William and Przedborski, 2003).

The loss of dopamine signalling is associated mainly with PD (Chinta and Anderson, 2006). Dopamine is a key neurotransmitter, and more recently has been identified to be an important modulator of arterial blood pressure (Chinta and Anderson, 2006). The main source of dopamine is the nigrostriatal dopaminergic neurons of the midbrain in the central nervous system (Greenamyre and Hastings, 2004; William and Przedborski, 2003). The SNpc contains the cell bodies of the nigrostriatal neurons which project to the putamen and caudate as shown in **Figure 1** (William and Przedborski, 2003). The SNpc is the main producer of dopamine (Chinta and Anderson, 2006). The dopaminergic neurons play an important role in controlling voluntary movement and behavioural processes including mood, reward, addiction and stress (Lotharius and Brundin 2002). In PD, the SNpc degenerates, and thus the dopaminergic input to the corpus striatum (caudate and putamen), fails as illustrated in **Figure 1** (Greenamyre and Hastings, 2004; Sawle, 2004; William and Przedborski, 2003).

Figure 1: A schematic representation of the neuropathology of PD and the accumulation of LBs in SNpc region: Comparison between **A)** The normal nigrostriatal pathways composed of dopaminergic neurons with cell bodies located in the SNpc projecting with solid red lines to the corpus striatum (caudate and putamen) versus **B)** The nigrostriatal degenerative pathway in PD with dashed lines indicating loss of projections from the SNpc to the corpus striatum and loss of dark-brown pigment (depigmentation) marked by arrows in SNpc, indicating loss of dopaminergic neurons. **C)** The immunohistochemical labelling of the intraneuronal inclusions, or LBs, in SNpc neurons using immunostaining with a specific antibody against the α -synuclein protein (left photograph) and against ubiquitin (right photograph). Both show an immunoreactive zone within the LBs containing both synuclein and ubiquitin (William and Przedborski, 2003).

PD is mainly a sporadic neurodegenerative disease with no apparent genetic linkage; but in about 5% of the cases, the disease is caused by inheritance of defective genes (Feigin and Eidelberg, 2007; Przedborski, 2005; Van de Warrenburg et al., 2007; Kang, et al., 2001). The cause of sporadic PD is unknown. However, there are currently two hypotheses regarding the pathogenesis of the disease (Kirik et al., 2002). One hypothesis speculates that the misfolding and the aggregation of proteins are the main cause for the death of SNpc dopaminergic neurons as shown in **Figure 2** (William and Przedborski, 2003). The initiation of aggregations can occur by covalent modifications (phosphorylation and protein adducts made with oxidized products of dopamine) (Ross and Poirier, 2004). Aging can also play a role in facilitating aggregation due to the decrease in the cell's ability to degrade misfolded proteins (Ross and Poirier, 2004). The second hypothesis claims that mitochondrial dysfunction, oxidative stress including toxic radicals, environmental toxins and toxic products of oxidized dopamine (resulting from normal dopamine metabolism generating reactive oxygen species (ROS)) are the sources for the loss of dopaminergic neurons (as illustrated in **Figure 2**) (Brown et al., 2005; Fukae et al., 2007; Chinta and Anderson, 2006; Lin and Beal, 2006; Saito et al., 2005; Schapira, 2007; William and Przedborski, 2003, Yoo et al., 2003).

The interaction between these two proposed mechanisms is possibly a third hypothesis regarding the pathogenesis of PD as illustrated in **Figure 2** (William and Przedborski, 2003). To date, findings show that oxidative damage can cause the misfolding and aggregation of α -synuclein in LBs (Mori et al., 2006). These aggregations of misfolded proteins can be neurotoxic to cells and can initiate damage by deforming the neurons and interfering the intracellular trafficking leading to cell death mechanisms such

Figure 2: Proposed mechanisms of neurodegeneration in PD: Schematic presentation showing the possible points of interactions between the two proposed hypotheses of misfolding and aggregation of proteins, combined with mitochondrial dysfunction, oxidative damage and abnormal dopamine metabolism along with mutations in PD-linked genes. Pathogenic mutations can interfere with the cellular degradation mechanism of misfolded proteins in the case of parkin and UCHL-1 as well as promote the accumulation of misfolding proteins in case of α -synuclein. Further, the oxidative damage from either dopamine neurotoxic adducts or mitochondrial dysfunction leads to toxicity and promotes the aggregation of misfolded proteins. The accumulation of misfolded proteins and formation of LBs are key events in pathogenesis of PD, initiating programmed cell death of dopamine neurons. However, LBs may also be neuroprotective by sequestering misfolded proteins in insoluble forms away from cellular elements (William and Przedborski, 2003).

as apoptosis (Taylor et al., 2002; William and Przedborski, 2003). The remaining rare cases of inherited PD are due to mutations in the identified PD-linked genes, such as α -synuclein, parkin, and ubiquitin C-terminal hydrolase L1 (UCHL-1) which functions in the ubiquitin-proteasome pathway (Van de Warrenburg et al., 2007; Takahashi et al., 2007; Kang et al., 2001).

Recent studies have focused on understanding the rare familial forms of PD, determining the role of genetics and further identifying PD-associated genes. Research on PD genes examines their expression and mutation and strives to develop neuroprotective therapies against potential targets. Mutations have been identified in at least six genes causing the familial forms of PD (Bertram and Tanzi, 2005; Bogaerts et al., 2007; Cookson, 2005). Autosomal dominant missense mutations occur in *SNCA* and *LRRK2* genes while recessive missense, nonsense and frameshift mutations occur in *parkin*, *PINK1*, *DJ-1* and *ATP13A2* (encoding a lysosomal type 5 P-type ATPase) (Bogaerts et al., 2007; Ramirez et al., 2006). The *SNCA* gene encodes for α -synuclein protein which functions in synaptic vesicle formation; *Parkin* encodes for a E3 protein ubiquitin ligase, a component of ubiquitin-proteasome system; *PINK1* encodes for phosphatase and tensin homologue (PTEN)-induced kinase 1, which functions as mitochondrial kinase; *DJ-1* encoding for the DJ-1 protein, a component of redox sensor and mitochondrial antioxidant system; *LRRK2* encodes for leucine-rich repeat kinase 2, a multifunctional kinase in the polarized distribution of the synaptic vesicles to axons (Bogaerts et al., 2007).

The pathogenic mutations associated with inherited PD can both directly induce abnormalities by toxic protein conformations or indirectly by interfering with the process of degradation and thus cause accumulation of misfolded proteins (Taylor et al., 2002; William and Przedborski, 2003). The emerging knowledge about PD-associated genes is a key step in understanding the pathogenesis of PD. Also, understanding the factors that initiate PD is an important issue for searching therapeutic strategies. Environmental risk factors, such as the oxygen homeostasis, can play an important role in disease pathophysiology.

To further understand the etiology and the pathogenesis of PD, animal models were studied (Thomas and Beal, 2007; William and Przedborski, 2003). Recently, changes in the brain of these animal models of neurodegenerative diseases have been detected using technical advances in imaging modalities such as positron emission tomography (PET) and magnetic resonance imaging (MRI) (Strome and Doudet, 2007). Further, animal models were utilized by exposing mice and rats to a neurotoxin that mimics the neurological symptoms of PD such as 6-hydroxydopamine (6-OHDA) and 1-methyl -4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Björklund and Lindvall, 2000; William and Przedborski, 2003). 6-OHDA is a neurotoxin that was used 30 years ago to establish the first animal model of PD and is associated with a pathological mechanism of inducing dopaminergic neuronal death in SNpc region (William and Przedborski, 2003). However, 6-OHDA cannot cross the blood-brain barrier and thus local stereotaxic injection into the SNpc is required (Breese et al., 2005; William and Przedborski, 2003). Once 6-OHDA is injected, it induces dopaminergic neuronal death and pathological PD lasting for 1-3 weeks (William and Przedborski, 2003). However, 6-OHDA cannot lead

to the production of LB-like aggregations.

MPTP is a dopaminergic neurotoxin producing irreversible and severe PD (Przedbroski, 2005; William and Przedborski, 2003). MPTP is highly lipophilic and crosses the blood-brain barrier within minutes, as illustrated in **Figure 3** (William and Przedborski, 2003). The pro-toxin MPTP is oxidized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP⁺) by monoamine oxidase B (MAO-B) in glial cells, followed by spontaneous oxidation to give 1-methyl-4-phenylpyridinium (MPP⁺). MPP⁺ is a polar active molecule which penetrates to dopaminergic neurons and has a high affinity for dopamine transporters (DAT), as shown in **Figure 3** (William and Przedborski, 2003). MPTP functions by inhibiting mitochondrial respiration through the production of MPP⁺ binding to complex I of the electron transport chain, thus interrupting the flow of electrons and subjecting cells to oxidative stress and ATP energy depletion, as illustrated in **Figure 4** (Przedborski, 2005; William and Przedborski, 2003). MPTP causes the production of superoxide radicals initiating oxidative stress and the appearance of energy crisis activating neuronal cell death. Also, MPP⁺ interferes with synaptic vesicles by binding to the vesicular monoamine transporter-2 (VMAT2) and stimulating dopamine leakage from the synaptic vesicles to the cytosol leading to the increase of superoxide radicals through the autooxidation of dopamine (William and Przedborski, 2003). Moreover, excess dopamine stimulates the release of neuromelanin which acts as an interneuronal storage of toxins (William and Przedbroski, 2003). Also, the MPP⁺ can remain in the cytosol, interacting with cellular enzymes, and causing toxicity, as shown in **Figure 4** (William and Przedbroski, 2003). It can also interfere with synaptic vesicles through its interactions with vesicular monoamine transporter-2 (VMAT2)

Figure 3: Schematic representation of MPTP metabolism as a toxic model in studying PD pathology: This illustrates the MPTP toxic mechanism in causing the death of dopaminergic neurons. The metabolic pathway is initiated by the crossing of MPTP through the blood-brain barrier followed by multiple steps of modifications and oxidations. MPTP is converted to MPDP⁺ by MAO-B in glial cells followed by series of oxidations leading to the formation of MPP⁺ and its release to the extracellular space. MPP⁺ penetrates to the dopamine neurons through dopamine transporters DAT (William and Przedbroski, 2003).

Figure 4: The MPP⁺ intracellular pathway in dopaminergic neurons: The active toxic molecule of MPP⁺ enters the dopaminergic neurons and causes toxicity by interfering with the mitochondria and blocking electron transport chain at complex I, as shown by X. This causes the build up of ROS and the depletion of ATP. Another potential toxic route of MPP⁺ is through interactions with cytosolic enzymes and binding to the synaptic vesicles through the interactions with vesicular monoamine transporter-2 (VMAT) (William and Przebrocki, 2003).

(William and Przedbroski, 2003). Thus, MPTP allows us to assess the role of oxidative stress in the development of PD.

Moreover, the use of MPTP in model animals enabled us to discover an elevation of the proinflammatory cytokine tumor necrosis factor (TNF) (McCoy et al., 2006). Further research was carried out to determine the functional effect of TNF in relation to PD. TNF was found to be a major mediator of neurotoxin mechanism in the loss of dopaminergic neurons by causing mitochondrial dysfunctions and activation of apoptotic death cascades. Thus, TNF may be a therapeutic target in the treatment of PD. However, one limitation is the fact that clinical diagnosis for the early stages of PD is currently very challenging. The identification of PD in humans is achieved after a significant loss of about 80% of dopamine neuronal cells (Björklund and Lindvall, 2000). Also, the exact pathway for TNF action is still ambiguous and further tests are required using different animal models. Nevertheless, the chronic use of anti-inflammatory drugs can eliminate the risks of getting PD by 46% (McCoy et al., 2006).

Furthermore, the recent discovery of PD-causing genes and the associated mutations in α -synuclein guided the focus toward a new aspect of PD pathogenesis, the cascade of events in an abnormal biochemical pathway. Thus, most of the current research changed direction to study the functional interactions of PD proteins and the aggregations of misfolded proteins forming LBs. Parkin mutations were found to be associated with a loss of E3 ubiquitin ligase activity leading to the accumulation of misfolded parkin substrates (Perez and Palmiter, 2005). Despite the fact that researchers were able to discover the novelty in these PD-causing genes, they were still unable to find the direct linkage between these biochemical abnormalities and the actual depletion of

dopamine-secreting neuronal cells. Nevertheless, further reports suggested a relationship between parkin, synuclein function, and proteasome inhibition (Perez and Palmiter, 2005). Recent research has focused on the protein aggregations and filaments formations found in PD and understanding the role of α -synuclein in the etiology of both familial and sporadic forms of PD.

6.3. α -Synuclein Gene (*SNCA*) and Protein (α SNCA)

The α -synuclein gene (*SNCA*), also known as *PARK1*, encodes a natively unfolded protein that has been found to be the main constituent of LBs in PD (Bogaerts et al., 2007; Cookson, 2005; Thomas and Beal, 2007). It is known to be abundant in neurotransmitter synapses, being associated with membranous structures such as vesicles and is involved in regulating vesicular release of neurotransmitters. Studies have shown that mice lacking the α -synuclein protein (α SNCA) suffer from a loss in vesicular dopamine release after electrical stimulation (Halliday and McCann, 2008; Stefanis et al., 2001b). α SNCA is bound to lipid membranes, such as those found in synaptic vesicles, by forming an amphipathic helix (Cookson, 2005; Volles et al., 2001). Furthermore, it is known to regulate the reserve pool of synaptic vesicles in the brain and to play a role in neuronal plasticity as illustrated in **Figure 5** (Lotharius and Brundin, 2002). α SNCA plays a role in presynaptic terminals where 50% of the protein is associated with synaptic membranes from early endosome (which stores dopamine) through interactions with phospholipase D2 (PLD2) and 50% in the cytosol, as shown in **Figure 5** (Lotharius and Brundin, 2002). It is essential for dopamine to be immediately sequestered in synaptic vesicles due to its potential to autooxidize, producing hydrogen peroxides (H_2O_2),

superoxide radical ($O_2^{\bullet-}$) and dopamine-quinone (DA-Q) radicals (Lotharius and Brundin, 2002).

The *SNCA* gene family encode for various isoforms of the protein, including α , β , γ -synucleins as well as synoretin (Bogaerts et al., 2007; Cookson, 2005; Cookson and Van der Brug, 2007; Dauer and Przedborski, 2003). The structural forms of all synucleins have, in common, a series of imperfect repeats characterized by a specific sequence of amino acids (KTKEGV). However, it is the fibrillar α SNCA aggregates that form the major component of LBs in PD (Masliah et al., 2000). Also, LBs might contain other proteins such as neurofilaments and cytoskeletal proteins. The structural form of α SNCA includes a N-terminal amphipathic region, phosphorylation sites at serine and tyrosine residues, a hydrophobic middle region promoting aggregation and a highly acidic C-terminal region as illustrated in **Figure 6** (Cookson, 2005; Thomas and Beal, 2007).

Three missense mutations (A53T, A30P and E46K) cause the autosomal dominant form of PD to be expressed (Cookson, 2005). A53T was the first point mutation of α SNCA discovered, followed by A30P (German kindred), E46K (Spanish kindred; also known as “LB dementia”) and finally the triplication of the wild type *SNCA* gene (Iowa kindred). Therefore, it is very clear that *SNCA* gene mutations play a role in the pathology of the familial PD promoting aggregations as shown in **Figure 5** (Lotharius and Brundin, 2002).

Figure 5: The potential function of α SNCA in formation of synaptic vesicles for dopamine storage in presynaptic terminals: Dopamine (DA) is synthesized from 3,4-dihydroxyphenylalanine (L-DOPA) and is directly sequestered in synaptic vesicles. Alternatively, DA can be autooxidized to produce hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^{\bullet-}$) and DA-quinone (DA-Q). Also, it can be oxidized by monoamine oxidase to produce H_2O_2 and 3,4-dihydroxyphenylacetic acid (DOPAC). α SNCA plays a role in formation of these vesicles by interacting with early endosome and PLD2 proteins. However, mutations in the *SNCA* gene, such as Ala30Pro and Ala53Thr, can inhibit the association of α SNCA with lipid membranes, causing accumulations of DA in the cytoplasm (Lotharius and Brundin, 2002).

Figure 6: The motif structure and functional regions of α SNCA: A schematic representation of the natively unfolded α -synuclein protein including sites of phosphorylations at both the serine (S) and tyrosine (T) and possible sites of mutation within the imperfect repeats KTKEGV region. The C-terminal region contains the acidic tail and calpain cleavage sites. The presence of the C-terminus tends to decrease aggregation and the presence of the hydrophobic region tends to increase aggregation in the imperfect repeat region (Cookson, 2005).

However, recent studies have raised the possibility that *SNCA* is also associated with the pathology of sporadic PD. These studies characterized the *SNCA* enhancer and promoter regions (Chiba-Falek and Nussbaum, 2001; Hashimoto et al. 1998, Holzmann et al., 2003). The analysis showed the presence of a polymorphic repeat site, known as NACP-Rep1, which is located approximately 10.7 Kb upstream of the translational start site. The human NACP-Rep1 sequence contains the following dinucleotides (TC) (TT) (TC) (TA) (CA) repeated a variable number of times. Regulation of *SNCA* by NACP-Rep1 was determined computationally as well as by luciferase reporter assay in mammalian cell lines such as human embryonic kidney (HEK293T) and human neuroblastoma (SH-SY5Y) cells. It was concluded that altered regulation of *SNCA* expression by these polymorphic microsatellite repeats may result in the development of sporadic PD.

Figure 6 shows the structure of the α SNCA protein (Cookson, 2005). Recent studies have shown that, upon deletion of residues 71-82, the α SNCA protein failed to aggregate and PD pathology was not observed (Cookson, 2005). This indicates the importance of the hydrophobic regions in self-association, aggregation and further accumulation of α SNCA. Also, it is important to note that the hydrophobic region only exists in the alpha form of SNCA and is not found in the other isoforms. Other studies suggested a major role of the C-terminal domain in regulating α SNCA aggregation by expressing C-terminal-truncated protein which resulted in α SNCA aggregation, dopaminergic loss and toxicity (Cookson, 2005; Murray et al., 2003). β SNCA has been found to provide protection against α SNCA toxicity by lowering its expression and aggregation and promoting the AKT pathway (Cookson, 2005). Furthermore, recent

studies have shown that phosphorylation sites play a role in increasing the aggregation of α SNCA. An example is the phosphorylation of Ser129, which has been shown to cause α SNCA aggregation in humans, primates, rats, mice and SH-SY5Y cells overexpressing α SNCA (Takahashi et al., 2007). Also, exposure to iron (II) leads to an increase in the aggregation of α SNCA, catalyzed by casein kinase 2 (CK2) induced phosphorylations (Takahashi et al., 2007). Retroviruses have also been utilized to overexpress α SNCA, creating an ideal model to study the loss of dopaminergic neurons and the formation of LBs (Yamada et al., 2004). In these studies, immunohistochemistry showed the aggregation of α SNCA and phosphorylations of Ser129 (Yamada et al., 2004). Recent studies and experiments in cell culture have shown that phosphorylation plays an important role in modulating the aggregation of α SNCA (Ross and Poirier, 2004).

Recent studies have shown that over-expression of α SNCA, under certain conditions, causes the formation of LBs (Stefanis et al., 2001a). Also, studies in PC12 pheochromocytoma cells (rat) have examined the upregulation of synuclein-1 (the rat homolog of human α SNCA) under different conditions, such as treatment with nerve growth factor (NGF) which promotes differentiation, release of neurotransmitters and neuroplasticity. With NGF treatment, the protein expression of synuclein-1 was found to increase, supporting its role in neuroplasticity (Stefanis et al., 2001a). Other studies looked at the relationship among α SNCA aggregates, dopamine metabolism (regulated by tyrosine hydroxylases (TH), which functions in converting tyrosine to L-DOPA (dopamine precursor)) and neuronal loss (Mori et al, 2006). The results concluded that α SNCA oligmerization is promoted by excessive dopamine and further formations of

protofibrils and aggregates are promoted by oxidative stress resulting from dopamine-quinone (DA-Q) and ROS production. Further, reduction in TH activity reduces dopamine metabolism and thus reduces cytoplasmic dopamine and cytotoxic DA-Q, protecting against α SNCA aggregations (Mori et al., 2006).

Recent research focuses on understanding the molecular mechanism and the biochemistry behind the formation of the insoluble fibrils of α SNCA (Cookson, 2005; Cookson and Van der Brug, 2007). **Figure 7** presents the cascade of events that precede the formation of α SNCA aggregates. Normally, an equilibrium exists between the linear and α -helical, membrane-associated monomers of α SNCA (the membrane associated form contains charged residues (red and blue) as well as nonpolar and hydrophobic amino acids (gray)) (Cookson, 2005). However, the A30P mutation favours the linear monomer, which leads to the formation of aggregates and protofibrils in the presence of other mutations such as E46K, A53T, and WxT3 (known as the Iowa triplication). Dopamine stabilizes aggregates and causes α SNCA to form ring-like structures, linking dopamine to aggregate formation. ROS and certain chemicals can act as cross-linkers, inducing aggregation (Cookson, 2005; Halliday and McCann, 2008; Lücking and Brice, 2000; Uversky et al., 2001).

Figure 7: The pathological pathway involved in the aggregation of α SNCA and the formation of LBs: A schematic representation of the proposed cascade events leading to α SNCA aggregation and formation of highly insoluble oligomers and fibrils with β -sheet like interactions. α SNCA exists in an equilibrium between the monomeric unfolded structure and the membrane associated forms with amphipathic helices; grey circles = nonpolar and hydrophobic amino acids, blue and red circles = charged residues. In the presence of mutations, there are cellular dopamine-induced aggregations and the formation of oligomers and ring-like structures which are kinetically stabilized by DA. The end product is the formation of highly insoluble fibrils and LBs (Cookson, 2005).

It is also interesting to note that different mutations will eventually have different effects. For example, A30P slows down the formation of β -sheet fibrils in comparison to other mutations. The β -pleated sheet-like bonding, along with covalent modifications of α SNCA, promote the conversion of aggregates into fibrils (Ross and Poirier, 2004; Cookson, 2005; Tsigelny et al., 2007). Fibrils are heavily insoluble polymers of proteins that aggregate to form LBs. Ubiquitin is attached to these proteins prior to their aggregation. Recent studies using molecular modelling and molecular dynamics showed that α SNCA can form higher order of aggregates that can propagate head to head and head to tail interactions from dimers, tetramers, hexamers and possible formation of ring-structures which are not available to bind to membranes (Tsigelny et al., 2007).

6.4. Oxidative Stress in PD and Hypoxia

Oxidative stress is considered to be a major player in several neurodegenerative disorders including Parkinson's disease (Beal, 1995; Saito et al., 2005; Simonian and Coyle, 1996; Yoo et al., 2003). In particular, oxidative stress leads to the generation of ROS, and reactive nitrogen species (RNS) associated with mitochondrial dysfunction (Bogaerts et al., 2007; Fukae et al., 2007; Yoo et al., 2003). The hallmark pathology of PD, including LB formation, is found to be mainly linked to oxidative damage and decrease in mitochondrial complex I activity in the SNpc (Beal, 1995; Fukae et al., 2007). In PD, oxidative stress can also be induced from the dopamine metabolism by autooxidations to dopamine-quinone (DA-Q), superoxide radicals ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2) as illustrated in **Figure 8** (Lotharius and Brundin, 2002). The increase in ROS and

Figure 8: The role of oxidative stress in the pathogenesis of PD: The cross-talk process for interactions of the familial forms of PD (due to mutations that are linked to α -synuclein (*PARK1*), parkin (*PARK2*) and ubiquitin carboxyl-terminal hydroxylases L-1 (*PARK5*)) and of the sporadic forms of the disease (due to defects in the ubiquitin-proteasome pathway (UPP) along with the increase of cytotoxic dopamine and catecholamines). These events enhance oxidative stress and promote aggregation of misfolded proteins leading to cell death and neurodegeneration (Lotharius and Brundin, 2002).

cytoplasmic dopamine induce modifications in α SNCA, aggregation of the protein and the production of LBs. Interactions between the sporadic and familial forms of PD pathways, along with oxidative stress and mitochondrial dysfunction, promote the pathogenesis of PD and cell death (Lotharius and Brundin, 2002).

Current studies aim to determine if changes in gene expression are induced by oxidative stress in PD (Bogaerts et al., 2007; Thomas and Beal, 2007). Oxidative stress results from the imbalance between the production of the ROS and the decline in antioxidant defenses. **Figure 9** shows the schematic representation of the formation of the highly reactive unpaired species such as the hydroxyl, nitric oxide and superoxide radicals (Lotharius and Brundin, 2002). Moreover, possible Fenton reactions might occur in the presence of Fe^{+2} , a reduced transition metal, leading to further propagating reactions. In order to test for this hypothesis; researchers have applied microarray analysis to monitor gene expressions in the nigral DA cell line (Yoo et al., 2003). It has been found that, under oxidative stress conditions, expression of specific genes related to the nuclear-encoded subunits of mitochondrial complex I were significantly downregulated whereas heme oxygenase-1 (HO-1), a cytoplasmic constituent of Lewy bodies, was significantly upregulated (Yoo et al., 2003). More HO-1 was also demonstrated to be complexed with heat shock proteins (HSP) which are synthesized under intracellular conditions of oxidative stress and thus protect brain tissue from damaging radicals (Fukae et al., 2007).

In PD, several studies have shown a decrease in mitochondrial complex I activity, an increase of oxidative stress, and an alteration in the activities of antioxidant defence systems (Fukae et al., 2007; Chinta and Anderson, 2006). Thus, the progressive course of

a **b**

Figure 9: The imbalance between the production and the elimination of ROS leading to the pathogenesis of PD: Abnormal increase in ROS from dopamine metabolism, as shown in **a)** through the production of DA-quinone and H_2O_2 radicals that can be harmful by producing hydroxyl radicals from Fenton reactions and **b)** through the increase in the ROS production in PD, overcoming the eliminations processes by catalase, superoxide dismutase and glutathione (GSH) systems, and enhancing cell death by oxidative stress (Lotharius and Brundin, 2002).

these neurodegenerative diseases may be due to the dual effects of impaired energy metabolism, depletion in ATP production and oxidative stress. Therefore, oxidative stress and mitochondrial dysfunction have been suggested as important factors in causing neuronal death in SNpc of PD patients (Beal, 1995). Lipid peroxidation, along with several DNA repairing enzymes, are upregulated in the SNpc of PD patients suggesting their cooperation in the repair of mitochondrial DNA in the brains of PD patients (Beal, 1995; Przedborski, 2005).

Moreover, aging is considered to act as another important factor in neurodegenerative disease, especially PD (Beal, 1995). Aging reduces the ability of cells to produce a variety of chaperones, resulting in an increase in protein misfolding and an activation of the proteasome system (Beal, 1995). Therefore, the incidence of proteasomal dysfunction increases with age, resulting in accumulation of misfolded proteins which may further inhibit the activated proteasome.

Several studies have also demonstrated that PD is linked to alterations in the activity of antioxidant defence systems (Chinta and Anderson, 2006). These studies suggest that depletion of reduced glutathione (GSH), which plays an important role in scavenging ROS and RNS within both the cytosol and the mitochondria of SNpc, will lead to a selective inhibition of complex I activity resulting in mitochondrial dysfunction (Lotharius and Brundin, 2002; Chinta and Anderson, 2006).

Thus, in order to test their hypothesis, a chronic depletion of GSH was induced in dopaminergic N27 cells by buthionine sulfoxamine treatment and complex I inhibition was measured by the rate of oxygen consumption (Chinta and Anderson, 2006). The results showed that, upon the induction of chronic dopaminergic GSH depletion, complex I inhibition was observed leading to mitochondrial dysfunction and further production of ROS impacting the progression of PD (Chinta and Anderson, 2006). Moreover, these effects can be reversible upon the return of GSH to normal levels. Therefore, these findings supported the evidence implicating the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of PD and that maintenance of cellular GSH levels within dopaminergic neurons can be a potential therapeutic target for treating the progression of PD.

6.5. Environmental Stressors: Stroke and Hypoxia

Humans encounter a variety of environmental challenges and stresses due to exposure to both physiological and clinical conditions during their lifespan. One such condition is low oxygen or hypoxia. Hypoxia is defined as any concentration of oxygen (O_2) below normal atmospheric concentration (21%) as shown in **Figure 10** (Kilmova and Chandel, 2008; Semenza, 2000a). Humans encounter hypoxic environments at high altitudes, during development *in utero*, and in ischemia-associated diseases such as cardiac arrest or stroke. Ischemia is a restriction in blood supply to a portion of a tissue or organ, creating a complex hypoxic environment possibly due to inability of tissues to sufficiently meet metabolic needs (Semenza, 2000b; Semenza, 2000d). An example is ischemic stroke which results from an occlusion in a blood vessel to the brain, causing a

disruption of cerebral blood flow through the middle cerebral artery (Lopez-Neblina et al., 2005; Shi and Liu, 2007; White et al., 2000). Ischemia and subsequent reperfusion with oxygenated blood initiates a cascade of apoptotic events within the hypoxic region affected by the stroke (Carbonell and Rama, 2007; Lopez-Neblina et al., 2005). Other types of stroke include thrombotic and embolic stroke, caused either by the build-up of clots inside the artery of the brain (thrombotic) or by clots that form in the circulatory system and travel to the brain (embolic), resulting in rupture of blood vessels, escape of blood into surrounding tissues, and a disruption of blood flow to specific brain regions (Lipton, 1999; Wardlaw et al., 2004).

Furthermore, studies have demonstrated that ischemic stroke increases the subsequent risk of developing neurodegenerative diseases (Del Barrio et al. 2005; Hu et al., 2006; Lee et al., 2006; Moroney et al., 1996; Struck et al., 1990; Tatemichi et al., 1994). Cerebral ischemia (focal and global) reduces both oxygen and glucose supply to brain tissue, resulting in brain injury and impairment of cellular function (Traystman, 2003). Neurons have the potential to exhibit all forms of cellular death upon an ischemic insult; these include a variety of changes such as the production of ROS, a decrease in ATP and eventually apoptosis in the periinfarct region (the area surrounding the infarct) and necrosis in the infarct region (Lipton, 1999).

Currently, there are two major animal models of cerebral ischemia a) the focal ischemia model which mimics thrombosis induced by the occlusion of the middle cerebral artery, and thus a reduction in blood flow, to a very specific region of brain, and b) the global ischemia model which mimics cardiac arrest and restrictions in blood flow to all the brain regions resulting in a severe impairment of hippocampal function

Figure 10: The adaptation process of mammalian cells in response to hypoxia (O_2 <21 %): The schematic representation of the mammalian cells in response to hypoxia (0.5 to 5 % O_2) and anoxia conditions (0 % O_2) through the activation of stress signals which trigger the initiation of the adaptation process by the activation of Hypoxia-Inducible Factors (HIFs) (Klimova and Chandel, 2008).

(Traystman, 2003). Recent studies have shown that ischemic neuronal insult increases the formation of A β peptide through increased production of the amyloid precursor protein (APP), thus providing a link to the pathogenesis of AD (Lee et al., 2006). These studies used *in vitro* models of cerebral ischemia (oxygen-glucose deprivation) to understand the relationship between ischemic neuronal insults and the amyloidogenic and non-amyloidogenic pathways. This was shown in both a human neuroblastoma cell line (SH-SY5Y) and cultured primary cells isolated from transgenic mice expressing human APP (Lee et al., 2006). Results showed that during oxygen-glucose deprivation, the amyloidogenic pathway of γ -secretase is activated with a corresponding decrease in non-amyloidogenic pathways, leading to an increase in the production of A β plaques. Thus, these studies demonstrated the possible role of ischemic insults in the development of Alzheimer's disease.

Other studies have shown that, upon reperfusion in an ischemic environment, ROS are generated, activating apoptotic pathways and decreasing ROS scavenger systems (such as intracellular GSH) in a manner similar to that observed in PD (Saito et al., 2005). Also, application of ischemic insults in areas close to or surrounding the SNpc increases the incidence of initiating PD due to its apoptotic effect on the dopaminergic neurons. Therefore, understanding the molecular links between ischemic stroke insult in specific regions of the brain and the development of neurodegenerative disease will enable us to develop future therapeutic interventions. According to the National Institute of Neurological Disorders and Stroke (NINDS), 50,000 Canadian suffer from stroke each year; Ischemic stroke account for 80% of all other types of strokes, and approximately 300,000 Canadians are living with stroke-related disabilities (NINDS, 2002). According

to the Canadian Study of Health and Aging (CSHA), one million Canadians will suffer from neurodegenerative disease by the year of 2025 (CSHA Working Group, 2000).

Insights into the effects of hypoxia on the levels of α SNCA will further our understanding of associations between cardiovascular disease and the development of PD. Basic knowledge of the effects of this stress on the accumulation of α SNCA may lead to the development of novel therapeutics and preconditions which minimize and prevent the formation of LBs.

My primary hypothesis is that there are molecular links between ischemic stroke and the development of neurodegenerative diseases; specifically PD. My approach is to examine the expression and activity of α SNCA in neuronal cells exposed to hypoxia. To elucidate these links, it is important to understand how organisms respond to hypoxia in terms of gene and protein expression.

The human response to hypoxia has been the focus of recent intensive basic and clinical research. The cellular response to hypoxia involves adaptive mechanisms at the level of both the organism and the cell (Chun et al., 2002). At the level of the organism, there is an increase in the production of red blood cells and formation of new blood vessels in order to increase the blood flow and delivery of oxygen to organs and tissues. At the cellular level, there is a switch from aerobic (O_2 -dependent) to anaerobic (O_2 -independent) metabolism. Furthermore, there is an induction of gene and protein expression as well as posttranslational modifications of hypoxia-responsive controls of both transcription and translation (Chun et al., 2002). One of the major regulators of gene expression in response to lack of oxygen are the transcription factors known as Hypoxia-

Inducible Factors (HIFs) illustrated in **Figure 10** (Semenza, 2000a; Semenza, 2000c; Semenza, 2007; Klimova and Chandel, 2008).

6.6. HIF-1 Transcription Factors

HIF-1 plays a critical role in maintaining oxygen homeostasis. It is a heterodimeric transcription factor that consists of two basic helix-loop-helix (bHLH) subunits known as HIF-1 α and HIF-1 β with molecular masses of 120 to 130 kDa and 91 to 94 kDa respectively (Chun et al., 2002; Semenza, 2000c). There are also three isoforms of the α subunit including HIF-1 α , HIF-2 α and HIF-3 α and they can interact with one of the three isoforms of the β -subunit including ARNT (HIF-1 β), ARNT (HIF-2 β) and ARNT3 (HIF-3 β) forming HIFs heterodimers (Semenza, 2001). HIF β , or ARNT, is also known to dimerize with the aryl hydrocarbon receptor mediating the transcription of genes encoding detoxification enzymes (Semenza, 1999). HIF-1 α has a higher affinity to bind to HIF-1 β and it is associated with the transcription of hypoxia-inducible genes (Bergeron et al., 1999). **Figure 11** represents, schematically, the domain structure of the HIF-1 subunits. The HLH domain mediates the dimerization of the HIF α and HIF β subunits and the basic domain mediates DNA binding (Semenza, 2000c). Nuclear localization signals (NLS), found in both subunits, mediate their translocation to the nucleus. Furthermore, there is an additional dimerization motif known as the PAS domain (named after the first proteins in which this domain was discovered; Period, Aryl hydrocarbon nuclear translocator and Similar) (Semenza, 2000c). The PAS domain, found in the N-terminal of both subunits, is divided into two subunits PAS-A and PAS-B mediating heterodimerization and DNA binding as shown in **Figure 11** (Chun et al.,

2002; Semenza, 2000c). HIF-1 α also contains transactivation domains (TAD) at both the N-terminus (NAD) and the C-terminus (CAD) separated by an inhibitory domain (ID), which is suppressed under hypoxic conditions leading to HIF-1 transcriptional activation (Chun et al., 2002). HIF-1 β contains a TAD near the C-terminus but it does not mediate transcriptional activation (Huang et al., 1998).

HIF-1 can be stabilized by both O₂-dependent and O₂-independent mechanisms as illustrated in **Figure 12** (D'Angio and Finkelstein, 2000; Chun et al., 2002; Semenza, 2000a; Wenger, 2000). O₂-independent mechanisms utilize chemical treatments that mimic the hypoxic environment, such as the iron chelator desferrioxamine and transition metals such as cobalt, nickel and manganese (Chachami et al., 2004; Chun et al., 2002; Clavijo et al., 2007). Chemical inhibition of the proteasome can also mimic the hypoxic condition (Chun et al., 2002). HIF-1 β is constitutively expressed and always present in the nucleus, while HIF-1 α highly regulated through its oxygen dependent degradation domain (ODDD) (Chun et al., 2002). The ODDD contains two PEST-like motifs that are rich in proline (P), glutamic acid (E), serine (S), and threonine (T), proposed to expedite degradation of the proteins in which they are contained (Safran and Kaelin, 2003). Under normoxic conditions, HIF-1 α is degraded in less than 10 minutes by the ubiquitin-proteasome pathway (Chun et al., 2002; Semenza, 2007).

A)

B)

Figure 11: The schematic representation of the HIF α and HIF β subunits and their important functional domains: **A)** The structure of HIF α and HIF β (ARNT) in linear form highlighting the main domains including the basic-helix-loop-helix (bHLH) domain mediating DNA binding; the inhibitory domain (ID) containing the oxygen dependent degradation domain (ODDD); amino (NLS-N) and carboxyl-terminal nuclear localization signals (NLS-C) mediating translocation of the subunits to nucleus; proline-serine-threonine-rich protein stability (PSTD) and Period/ARNT/Single minded (PAS) domains mediating dimerization. HIF-1 α is an 826 amino acid protein containing two transactivation domains (N- and C-terminal TADs) which activate transcription through the interactions of HIF-1 with the basal transcription machinery by recruiting CBP/p300 to activate transcription of target genes (Semenza, 2000c). **B)** The structure of the heterodimeric transcription factor HIF comprised of HIF α and HIF β subunits. Various heterodimers can be formed from interaction of one of three alpha subunits (HIF-1 α , HIF-2 α and HIF-3 α) with one of the three beta subunits (HIF-1 β , HIF-2 β , and HIF-3 β). HIF-1 α also contains the ODDD containing two LXXLAP sequences (where L is leucine, X is any amino acid, A is alanine and P is proline). HIF-1 β lacks the N-TAD and ODDD domains (taken from Willmore, 2004).

Figure 12: Schematic representation of HIF-1 α stabilization versus degradation pathways: Under normoxic conditions, HIF-1 α is destabilized by hydroxylation, ubiquitination and subsequent degradation by the proteasome. Under hypoxic conditions, HIF-1 α is not hydroxylated or ubiquitinated, stabilized and translocated to the nucleus to dimerize with HIF-1 β . The stable HIF-1 heterodimer will bind to Hypoxia Response Elements (HREs) in the promoters and enhancers of hypoxia-inducible genes, promoting gene transcription (D'Angio and Finkelstein, 2000).

The O_2 -dependent regulation of HIF α is initiated by the posttranslational modification known as hydroxylation. Hydroxylation is the addition of an OH group to specific amino acids by means of families of enzymes known as hydroxylases. HIF α hydroxylation is regulated by both proline and aspartate/asparagine hydroxylases (Lando et al., 2002; Safran and Kaelin, 2003). HIF α proline hydroxylases (proline hydroxylase domain-containing hydroxylases or PHDs) recognize a specific sequence of amino acids (LXXLAP where L = leucine, X = any amino acid, A = alanine and P = proline), found twice within the ODDD, within which PHDs hydroxylate the proline residues at amino acids 402 and 564 (Chun et al., 2002). Hydroxylases (dioxygenases) depend upon oxygen, iron (II) and ascorbate as cofactors as well as 2-oxoglutarate (α -ketoglutarate) and amino acids such as lysine, aspartate, asparagines, phenylalanine, tyrosine, tryptophan and proline as substrates (Lando et al., 2002; Safran and Kaelin, 2003). Hydroxylated HIF α interacts with the von Hippel-Lindau tumour suppressor protein (pVHL), which is the substrate recognition subunit of the E3 ubiquitin ligase (Safran and Kaelin, 2003). pVHL only binds substrate proteins containing hydroxylated LXXLAP sequences, resulting in the substrate protein's polyubiquitination by the E3 ubiquitin ligase complex (Safran and Kaelin, 2003). pVHL cannot bind to non-hydroxylated LXXLAP sequences (Safran and Kaelin, 2003). Hydroxylated and polyubiquitinated HIF α is degraded by the 26S proteasome. Thus, the degradation of HIF α is dependent upon its hydroxylation and requires one atom of molecular O_2 to be incorporated into the OH groups attached to the protein (Safran and Kaelin, 2003).

With the removal of oxygen, one PHD substrate is lacking and HIF α is not hydroxylated, ubiquitinated or degraded. It may then be transported to the nucleus and

dimerize with HIF β to transactivate hypoxia-dependent gene expression as shown in **Figure 12** (D'Angio and Finkelstein, 2000). Moreover, HIF α contains two TADs in which the C-TAD contains an asparagine residue (Asn803) that is hydroxylated by an asparagine hydroxylase known as Factor Inhibiting HIF (FIH) (Lando et al., 2002; Safran and Kaelin, 2003). In the presence of oxygen, Asn803 is hydroxylated and this prevents the recruitment of the coactivators CREB-binding protein (cAMP-responsive element-binding-1; CBP or p300) leading to a repression of HIF transactivation (Sharp et al., 2004; Wenger, 2000). Therefore, hydroxylation is considered to be a key event in regulating HIF α by modulating its interaction with both pVHL and CBP/p300 in an oxygen-dependent manner.

Recent studies have uncovered other proteins that are regulated by hydroxylation in a similar manner as HIF α . These proteins contain the same LXXLAP sequence found in HIF α . Examples of these include the Rpb1/Rbp2 subunits of RNA polymerase II, which are hydroxylated by PHDs in a manner similar to HIF α (Kuznetsova et al., 2003). This was discovered by studying computational models of Rpb1 subunits, followed by immunoprecipitation assays and *in vitro* ubiquitination. Another study was performed on erythroid-specific aminolevulinic acid synthase (ALAS2), which is the first and rate-limiting enzyme responsible for the synthesis of heme for hemoglobin. ALAS2 was shown to be regulated in a HIF- independent manner due to the presence of a LXXLAP motif found within the protein (Abu-Farha et al., 2005). Therefore, identification of LXXLAP sequences in other proteins can identify other candidates that may be regulated and/or degraded in a manner similar to HIF α .

HIF α degradation can also be an O₂-independent process in the presence of hypoxic mimics such as the transition metals cobalt, nickel and manganese. These metals have the ability to substitute for iron in the PHD enzymes, thus lowering their affinity for oxygen (Chachami et al., 2004; Chun et al., 2002; Wang et al., 1995). Likewise, iron chelation inactivates hydroxylases as active enzymes have an iron-sulfur cluster (thus the requirements for iron) at their active site.

In the absence of oxygen, HIF α is stabilized, travels to the nucleus where it dimerizes with HIF β . The dimer may then bind to HIF DNA recognition sequence (5'-RCGTG-3') known as the Hypoxia Response Element (HRE) located in the cis-regulatory regions of hypoxia-regulated genes (Chun et al., 2002; D'Angio and Finkelstein, 2000; Safran and Kaelin, 2003; Semenza 2000c; Wenger, 2000). HIF is known to be directly associated with the transcription of over 60 hypoxia-inducible genes including those that play major roles in metabolism (energy, iron, glucose, extracellular-matrix, amino acid, nucleotide metabolisms), cell survival, angiogenesis, apoptosis, cell motility and adhesion, erythropoiesis, vascular tone, etc (Semenza, 2003). These include genes such as vascular endothelial growth factor (*VEGF*) and erythropoietin (*EPO*) (Semenza, 2003; D'Angio and Finkelstein, 2000; Safran and Kaelin, 2003). HREs were first discovered when studying *EPO* gene expression; the *EPO* enhancer, placed into a reporter gene vector followed by transient transfection into mammalian cells and testing reporter gene function, expressed the reporter differentially under different oxygen conditions (Semenza, 2000b; Semenza, 2003; Wang and Semenza, 1993). Mutations in the *EPO* HRE sites prevented the binding of HIF and eliminated the transcription of the reporter gene (Semenza, 2000b; Semenza, 2003; Wang and Semenza, 1993). Therefore,

identification of HREs in the promoters and enhancers of genes suggest potential hypoxia regulation by HIFs.

6.7. Hypothesis and Objectives

The development of neurodegenerative diseases has been shown to be more prevalent in patients that have suffered from cardiovascular diseases such as stroke (Del Barrio et al. 2005; Hu et al., 2006; Lee et al., 2006; Moroney et al., 1996; Struck et al., 1990; Tatemichi et al., 1994). Associated with stroke is a cessation of blood flow to a portion of the brain (ischemia) resulting in a decrease in oxygen (hypoxia) to the affected tissues. Proteomic studies have shown that the amount of α SNCA increases in response to perinatal hypoxia/ischemia in children (Hu et al., 2006). The possibility exists that hypoxia upregulates expression of α -synuclein at both the gene and protein levels. The possibility that hypoxia regulates *SNCA* and α SNCA expression remained to be tested. Identification of HREs in the *SNCA* promoter suggested that α -synuclein expression may be oxygen-dependent. HIF may potentially regulate α -synuclein expression, under hypoxic conditions, leading to the eventual development of PD.

The research for my Masters thesis attempted to link hypoxia-associated ischemic cardiovascular diseases, such as stroke, to the development of PD. My project focused on studying the expression of α -synuclein in response to hypoxia. **My hypothesis was that hypoxia may promote both gene and protein expression of α -synuclein in a HIF-dependent manner.** My approach a) identified potential HRE-Cis acting domains in the α -synuclein promoter, b) examined α SNCA expression of both soluble and insoluble forms of the protein by Western blot in mammalian cell lines in response to hypoxia, c)

determined *SNCA* expression in response to hypoxia by real-time RT-PCR, d) produced knockdowns of *SNCA* gene expression by siRNA and assessed cell viability under hypoxic conditions and e) assessed α SNCA and *SNCA* knockdown by Western blot and real-time RT-PCR respectively. The mammalian cell lines utilized for this study included human embryonic kidney (HEK293T), an adherent clone of the rat pheochromocytoma (PC12-AC) and human neuroblastoma (SH-SY5Y) cell lines.

7. MATERIALS AND METHODS

7.1. Promoter Search for Hypoxia-Inducible α -Synuclein Cis-acting Elements

The genomic sequence (promoter and enhancer) of *SNCA* (-10,700 to +443) was analyzed for potential HRE sites. This was done using the Human Genome Resources under the NCBI website and downloading the α -synuclein genomic sequence using Sequence Viewer. This sequence was searched for cis-acting elements utilizing the online program Transcription Factor Binding Sites Search (TFSearch; Threshold value = 85) (www.cbrc.jp/htbin/nph-tfsearch).

7.2. Chemicals and Cell Culture

All chemicals and kits were purchased from BioShop Canada Inc. (Burlington, ON) unless otherwise specified. Human embryonic kidney cells (HEK293T), as shown in **Figure 13**, were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Eagle's modified Dulbecco's medium (DMEM) supplemented with 10% heat-inactivated horse serum, 3% antibiotics-antimycotic (10,000 Units/mL penicillin G (sodium salt), 10,000 μ g/mL streptomycin sulphate, and 25 μ g/mL amphotericin B as Fungizone® in 0.85% saline) (Invitrogen, Carlsbad, CA). The T-subtype of HEK293 cells express the SV40T antigen which permits the extrachromosomal replication of transfected plasmids containing a SV40 origin of replication. The plasmid also confers neomycin resistance to allow antibiotic selection in HEK293T cells (Cooper et al., 1997).

A

High Density Scale Bar = 100µm

B

High Density Scale Bar = 100µm

C

High Density Scale Bar = 100µm

Figure 13: The three cell lines used to study α -synuclein gene and protein expression under normoxic and hypoxic conditions: The images represent high density of all three cell lines: **A)** human embryonic kidney cells, T-subtype (HEK293T) adherent cells; **B)** an adherent clone (AC) of rat adrenal medullary pheochromocytoma (PC12-AC) cells; **C)** human neuroblastoma (SH-SY5Y) adherent and floating cells (the American Type Culture Collection (ATCC), Manassas, VA).

An adherent clone of the rat adrenal medullary pheochromocytoma cell line (PC12-AC), as shown in **Figure 13**, was obtained as a gift from S.A.L. Bennett (Department of Biochemistry, University of Ottawa, Ottawa, ON) and maintained in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum and 3% antibiotics-antimycotic (10,000 Units/mL penicillin G (sodium salt), 10,000 µg/mL streptomycin sulphate, and 25 µg/mL amphotericin B as Fungizone® in 0.85% saline) (Invitrogen, Carlsbad, CA).

Human neuroblastoma cell line (SH-SY5Y) derived from bone marrow, as shown in **Figure 13**, was obtained as a gift from S.T. Hou (Institute for Biological Sciences, National Research Council of Canada, Ottawa, ON) and maintained in a 1:1 mixture of Earl's minimal essential medium (EMEM) and nutrient mixture F-12 supplemented with 10% heat-inactivated fetal bovine serum and 3% antibiotics-antimycotic (10,000 Units/mL penicillin G (sodium salt), 10,000 µg/mL streptomycin sulphate, and 25 µg/mL amphotericin B as Fungizone® in 0.85% saline) (Invitrogen, Carlsbad, CA).

Cells were grown in 75 cm² flasks at a seeding density of 1×10^6 cells/mL at 37°C, 21% O₂ and 5% CO₂ in a humidified tissue culture incubator (Thermo Forma, Marietta, OH). Confluent cultures were maintained weekly by initially washing in 1 X phosphate buffered saline (10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, pH 7.4; Sigma, St. Louis, MO) and followed by trypsinization. Trypsinization was stopped with the addition of media and cells were diluted to the appropriate concentrations in media.

7.3. Hypoxia Treatments and Chemical Hypoxic Mimetics

Prior to seeding of cells for experiments, the cell density was determined by counting the cell number on a haemocytometer and assaying cell survival by the trypan blue exclusion method (Altman et al., 1993). This method was used to assess the proportion of live to dead cells. Living cells are colourless while dead cells are stained blue. Briefly, 90 μ l of cells were mixed with 10 μ l trypan blue solution (0.4%) (Sigma Chemical Co, St. Louis, MO) in an Eppendorf tube and cells were counted using haemocytometer. Cells were seeded at 1.5×10^5 cells per 6 cm plate 24 hrs prior to treatments. Cells treated with hypoxic conditions (1% O₂, 5% CO₂, 37°C) were placed in a triple-gas incubator (Thermo Forma, Marietta, OH) for the time of the treatment. Cells were also treated with chemical treatments that mimic hypoxic conditions including cobalt (100 μ M cobalt chloride; Sigma, St. Louis, MO), desferoxamine (100 μ M desferoxamine mesylate; Sigma, St. Louis, MO) and proteasome inhibitor (PI; 10 μ M MG132 (Cbz-Leu-Leu-Leu-al); Sigma, St. Louis, MO) (Lee and Goldberg, 1998).

7.4. Cell Harvest, Whole Cell Lysis and Isolation of Soluble α SNCA

After treatment, cells were harvested by washing with ice cold 1 X phosphate buffered saline (10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, pH 7.4; Sigma, St. Louis, MO), removed from plates with cell scrapers, pelleted by centrifugation at 5,000 rpm for 3 minutes at 4°C using IEC-Multi RF centrifuge (Thermo IEC, Needham Heights, MA) and flashed frozen in liquid nitrogen. Cell pellets were stored at -80°C until lysis. Cells were frozen within five minutes of removing them from hypoxic conditions.

For whole cell extraction and isolation of soluble α SNCA, cell pellets were lysed in a whole cell lysis buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 25% glycerol, 500 μM PMSF and 500 μM dithiothreitol. Cell pellets were then incubated on ice for 5 min. Cell lysates were then centrifuged at 5,000 rpm for 30 sec at 4°C using IEC-Multi RF centrifuge (Thermo IEC, Needham Heights, MA) and the supernatant was removed and centrifuged once more at 13,000 rpm for 15 min at 4°C. The remaining pellet was stored at -80°C for further urea extraction. Following centrifugation, the supernatant containing the soluble protein was aliquotted and stored at -80°C.

7.5. Urea Extraction of Insoluble α SNCA

The urea extraction procedure was done on the remaining pellet stored at -80°C from whole lysis extraction. The solubilization procedure was performed according to that found in *Expression of Cloned Genes in Escherichia coli* protocol manual (Cold Spring Harbor Laboratory, NY, 1982). Cell pellets were resuspended in urea lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.1 μM PMSF and 8 M urea. Resuspended cell pellets were incubated for 1 hr at room temperature and then mixed with one volume of a buffer containing 50 mM KH_2PO_4 (pH 10.7), 1 mM EDTA and 50 mM NaCl. The pH of the final mix was adjusted to 8.0 with HCl and incubated at room temperature for 30 min and then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant containing the insoluble protein was aliquotted and stored at -80°C.

7.6. Protein Determination of Soluble and Insoluble α SNCA

The protein concentration of whole cell lysates and urea extracts were determined using the Bio-Rad Protein Dye Reagent (Bio-Rad, Hercules, CA), with bovine serum albumin (Sigma, St. Louis, MO) as a standard, according to the manufacturer's protocol.

7.7. Western Blot Analysis

α SNCA protein level was analyzed in normoxic and hypoxic HEK293T, PC12-AC and SH-SY5Y whole cell and urea extracts. Protein samples of 68 to 100 μ g were resuspended in equal amounts of Laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% β -mercaptoethanol (Sigma, St. Louis, MO). The protein mixtures from different treatments were boiled for 3 min, and loaded on a 12% (w/v) sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) in running buffer (25 mM Tris (pH 8.3), 250 mM glycine, and 0.1 % SDS) and 4 % stacking gel to separate the proteins. Protein standards for SDS-PAGE were the Precision Plus Kaleidoscope Standards with broad range (10 to 250 KDa) (Bio-Rad, Hercules, CA). Proteins were transferred from the gel to an Immobilon-P PVDF transfer membrane (Millipore, Bedford, MA) overnight at 4°C at 180 mA using transfer buffer (20 mM Tris-HCl (pH 8.0), 150 mM glycine and 20% methanol). The separation and transfer of proteins were performed using a Bio-Rad mini-gel and transfer apparatus (Bio-Rad, Hercules, CA).

Non-specific binding of proteins to the PVDF membrane was blocked by incubating the membrane with 5 % non-fat powdered milk in TBST (20 mM Tris-HCl

(pH 7.6), 137 mM NaCl, and 0.1 % Tween 20) for 1 hr at room temperature with agitation. Western blot analysis for α SNCA was performed using monoclonal mouse antihuman α SNCA antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), mouse antihuman β -tubulin (1:1,000 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA), monoclonal horseradish peroxidase (HRP)-labelled mouse anti-Actin antibody (1:800 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish peroxidase-labelled antimouse IgG secondary antibody (1:2,000 dilution; DAKO Cytomation Inc., Mississauga, ON). Membranes were washed with TBST and proteins were detected using the Renaissance Western Blot Chemiluminescence Reagent (Millipore, Bedford, MA) followed by exposure to Kodak X-OMAT-AR film according to the manufacturer's protocol. Protein bands were quantified by densitometry (AlphaEaseFC V.3.1.2., Alpha Innotech Co., San Leandro, CA).

7.8. RNA Isolation and Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the treated cells using the RNeasy Mini kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. The only modification made was the use of the spin technology with centrifugation at maximum speeds 13,000 rpm for 25 sec instead of the suggested speed at 10,000 rpm for 15 sec because this procedure produces higher RNA yield. RNA integrity was verified by OD_{260}/OD_{280} nm absorption ratio > 1.7. Gene specific primers were used for the amplification of the target gene *SNCA* (134 bp amplicon):

Forward primer, 5'-ACAAGTGCTCAGTTCCAATGTGCC-3'

Reverse primer, 5'-GTGAAAGGGAAGCACCGAAATGCT-3'

As well for the amplification of the control housekeeping gene β -actin (279 bp amplicon):

Forward primer, 5'-GCGGGAAATCGTGCGTGCATT-3'

Reverse primer, 5'-GATGGAGTTGAAGGTAGTTTCGTG-3'

SNCA primers were generated by Integrated DNA Technology (Coralville, IA) and β -actin primers were generated by Sigma Genosys (Oakville, ON). One hundred ng of total RNA was reverse transcribed into cDNA and PCR amplified in the same tube using the iScript™ One-Step RT-PCR kit with SYBR® Green (Bio-Rad, Hercules, CA) containing combination of iScript RNase H⁺ reverse transcriptase and iTaq™ DNA polymerase. Further, the 2 X SYBR® Green RT-PCR Reaction Mix contains the iTaq antibody-mediated Hot-Start DNA polymerase that denatures irreversibly upon heat activation and releases active iTaq DNA polymerase (Bio-Rad, Hercules, CA).

Real-time RT-PCR was used to quantify the relative changes in *SNCA* expression in comparison to a reference gene (β -actin). Real-time RT-PCR reactions were performed on the Rotor-Gene 6000 (Corbett Research, Montreal, QC). A 50 μ l reaction mixture was prepared to the indicated final concentration containing 25 μ l of SYBR® Green RT-PCR reaction mix (2X), 1.5 μ l of forward and reverse primers (10 μ M each), 1 μ l of iScript Reverse Transcriptase for one-step RT-PCR and nuclease free water. The components were added to 100 ng total RNA and the reaction mixture was incubated following

protocol; cDNA synthesis at 50°C for 10 min, iScript Reverse Transcriptase inactivation at 95°C for 5 min, PCR cycling and detection for 40 cycles at 95°C for 10 sec (denaturation), 60°C for 30 sec (annealing) and 72°C for 30 sec (primer extension). A melt curve step (95°C for 1 min, 55°C 1 min and 55°C for 10 sec, for 80 cycles, increasing each by 0.5°C each cycle) was included after each run. The fluorescence of each sample was analyzed to determine the initial copy number of genes using Rotor-Gene 6000 Software v.1.7. (Corbett Research, Montreal Biotech Inc., Montreal, QC). The critical threshold (Ct value; the point at which the fluorescence increased above the background fluorescence of the sample) was obtained manually for each run. A sample of total RNA was serially diluted in ten-fold dilutions and amplified to create the standard curve. RT-PCR products were resolved on a 2 % agarose gel with ethidium bromide and visualized using UV light. The resulting bands were analyzed with an AlphaEaseFC gel documentation system and quantified by densitometry (AlphaEaseFC v.3.1.2., Alpha Innotech Co., San Leandro, CA).

7.9. Design of siRNA Molecules

Small-interference RNA (siRNA) molecules were used to achieve the knockdown of α SNCA in HEK293T cells. RNAi technology is based on the preexisting protein expression inhibition pathway triggered via the insertion of an artificial double-stranded RNA (dsRNA), designed for a target gene (Dykxhoorn and Lieberman, 2005). The dsRNA molecule enters the cell and binds only to the RNA molecule of its complementary gene, creating a double stranded RNA molecule, triggering specialized

proteins to digest it. The pathway is initiated by the digestion of dsRNA's, by an enzyme called Dicer, into 20-25 nucleotide fragments, called small interfering RNAs (siRNA). Subsequently, the siRNA recruits RNA-induced silencing complexes (RISCs) that unwind the siRNA. This process of unwinding produces siRNA-RISC complex that targets complementary mRNA molecules and cleaves them. The destruction of the endogenous RNA template disables the production of the targeted protein.

The knockdown of α SNCA was carried out using synthesized siRNA molecules (Santa Cruz Biotechnology, Santa Cruz, CA), including a pool of 3 target-specific 20 to 25 nt siRNAs designed to knockdown gene expression in human cells. The siRNA vial contained 3 nmol of lyophilized siRNA making sufficient for a 10 μ M solution, once resuspended, following the manufacturer's protocol. A fluorescent control siRNA (Fluorescein Conjugate)-A non-targeting 20 to 25 nt siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control for transient transfection. The scrambled sequence, conjugated to fluorescein, would not lead to the specific degradation of any cellular mRNA. The transient transfection efficiency was monitored using fluorescence microscopy (Olympus BH2-RFCA, San Leandro, CA).

7.10. Transient Transfection of HEK293T Cells

HEK293T cells were plated in antibiotic-free growth medium at a density of 1.5×10^6 cells/mL in 6-well plates and 2 mL of DMEM supplemented with 10% horse serum for 24 hours. Transfections were carried out using Lipofectamine™2000 (Invitrogen, Carlsbad, CA). The siRNA molecules were diluted in 100 μ L of Opti-MEM® I Reduced

Serum Medium from GIBCO (Grand Island, NY) to 0.25 to 2 μg siRNA/mL and mixed with LipofectamineTM2000 in an equal volume of Opti-MEM medium according to the manufacturer's protocol. Cells were incubated with the siRNA/LipofectamineTM2000 solution for 24 hrs, after which regular growth media was added. After changing to regular growth medium, cells were treated with either normoxia or hypoxia (1 % O₂) for 3 to 24 hrs. Cells were harvested after treatments, centrifuged and cell pellets stored at -80°C until lysis for subsequent total protein isolation for Western blot or total RNA isolation for real-time RT-PCR analysis as previously described. Alternatively, cells were assessed for viability after treatment using the MTT assay as described below.

7.11. MTT Assay Cell viability under siRNA-SNCA and Hypoxic Conditions

The dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO) was used to assess cell viability in these studies. The MTT assay was used to measure the viability of HEK293T cells under both knockdown of α SNCA and exposure to hypoxia. The MTT assay system works on the principle of conversion of the soluble yellow tetrazolium salt (MTT) to blue-black insoluble formazan product (Mosmann, 1983). The formazan product is only formed when the MTT enters the mitochondria of metabolically active living cells and is reduced by dehydrogenases of the respiratory chain. The insoluble blue-black formazan product can then be solubilized in dimethylsulfoxide (DMSO) and the colour can be quantified by spectrophotometry with a strong absorption at 570 nm.

The MTT (Sigma, St. Louis, MO) stock solution was prepared to a final concentration of 5 mg/mL in PBS. Following siRNA transfection and hypoxia treatments, stock MTT was added to each plate at one tenth of the original media volume and the plates were incubated at 37°C in a tissue culture incubator for 2 hours. After incubation, the media was replaced with the same volume of DMSO (Sigma, St. Louis, MO) to dissolve the formazan precipitates, and incubated in the tissue culture incubator for another 10 min at 37°C. The absorbance of the blue solution was then measured in a 96-well microplate using a SpectraMax 340^{PC} microplate reader (Molecular Devices, Sunnyvale, CA) at 570 nm and corrected by subtracting the background absorption at 630 nm. The data analysis of the absorbance values were normalized to control cells under normoxic conditions with a value of 100%.

7.12. Statistical Data Analysis

Data analysis of α -synuclein gene and protein expression was quantified by densitometry (AlphaEaseFC v.3.1.2., Alpha Innotech Co., San Leandro, CA.). All results were expressed as means $\pm$ S.E.M. of at least three independent experiments unless otherwise stated. Data analysis was performed using the Student's t-test for two-sample assuming unequal variances (assuming the two data sets came from distributions with unequal variances representing measurements for each subject before and after treatment) with a value of $p \leq 0.05$ considered statistically significant in comparison to controls.

8. RESULTS

8.1. Analysis of Hypoxia-Inducible α -Synuclein Cis-acting Elements

The binding sites for various transcription factors, as well as twenty six potential HRE sites with the recognition sequence (5'-RCGTG-3'), were identified in the genomic sequence (promoter and enhancer) of *SNCA* (-10,700 to +443). Twelve of these potential HREs sites corresponded to the sequence 5'-ACGTG-3' repeated in positions -10,050 to -10,055; -9536 to -9541; -8796 to -8801; -8215 to -8220; -6042 to -6047; -5471 to -5476; -3715 to -3720; -3616 to -3621; -2712 to -2717; -1894 to -1899; -1674 to -1679; -130 to -135, nine of the HREs sites corresponded to the sequence 5'-GCGTG-3' repeated in positions -9527 to -9532; -6775 to -6780; -5667 to -5672; -2490 to -2495; -2465 to -2470; -2142 to -2147; -1374 to -1379; -741 to -746; -150 to -155, and five HRE site correspond to 5'-TCGTG-3' repeated in positions -9757 to -9762; -3604 to -3609; -1691 to -1696; -1278 to -1283; -953 to -958 in the 5'- regulatory region of the α -synuclein promoter.

8.2. Changes of α SNCA Soluble Protein Levels in HEK293T Cells under Hypoxic Conditions

To investigate whether hypoxia promotes an increase in expression of α SNCA, α SNCA protein levels, were analyzed in whole cell extracts from HEK293T cells treated with normoxic (21% oxygen) and hypoxic (1% oxygen) conditions using Western blots and a monoclonal mouse antihuman α SNCA antibody (1:500 dilution; recognizes human, rat and mouse proteins). Cells were also treated with chemical treatments that mimic

hypoxic conditions including cobalt (100 μ M cobalt chloride), desferoxamine (100 μ M desferoxamine mesylate), and proteasome inhibitor (PI; 10 μ M MG132; Cbz-Leu-Leu-Leu-al) (Clavijo et al., 2007; Lee and Goldberg, 1998 ; Wang et al., 1995; Wang and Semenza, 1993). The immunoblot analysis showed three bands of approximately 19, 75 and 130 kDa in molecular weight (**Figure 14A**). Prestained Broad Range Protein Markers (Bio-Rad, Hercules, CA) were used for molecular weight determination. The 19 kDa band represents, specifically, the presence of α SNCA as a monomer (Cookson, 2005). However, α SNCA is known to aggregate and form oligomers (Tsigelny et al., 2007). Thus, the 75 and 130 kDa bands were determined to be the tetramer and hexamer aggregated forms of α SNCA respectively.

Visual inspection, along with densitometric analysis (**Figure 14B**), showed a significant increase in the monomer form of α SNCA under short-term hypoxia (3 to 6 hours of hypoxia), proteasome inhibitor (MG132) and an iron chelator (desferoxamine (DFX); $p \leq 0.05$). Under long-term hypoxia exposure (16 to 24 hours of hypoxia), there was a significant shift from the monomeric to the aggregated forms (tetramer and hexamer) of α SNCA ($p \leq 0.05$). However, variable results were obtained under 48 hours of hypoxia treatment, showing no significant changes in both the monomer and the aggregated forms of α SNCA. Changes in α SNCA protein expression were normalized to levels of β -tubulin (detected at 50 kDa) by mouse antihuman β -tubulin antibody (1:1,000 dilution; Developmental Studies Hybridoma Bank, Iowa City, Iowa).

A

B

Figure 14: Western blot analysis of α SNCA soluble protein expression in HEK293T cells under hypoxic conditions. Top panel: Western blots for α SNCA were performed using an antibody specific to α SNCA and whole cell extracts for soluble protein prepared from HEK293T cells after normoxia and hypoxia exposures. **A)** Representative Western blot with anti-human- α SNCA and anti-human- β -tubulin. Top panel: the bands at 19, 75 and 130 kDa represent the monomeric (M), tetrameric (T) and hexameric (H) forms of α SNCA respectively as indicated on the right. Position of the molecular weight marker (MW) in kDa is indicated on the left. Bottom panel: the band at 50 kDa is β -tubulin, used as a control for loading and normalization. **B)** Densitometry analysis of changes in α SNCA soluble protein expression after treatments. Data represent means $\pm$ S.E.M. from six independent experiments with * = $p \leq 0.05$, compared to normoxia.

8.3. Changes of Insoluble α SNCA Protein in HEK293T Cells Under Hypoxic Conditions

To further investigate the effect of hypoxia exposure on α SNCA protein expression, insoluble forms of the protein were analyzed in urea extracts from HEK293T lysates using Western blotting and the specific antibody to the α SNCA protein as previously mentioned. The results showed one band at 130 kDa (**Figure 15A**). The band was consistent with previous soluble extraction and thus was labelled as the hexamer form of α SNCA. Visual inspection, along with densitometry, showed significant increase in the insoluble hexamer during long-term hypoxia of 16 hours ($p \leq 0.05$, **Figure 15B**). Further, significant increase in the insoluble hexamer form was also seen under 24 to 48 hours hypoxia treatments ($p \leq 0.05$). No changes were seen under short term hypoxia (3 to 6 hours) and chemical treatments that mimics hypoxia. All results were normalized to β -tubulin which was detected at 50 kDa.

8.4. Changes of Insoluble α SNCA Protein in PC12-AC in Comparison to Soluble Levels Under Hypoxic Conditions

The protein levels of soluble and insoluble α SNCA were further studied in the whole cell extracts from PC12-AC exposed to long-term hypoxia and cobalt treatments using Western blotting. The results showed two bands with molecular weights of 37 and 75 kDa (**Figure 16A**) which represented the dimeric and tetrameric forms of α SNCA. Visual detection showed an oversaturation of the signals on Western blots, in both bands, under all conditions. The densitometric analysis showed no significant changes for both the dimeric and tetrameric soluble forms under long term hypoxia (16 to 24 hours) and

cobalt treatments (**Figure 16B**, $p \leq 0.05$). These results were normalized to β -tubulin, which was used as loading control.

To further investigate the expression and aggregation of the α SNCA protein, the insoluble protein levels during normoxia and hypoxia were analyzed in urea extracts from PC12-AC cells using Western blotting. The results showed two bands at 37 and 75 kDa in molecular weight (**Figure 16A**). These two bands represented the dimeric and tetrameric forms of α SNCA respectively. Oversaturation of signal was detected in the insoluble tetramer bands versus a weaker signal detected in the insoluble dimer bands. Densitometry analysis showed a significant increase in the insoluble tetrameric form of α SNCA under cobalt chloride treatments (**Figure 16B**). These results were normalized to β -tubulin, which was used as loading control.

A

B

Figure 15: Western blot analysis of insoluble α SNCA protein expression in HEK293T cells under hypoxic conditions: Westerns for α SNCA were performed using an antibody specific to α SNCA with urea cell extracts for insoluble protein prepared from HEK293T cells after normoxia and hypoxia exposures. **A)** Representative Western blot with anti-human- α SNCA and anti-human- β -tubulin. Top panel: the bands at 130 kDa represent the hexameric (H) forms of α SNCA as indicated on the right. Position of the molecular weight marker (MW) in kDa is indicated on the left. Bottom panel: the band at 50 kDa is β -tubulin. β -Tubulin was used as a control for loading and normalization. **B)** Densitometry analysis of changes in α SNCA soluble protein expression after treatments. Data represent means $\pm$ S.E.M. from three independent experiments with * = $p \leq 0.05$, compared to normoxia.

A

B

Figure 16: Western blot analysis of soluble versus insoluble α SNCA protein expression in PC12-AC cells under hypoxic conditions: Western blots for α SNCA were performed using an antibody specific to α SNCA and urea cell extracts for insoluble protein prepared from PC12-AC cells after normoxic and hypoxic exposures. **A)** Representative Western blot with anti-human- α SNCA and anti-human- β -tubulin. Top panel: the bands at 37 and 75 kDa represent the dimeric (D) and tetrameric (T) forms of α SNCA respectively, as indicated on the right. Position of the molecular weight marker (MW) in kDa is indicated on the left. Bottom panel: the band at 50 kDa is β -tubulin. β -Tubulin was used as a control for loading and normalization. **B)** Densitometry analysis of changes in α SNCA soluble protein expression after hypoxia treatment. Data represent means $\pm$ S.E.M. from three independent experiments with * = $p \leq 0.05$, compared to normoxia.

8.5. Changes of Soluble α SNCA Protein in SH-SY5Y Cells Under Hypoxic Conditions

The protein levels of soluble α SNCA in SH-SY5Y cells were studied during normoxic and hypoxic conditions in whole cell extracts from SH-SY5Y lysates using Western blotting. The results showed the presence of one band at 75 kDa in molecular weight (**Figure 17A**). This one band represented the tetrameric soluble form of α SNCA and densitometric analysis showed no significant changes ($p \leq 0.05$) under long-term hypoxia exposure. However, under short-term hypoxia (6 hours), there was a significant increase in the tetrameric soluble form of α SNCA protein (**Figure 17B**). The results were normalized to β -tubulin which was used as a control for loading.

8.6. Protein Expression of Insoluble α SNCA in SH-SY5Y Cells Under Hypoxic Conditions

To further understand the changes in α SNCA in SH-SY5Y cells, urea extracts were used to study the insoluble forms of α SNCA by Western blot. The results showed two bands at 75 and 130 kDa in molecular weight (**Figure 18A**). The two bands were consistent with the previous results and were labelled as tetramer and hexamer forms of α SNCA respectively. Visual inspection along with densitometry analysis showed a significant increase ($p \leq 0.05$) in the tetrameric form of the insoluble α SNCA under long-term hypoxia exposure (between 16 and 48 hours of hypoxia) in comparison to 24 hours of normoxia (**Figure 18B**). However, the hexameric form showed a significant decrease

under short-term hypoxia exposure, long-term hypoxia, cobalt treatment and proteasome inhibitor treatment compared to normoxia. All α SNCA changes were normalized to tubulin which was used as a loading control (which was detected at 50 kDa in molecular weight).

8.7. Optimization For Total RNA Amount by Real-Time RT-PCR

The gene expression of *SNCA* was measured using iScript One-Step RT-PCR with SYBR Green (Bio-Rad, Hercules, CA) which contains a master mix of iScript RNase H⁺ reverse transcriptase and iTaq antibody-mediated hot-start DNA polymerase which releases fully active iTaq DNA polymerase upon heat activation. Real-time RT-PCR was used to relatively quantify the changes in *SNCA* gene expression in comparison to reference gene (β -actin) using gene specific primers. Real-time RT-PCR reactions were performed on the Rotor-Gene 6000 (Corbett Research, Montreal, Quebec).

A

B

Figure 17: Western blot analysis of soluble α SNCA protein expression in SH-SY5Y cells under hypoxic conditions: Western blots for α SNCA were performed using an antibody specific to α SNCA and whole cell extracts for soluble protein prepared from SH-SY5Y cells after normoxic and hypoxic exposures. **A)** Representative Western blot with anti-human- α SNCA and anti-human- β -tubulin. Top panel: the band at 75 kDa represents the tetrameric (T) form of α SNCA as indicated on the right. Position of the molecular weight marker (MW) in kDa is indicated on the left. Bottom panel: the band at 50 kDa is β -tubulin. β -Tubulin was used as a control for loading and normalization. **B)** Densitometry analysis of changes in α SNCA soluble protein expression after hypoxia treatments. Data represent means $\pm$ S.E.M. from three independent experiments with * = $p \leq 0.05$ compared to normoxia.

A

B

Figure 18: Western blot analysis of insoluble α SNCA protein expression in SH-SY5Y cells under hypoxic conditions: Western blots for α SNCA were performed using an antibody specific to human α -SNCA and urea cell extracts for insoluble protein prepared from SH-SY5Y lysates after normoxic and hypoxic exposure. **A)** Representative Western blot with anti-human- α SNCA and anti-human-tubulin. Top panel: the band at 75 kDa represents the tetrameric (T) form of α SNCA as indicated on the right. Position of the molecular weight marker (MW) in KDa is indicated on the left. Bottom panel: the band at 50 KDa is tubulin. Tubulin was used as a control for loading and normalization. **B)** Densitometry analysis of changes in α SNCA soluble protein expression after hypoxia treatments. Data represent means $\pm$ S.E.M. from three independent experiments with $p \leq 0.05$ compared to normoxia; * = increase in tetramer, ** = decrease in hexamer.

In order to optimize for gene expression, total RNA was obtained from normoxic HEK293T cells. Real-time RT-PCR was performed across a broad range of total RNA concentrations (1 pg to 100 ng) to determine the optimum amount of total RNA to use for comparisons. The log-phase of cDNA amplification, with a manual set of critical threshold (Ct) value (the point at which the fluorescence increased above the background fluorescence of the sample) (**Figure 19A**), was taken for analysis. The melting curve showed a single specific peak at higher concentrations of total RNA (100 pg to 100 ng) (**Figure 19B**). Furthermore, the standard curve showed a best fit line with a continuous decrease of Ct values correlated with an increase of total RNA utilized in the reaction (**Figure 19C**). From the real-time RT-PCR profiles, it was concluded that the optimum amount of total RNA was 100 ng for further analysis of *SNCA* gene expression.

8.8. Gene Expression of *SNCA* in HEK293T, PC12-AC, and SH-SY5Y Cells Under Long-Term Hypoxic Versus Normoxic Conditions

To compare *SNCA* gene expression in different mammalian cells, real-time RT-PCR was utilized to assess *SNCA* gene expression in three different cell lines using gene-specific primers. The quantification profiles, along with the melt curve analysis, showed an average Ct value of 20 for both HEK293T and SH-SY5Y cells under normoxic and hypoxic conditions (**Figure 20**). However, there were no changes of *SNCA* gene expression during long-term hypoxia when compared to normoxia. Furthermore, the *SNCA* gene expression profile in PC12-AC cells had a significantly higher Ct value than SH-SY5Y cells, indicating lower gene expression. However, the *SNCA* gene-specific

primers were designed specifically to human gene and not the rat gene and thus primers may not have bound as tightly.

8.9. Gene Expression of *SNCA* in HEK293T Under Hypoxic Conditions by Real-Time RT-PCR

To investigate whether increased α SNCA protein expression during hypoxia correlated with increased expression of the *SNCA* gene, *SNCA* gene levels during normoxic and hypoxic conditions were analyzed using total RNA isolated from HEK293T cells, the iScript One-Step RT-PCR kit with SYBR Green and gene-specific primers. The results of the quantification profiles for Ct values, along with the densitometric analysis, showed no significant changes during short- and long-term hypoxia treatments (**Figure 21**). All results were normalized to the internal reference gene of β -actin for real time RT-PCR. Thus, *SNCA* gene expression is unaffected in HEK293T cells during hypoxic conditions.

A

B

C

Figure 19: Real-time RT-PCR analysis of *SNCA* gene expression using different amounts of total RNA isolated from HEK293T cells under normoxic conditions: (■ 1 pg to ■ 100 ng) of total RNA from normoxic HEK293T cells was reverse transcribed and amplified using gene-specific primers for the *SNCA* gene. **A)** Quantification analysis performed using the iScript One-Step RT-PCR with SYBR Green and gene-specific primers across a range of concentrations from 1 pg to 100 ng of total HEK293T RNA. **B)** Melt curve analysis of RT-PCR reactions showing no nonspecific PCR artefacts and revealing a single clean melting peak for all high-concentration samples. **C)** The standard curve analysis of Ct values across a range of concentrations (1 pg to 100 ng) of total RNA isolated from HEK293T cells.

A **B**

Figure 20: Real-time RT-PCR analysis of *SNCA* gene expression at 100 ng of total RNA from HEK293T, PC12-AC, and SH-SY5Y cells during hypoxic and normoxic conditions: iScript One-Step RT-PCR with SYBR Green with gene-specific primers for the *SNCA* gene were used to analyze 100 ng of total RNA from 24 hours normoxic and 24 hours hypoxic HEK293T, PC12-AC and SH-SY5Y cells. **A)** Quantification analysis of α -SNCA gene expression in HEK293T RNA ■ 24 hours normoxic; ■ 24 hours hypoxic, PC12-AC RNA ■ 24 hours normoxic; ■ 24 hours hypoxic, SH-SY5Y RNA ■ 24 hours normoxic; ■ 24 hours hypoxic. **B)** Melt curve analysis of real-time RT-PCR reactions showing no nonspecific PCR artefacts and a single clean melting peak for all high concentrated samples.

A **B** **C**

Normoxia (h)	24	—	—	—	—	24	24
Hypoxia (h)	—	6	16	24	48	—	—
Cobalt (h)	—	—	—	—	—	24	—
MG132 (h)	—	—	—	—	—	—	6

SNCA (134 b.p.)

β -actin (279 b.p.)

D

E

Figure 21: Real-time RT-PCR analysis of *SNCA* gene expression in HEK293T cells under hypoxic conditions: the iScript One-Step RT-PCR kit with SYBR Green and gene-specific primers for the *SNCA* gene were used to analyze 100 ng of total RNA from HEK293T cells. **A)** Quantification analysis of *SNCA* gene expression in HEK293T RNA ■ 24 hours normoxic; ■ 6 hours hypoxic; ■ 16 hours hypoxic; ■ 24 hours hypoxic; ■ 48 hours hypoxic; ■ cobalt chloride (24 hours normoxic); ■ proteasome inhibition (6 hours normoxic). **B)** Melt curve analysis of real-time RT-PCR reactions showing no nonspecific PCR artefacts revealing a single, clean melting peak for all high concentration RNA samples. **C)** Analysis of gene products resolved via gel electrophoresis revealing a single amplicon of correct molecular weight (*SNCA*, 134 bp; β -actin, 279 bp). **D)** Analysis of the changes in Ct values of the treatments shown in **A)**. **E)** Densitometry analysis of changes in *SNCA* gene expression of the treatments shown in **C)**. β -actin is used as internal reference gene for real-time RT-PCR. Data represent means $\pm$ S.E.M. from four independent experiments with $p \leq 0.05$ compared to normoxia.

8.10. Gene Expression of *SNCA* in SH-SY5Y Cells Under Hypoxic Conditions by Real-Time RT-PCR

To further study *SNCA* gene expression under hypoxic conditions, gene levels were analyzed from total SH-SY5Y RNA under normoxic and hypoxic conditions by real-time RT-PCR using *SNCA* gene-specific primers. The results, including the quantification profiles with Ct values and the densitometry analysis, showed no significant changes in the *SNCA* gene between normoxia and hypoxia (**Figure 22**). All results were normalized to the β -actin gene as an internal reference. Thus, the gene expression of α SNCA does not change in SH-SY5Y cells between normoxic and hypoxic conditions.

8.11. Knockdown By siRNA Molecules Targeting *SNCA*

The knockdown of *SNCA* was carried on using synthesized siRNA molecules (Santa Cruz Biotechnology, Santa Cruz, CA), including a pool of 3 target-specific 20-25 nt siRNAs designed to knockdown α -synuclein gene and protein expression in human cells.

A **B** **C**

Normoxia (h)	24	—	—	—	—	24	24
Hypoxia (h)	—	6	16	24	48	—	—
Cobalt (h)	—	—	—	—	—	24	—
MG132 (h)	—	—	—	—	—	—	6

SNCA (134 b.p.)

β -actin (279 b.p.)

D

E

Figure 22: Real-time RT-PCR analysis of *SNCA* gene expression in SH-SY5Y cells under hypoxic conditions: the iScript One-Step RT-PCR kit with SYBR Green and gene-specific primers for the *SNCA* gene were used to analyze 100 ng of total RNA from SH-SY5Y cells. **A)** Quantification analysis of *SNCA* gene expression in SH-SY5Y cells RNA ■ 24 hours normoxic; ■ 6 hours hypoxic; ■ 16 hours hypoxic; ■ 24 hours hypoxic; ■ 48 hours hypoxic; ■ cobalt chloride (24 hours normoxic); ■ proteasome inhibition (6 hours normoxic). **B)** Melt curve analysis of real-time RT-PCR reactions showing no nonspecific PCR artefacts revealing a single clean melting peak for all high concentrated samples. **C)** Analysis of gene products resolved via gel electrophoresis revealing a single amplicon of correct molecular weight (*SNCA*, 134 bp; β -actin, 279 bp). **D)** Analysis of the changes in Ct values of the treatments shown in **A)**. **E)** Densitometry analysis of changes in *SNCA* gene expression of the treatments shown in **C)**. β -actin is used as internal reference gene for real-time RT-PCR. Data represent means $\pm$ S.E.M. from four independent experiments with $p \leq 0.05$ compared to normoxia.

8.12. Optimization of Amount of siRNA Molecules for Maximal *SNCA* Knockdown by Western Blot Analysis

To investigate the effects of knockdown by siRNA molecules on *SNCA*, the protein expression of both soluble and insoluble α SNCA during siRNA knockdown were studied in whole cell and urea cell extracts from HEK293T cells transfected with 0.25 to 1 μ g of siRNA using Western blot. The results for both soluble and insoluble protein showed the three bands at 19, 75 and 130 kDa in molecular weight (**Figure 23**). The three bands were consistent with previous results and were the monomeric, tetrameric and hexameric forms of α SNCA, respectively. The monomer was detected in both soluble and insoluble protein, the tetramer in soluble protein and the hexamer in insoluble protein. Visual inspection, along with densitometry analysis, showed a decrease in the monomer in both soluble and insoluble proteins under the transfection with 1 μ g of siRNA molecules, in comparison to controls without siRNA. Also, decreases in the monomer α SNCA were shown under the effect of both 0.25 and 0.5 μ g of siRNA, in both soluble and insoluble protein. However, no changes were detected in either the tetrameric or hexameric forms of soluble and insoluble α SNCA protein. All the results were normalized to either actin or β -tubulin (detected at 42 and 50 kDa respectively). These results concluded that 1 μ g of siRNA molecules were effective in causing a detectable knockdown of α SNCA at the protein level as shown by the monomer.

A **D** **B** **E** **C** **F**

Figure 23: Western blot analysis of α SNCA knockdowns by (0.25 to 1 μ g) siRNA molecules in HEK293T cells under normoxic conditions: Western blots for α SNCA were performed using an antibody specific to human α SNCA with both whole cell and urea cell extracts for soluble and insoluble proteins respectively, prepared from HEK293T cells after transfection with siRNA molecules targeting specifically α SNCA under normoxia. **A and D)** Representative Western blots with anti-human- α SNCA for soluble and insoluble proteins; **B and E)** anti- β -tubulin and anti-actin respectively. The bands at 19, 75 and 130 kDa represent the monomeric (M), tetrameric (T) and hexameric (H) forms of α SNCA as indicated on the right; β -tubulin and actin occur at molecular weights of 42 and 50 kDa respectively. β -tubulin and actin were used as controls for loading and normalization. Position of the molecular weight marker (MW) in kDa is indicated on the left. **C and F)** Densitometry analysis of changes in soluble and insoluble α SNCA respectively after transfection with different amounts (0.25 to 1 μ g) of siRNA molecules.

8.13. Optimization of Transfection Time for siRNA Knockdown by Western Blot Analysis

To further investigate the effect of siRNA molecules in knocking down α SNCA protein expression, HEK293T cells were transfected with optimal amount of siRNA (1 μ g) and harvested at different times of incubation under normoxic conditions. Both the soluble and insoluble proteins were studied from the whole cell and urea extracts of HEK293T cells respectively, using Western blots. The results showed three bands with molecular weights of 19, 75 and 130 kDa (**Figure 24**). These bands represented the monomeric, tetrameric and hexameric forms of α SNCA respectively. Visual analysis, along with the densitometry analysis, showed a decrease of the monomer form of α SNCA during short-term incubation with siRNA in comparison to controls. The 12 and 24 hour incubation periods showed clear effective knockdown of α SNCA in comparison to controls. However, the longer incubation times of 48 and 72 hours overcome the knockdown effect of siRNA and the monomeric form of the α SNCA increased in comparison to the shorter time of incubation and the controls. All results were normalized to β -tubulin.

A **D** **B** **E** **C** **F**

Figure 24: Western blot analysis of α SNCA knockdown by 1 μ g siRNA molecules in HEK293T cells under different durations of normoxic conditions: Western blots for α SNCA were performed using an antibody specific to α SNCA with both whole cell and urea cell extracts for soluble and insoluble proteins respectively, prepared from HEK293T cells after transfection with siRNA molecules targeting *SNCA* under normoxic conditions. **A and D)** Representative Western blots with anti-human- α SNCA in soluble and insoluble protein; **B and E)** anti-human- β -tubulin. The bands at 19, 75 and 130 kDa represent the monomeric (M), tetrameric (T) and hexameric (H) forms of α -SNCA respectively as indicated on the right; the band at 50 kDa is β -tubulin. β -tubulin was used as a control for loading and normalization. Position of the molecular weight marker (MW) in kDa is indicated on the left. **C and F)** Densitometry analysis of changes in soluble and insoluble α SNCA protein after transfection with 1 μ g siRNA for different times of normoxia.

8.14. Transfection Efficiency of siRNA Molecules by Fluorescence Microscopy

To further investigate and optimize the transfection method and ensure the delivery of siRNA molecules to the cytoplasm of HEK293T cells, cells were transfected with a fluorescent positive siRNA control (Fluorescein Conjugate-A non-targeting 20-25 nt siRNA; Santa Cruz Biotechnology, Santa Cruz, CA). The scrambled sequence conjugated to fluorescein would not lead to the specific degradation of any cellular mRNA. Fluorescent microscopy (Olympus BH2-RFCA, San Leandro, CA) was used to image the fluorescent siRNA (**Figure 25**). The results showed that the majority of HEK293T cells were transfected with control siRNA indicating high efficiency of delivery.

8.15. Changes of Soluble Monomer α SNCA Protein Expression in HEK293T Under siRNA Knockdown and Hypoxic Conditions

To investigate the effect of α SNCA knockdown with the optimum amount of siRNA molecules followed by hypoxia exposure in HEK293T cells, soluble α SNCA expression was measured using Western blots in whole cell extracts of HEK293T transfected with siRNA molecules and treated with hypoxia. The Western blot showed a lower band at 19 kDa in molecular weight and was labelled as the monomeric form of α SNCA (**Figure 26A**). Visual detection along with densitometry analysis showed a significant decrease in the monomer protein level ($p \leq 0.05$) during the 24 hours normoxic with siRNA knockdown to 58% of control levels. Also, due to short-term hypoxia treatments (6

hours), a significant increase in the monomeric protein level was detected compared to its 24 hours normoxia control ($p \leq 0.05$). Furthermore, during long-term hypoxia exposure, the monomer levels decreased significantly at 16 hours of hypoxia with siRNA by 50% compared to the controls ($p \leq 0.05$). Also, a significant decrease in the monomer level ($p \leq 0.05$) was shown at 24 hours hypoxia with siRNA by 65% compared to its controls. All the densitometry results were normalized to the levels of β -tubulin which was used as a loading control.

8.16. Changes of Soluble Tetramer α SNCA Protein Expression in HEK293T Cells Under siRNA Knockdown and Hypoxia

To further study the knockdown of α SNCA protein expression by siRNA molecules and the effect of the hypoxic conditions on the aggregation of α SNCA, the tetrameric soluble form was analyzed by Western blot from whole cell extracts of HEK293T cells transfected with the optimal amount of siRNA and further exposed to short- and long-term hypoxia. The results show that no significant changes ($p \leq 0.05$) occurred in the tetrameric soluble form of α SNCA protein (75 kDa in molecular weight) at 24 hours of normoxic siRNA treatment compared to nontransfected controls (**Figure 27**). There were no significant changes in the tetramer during both short- and long-term hypoxia with siRNA knockdown compared to their controls ($p \leq 0.05$). All the results were normalized to β -tubulin which was used as a loading control.

Figure 25: Localization of fluorescent small interference RNA (siRNA) molecules in the cytoplasm region of HEK293T cells: The transfection efficiency of siRNA molecules were analyzed using a fluorescent positive control siRNA (Fluorescein Conjugate-A) and imaged by fluorescence microscopy (Olympus BH2-RFCA, San Leandro, CA). The majority of the HEK293T cells were fluorescent showing efficient delivery of siRNA. **A)** fluorescent siRNA within cells (green) under fluorescence microscopy; **B)** HEK293T cells under brightfield microscopy; **C)** Overlay images with fluorescent siRNA (green). Bar = 50 μm .

A

B

Figure 26: Western blot analysis of soluble monomeric form of α SNCA protein expression in HEK293T cells under siRNA knockdown and hypoxia conditions:

Western blots for α SNCA were performed using an antibody specific to α SNCA and whole cell extracts for soluble protein prepared from HEK293T cells after transfection with 1 μ g siRNA molecules targeting *SNCA* under normoxic and hypoxic conditions. **A)** Representative Western blot with anti-human- α SNCA and anti-human- β -tubulin. (-) indicating no siRNA, (+) with siRNA. The band at 19 kDa is monomeric (M) form of α SNCA as indicated on the right; the band at 50 kDa is β -tubulin. β -tubulin was used as a control for loading and normalization. **B)** Densitometry analysis of changes in soluble α SNCA protein expression after siRNA knockdown and hypoxia treatments. Data represent means $\pm$ S.E.M. from five independent experiments with $p \leq 0.05$ compared to normoxia; * = hypoxia, ** = siRNA.

A

B

Figure 27: Western blot analysis of the soluble tetrameric form of α SNCA protein expression in HEK293T cells under siRNA knockdown and hypoxia treatments:

Western blots for α SNCA were performed using an antibody specific to α SNCA and whole cell extracts for soluble protein prepared from HEK293T cells after transfection with 1 μ g siRNA molecules targeting α -synuclein under normoxic and hypoxic conditions. **A)** Representative Western blot with anti-human- α SNCA and anti-human- β -tubulin. (-) indicating no siRNA, (+) with siRNA. The band at 19 kDa is monomeric (M) form of α SNCA as indicated on the right; the band at 50 kDa is β -tubulin. β -tubulin was used as a control for loading and normalization. **B)** Densitometry analysis of changes in soluble α SNCA protein expression after siRNA knockdown and hypoxia treatments. Data represent means $\pm$ S.E.M. from four independent experiments with $p \leq 0.05$ compared to normoxia.

8.17. Cell Viability of HEK293T Under siRNA Knockdown and Hypoxia by MTT Assay

The results showed a significant decrease in cell viability under all hypoxic treatments; including short- and long-term hypoxia exposure; in the absence of siRNA compared to 24 hours normoxia ($p \leq 0.05$) (**Figure 28**). Cell viability decreased significantly from 6 to 24 hours of hypoxia by 24% compared to normoxia. There was no significant change between 3 hours of hypoxia and 24 hours of normoxia. With α SNCA knockdown, HEK293T cell viability significantly decreased during 24 hours normoxia to 94% of normoxic control levels without siRNA. The absorbance values were normalized to the control cells under normoxic conditions with a value of 100% (**Figure 28B**). The results also showed a significant decrease in viability by 10% at 24 hours of hypoxia with knockdown compared to 24 hours of hypoxia without knockdown ($p \leq 0.05$). However, no significant changes were found at 3, 6 or 16 hours hypoxia with knockdown compared to 16 hours of hypoxia without knockdown.

These results indicated that hypoxic conditions, from 6 to 24 hours, had a significant effect on HEK293T cell survival. Furthermore, the α SNCA knockdown by siRNA molecules had a significant effect on reducing cell viability under 24 hours normoxic and hypoxic conditions compared to their controls. Thus, these findings suggested that α SNCA knockdowns had significant effect on reducing cell viability but not to the same extent as hypoxia.

A

B

Figure 28: Cell viability of HEK293T cells under α SNCA knockdown by siRNA molecules and hypoxia exposure as measured by MTT: The viability of HEK293T cells was measured under α SNCA knockdown and hypoxia treatments by MTT. **A)** Cell viability of HEK293T under hypoxia and siRNA treatments. **B)** Cell viability of HEK293T with the absorbance values being normalized to the control cells under normoxic conditions. Data represent means $\pm$ S.E.M. from four independent experiments with $p \leq 0.05$ compared to normoxia; * = hypoxia, ** = siRNA.

8.18. Gene Expression Analysis of *SNCA* in HEK293T Cells Under siRNA Knockdown and Hypoxia by Real-Time RT-PCR

To investigate whether decrease in the monomeric form of soluble α SNCA protein expression during siRNA knockdown and hypoxia exposure correlated with the gene expression profile, *SNCA* gene levels during transfection with 2 μ g of siRNA, followed by hypoxia, were analyzed from total HEK293T RNA using iScript One-Step RT-PCR kit with SYBR Green and gene-specific primers. The results of both the quantitative profiles with Ct values and the densitometry analysis showed no significant changes at the gene level by either siRNA knockdown or hypoxia exposure (**Figure 29**). All the results were normalized to β -actin used as an internal reference gene. These results indicate that the effect of siRNA knockdown, along with hypoxia treatment, was only significant at the level of the protein and not at the level of the gene.

A

B

C

D **E**

Figure 29: Real-time RT-PCR analysis of *SNCA* gene expression in HEK293T cells transfected with 2 μ g siRNA molecules under hypoxic conditions: iScript One-Step RT-PCR with SYBR Green and gene-specific primers for *SNCA* gene were used to analyze 100 ng of total RNA from HEK293T cells. **A)** Quantification analysis of *SNCA* gene expression in total RNA from HEK293T cells ■ 24 hours normoxia; ■ 24 hours normoxia/siRNA; ■ 6 hours hypoxia; ■ 6 hours hypoxia/siRNA; ■ 16 hours hypoxia; ■ 16 hours hypoxia/siRNA; ■ 24 hours hypoxia; ■ 24 hours hypoxia/siRNA. **B)** Melt curve analysis of RT-PCR reactions performed using iScript One-Step RT-PCR kit with SYBR Green showing no nonspecific PCR artefacts revealing a single clean melting peak for all high concentration RNA samples. **C)** Analysis of the gene products resolved via gel electrophoresis revealing a single amplicon of correct molecular weight (*SNCA*, 134 bp; β -actin, 279 bp); (-) indicating no siRNA; (+) with siRNA. β -actin was used as internal reference gene for real-time RT-PCR. **D)** Analysis of the changes in Ct values under siRNA/hypoxic conditions. **E)** Densitometry analysis of changes in *SNCA* gene expression after siRNA knockdown and hypoxia treatments. Data represent means $\pm$ S.E.M. from three independent experiments with $p \leq 0.05$ compared to normoxia.

9. DISCUSSION

The *SNCA* gene product plays a pivotal role in the pathogenesis of PD. The α SNCA protein is believed to be responsible for the neuronal death and resulting dementia in PD (Lotharius and Brundin, 2002). PD is characterized by the death of SNpc dopaminergic neurons (Sawle, 2004; Thomas and Beal, 2007). The pathological hallmark of PD is the formation of LBs which are highly dense spherical cytoplasmic insoluble protein aggregates (William and Przedborski, 2003). The major constituent of these LBs are the aggregated insoluble forms of α -synuclein protein (Cookson, 2005; Ross and Poirier, 2004; Sawle, 2004; Thomas and Beal, 2007). These heavily insoluble polymers and fibrils of α SNCA are highly enriched with β -pleated sheet-like interactions and covalent modifications (Cookson, 2005; Cookson and Van der Brug, 2007; Ross and Poirier, 2004; Tsigelny et al., 2007).

Recent studies have shown that α -synuclein protein can form higher order of aggregates of dimers, tetramers, hexamers and ring-structures that can propagate head to head and head to tail interactions (Tsigelny et al., 2007). Studies have shown that multiple factors play a role in initiating aggregations, such as covalent modifications and phosphorylations of proteins, the presence of ROS and protein aging due to the decrease in cell ability to degrade misfolded proteins (Ross and Poirier, 2004). All of these factors, along with various environmental elements, can facilitate the formation of toxic aggregates which eventually lead to cell death (apoptosis) (Taylor et al., 2002; William and Przedborski, 2003).

Ischemic stroke, which is a low oxygen (hypoxic) condition, plays a role in neuronal degeneration through ROS production (Del Barrio et al. 2005; Hu et al., 2006; Lee et al., 2006; Moroney et al., 1996; Struck et al., 1990; Tatemichi et al., 1994). Studies have shown that the amount of α SNCA increases in response to perinatal hypoxia/ischemia in children (Hu et al., 2006). It is therefore essential to examine α -synuclein mRNA levels and protein expression under short- and long-term hypoxic conditions.

This study shows that the levels of both soluble and insoluble α SNCA protein are significantly increased in response to hypoxia. Short-term hypoxia caused significant increases of the monomeric soluble form of α SNCA protein; whereas long-term hypoxia caused significant increases at the hexameric insoluble form of α SNCA protein. Despite the presence of putative HREs located within the α -synuclein promoter, gene expression profiles of *SNCA* were unchanged under short and long-term hypoxia. Increased aggregations of α SNCA due to long-term hypoxia may be one of the key factors linking the increased incidence of PD following ischemic stroke.

9.1. Putative HRE Sites Search

Analysis of the α -synuclein genomic sequence revealed twenty six potential HRE sites within the 5'-regulatory region of α -synuclein promoter. The aim of this search was to find potential cis-acting elements that can possibly be regulated by HIF-1 transcription factor under hypoxic conditions. HIF-1 is known to activate the transcription of over 60 hypoxia-inducible genes including those that play a role in cell survival, angiogenesis

(growth of new vessels), glucose metabolism, erythropoiesis, etc (D'Angio and Finkelstein, 2000; Safran and Kaelin, 2003; Semenza, 2003). These genes are regulated by the stabilization of HIF-1 heterodimer which bind to HIF DNA recognition sequences (5'-RCGTG-3' where R can be A, G, T) known by the hypoxia response elements (HREs) located in the promoters and enhancers of hypoxia-regulated genes (Chun et al., 2002; D'Angio and Finkelstein, 2000; Lee et al., 2006; Safran and Kaelin, 2003; Semenza, 2000c; Wenger, 2000). These HREs sites were first discovered when studying erythropoietin gene expression which stimulates erythroid cell production in a HIF-1-dependent mechanism (D'Angio and Finkelstein, 2000; Fandrey et al., 1994, Semenza, 2000b; Wang and Semenza, 1993). Therefore, the identification of HREs in the promoters and enhancers of genes suggest possible hypoxic regulation by HIFs. To investigate whether each HRE sequence is an actual binding site for HIF-1 under hypoxia, primary analysis of *SNCA* gene expression was performed later to verify the possible regulation of *SNCA* at the mRNA level under hypoxic conditions.

9.2. Protein Expression of α SNCA in HEK293T Cells Under Hypoxic Conditions

The protein expression of both soluble and insoluble forms of α SNCA was analyzed in response to short- and long-term hypoxic conditions in non-neuronal like cells HEK293T. The α SNCA protein was found in three possible forms as monomer, tetramer and hexamer in HEK293T cells. The results showed a significant increase in the soluble monomeric form of α SNCA protein expression under short-term hypoxia (3 to 6 hours) and chemical treatments such as proteasome inhibitor (MG132) and

desferoxamine (DFX) ($p \leq 0.05$). However, under long-term hypoxia (16 to 24 hours), a significant increase in both the soluble and insoluble hexameric form of α SNCA was detected ($p \leq 0.05$). Further, significant changes were also detected in the insoluble hexameric form of α SNCA protein under 48 hours hypoxic treatments ($p \leq 0.05$). Thus, these findings suggested the possibility of increased accumulation of α SNCA during short-term hypoxia leading to aggregation of the protein during long-term hypoxia in HEK293T cells. This supports the possibility that hypoxia may promote the production or stability of the monomeric linear structure of α SNCA protein under short-term condition followed by further formation of high molecular weight insoluble protein forms under longer hypoxic exposure.

Studies have shown that the monomeric form of α -synuclein is in equilibrium with the membrane-associated forms, which are characterized by higher helical content (Cookson, 2005). However, the presence of mutations, oxidative stress such as ROS, phosphorylations, dopamine (DA) and toxins, will eventually lead to oxidative modification of the monomeric unfolded protein and will increase its rate of aggregation, formation of oligomers or protofibrils and eventually highly insoluble polymers of protein known by fibrils (Cookson, 2005; Ross and Poirier, 2004; Uversky et al., 2001). Studies have characterized these aggregated forms through molecular modelling and molecular dynamics, indicating the various configurations of these propagating multimers that have unfavourable binding to membranes and can be formed by docking monomers to dimers or by addition of dimers to dimers resulting in the tetramer and the hexamer forms (Tsigelny et al., 2007).

α SNCA protein is known to play an important role in the formation of synaptic vesicles at the presynaptic terminals of dopaminergic neurons where 50% of the protein is associated with membrane and 50% is cytosolic (Lotharius and Brundin et al., 2002). Despite the fact that HEK293T cells are non-neuronal-like cells, studies have used these cells as models to look at α SNCA protein expression (Chen et al., 2008; Cookson and Van der Brug, 2007; Orth et al., 2003). Research has shown the possible functions of α SNCA protein in non-neuronal cells, indicating its important role in being associated with membrane structures such as mediating the trafficking of vesicles in between Endoplasmic Reticulum (ER) and Golgi apparatus, along with its association with the mitochondrial membrane and enhancing its susceptibility to toxic compounds (Cookson and Brug, 2007; Lucking and Brice, 2000; Orth et al., 2003).

Studying the protein expression of α SNCA at the cellular level under hypoxic conditions (1% O₂) and treatments that mimic hypoxia, such as iron chelation and proteasome inhibition, is essential in understanding the possible factors that initiate aggregation and the eventual formation of LBs in PD. Previous proteomics studies have showed that, upon the exposure to permanent ligation of the left carotid artery followed by 90 min hypoxia (7.8% O₂), an increase in the amount of α SNCA in perinatal rat brain was detected in the two-dimensional proteome analysis (Hu et al., 2006). Reduced oxygen levels, after an ischemic stroke, creates a hypoxic environment followed by subsequent reoxygenation of tissues can cause the generation of ROS and activation of the apoptotic events (Carbonell and Rama, 2007; Lopez-Neblina et al., 2005; Saito et al., 2005). ROS can react with lipids, DNA, and proteins causing cellular damage and subjecting the cells to oxidative stress (Przedborski, 2005). Furthermore, the presence of

ROS, along with chemicals such as metals, can trigger oxidative modifications of proteins and act as covalent cross-linkers, stabilizing the α SNCA aggregates and resulting in the formation of LBs (Cookson, 2005; Halliday and McCann, 2007; Lücking and Brice, 2000; Uversky et al., 2001).

At the cellular level, hypoxia exposure, which is characterized by reduced levels of oxygen, can lead to the build up of ROS generated by complex III of the mitochondria. Without oxygen as the final electron acceptor in the electron transport chain, electrons build up along the chain and the cytochromes become highly reduced. The presence of 1% O₂ in the intermembrane space will result in oxygen accepting extra electrons from cytochromes and the formation of superoxide, which moves from the mitochondria to the cytosol as illustrated in **Figure 30** (Klimova and Chandel, 2008). Mitochondrial superoxide is converted to H₂O₂ which activates oxidant-signalling pathways and can stabilize HIF factors, which play a critical role in maintaining oxygen homeostasis (Semenza, 2000d). Hypoxic-mimicking chemicals such as cobalt chloride and desferroxamine are clinically tested to induce hypoxic conditions (Clavijo et al., 2007; Wang et al., 1995; Wang and Semenza, 1993). Iron chelation can stabilize HIF factors by removing iron from the active sites of the PHD enzymes, lowering their affinity for oxygen and preventing degradation of HIFs (Chachami et al., 2004; Chun et al., 2002; Wang et al., 1995). Iron chelation can also affect the activity of enzymes in respiratory chain complex, causing a reduction in cellular ATP levels and cellular damage (Clavijo et al., 2007). Also, the use of proteasome inhibitor (MG132), which is a selective sequence of amino acids that blocks the proteasome and inhibits its function in breaking down damaged and inactive proteins, prevents HIF degradation and results in the activation of

hypoxia-inducible gene expression (Lee and Goldberg, 1998; Mircea et al., 2001, Panu et al., 2001).

Thus, understanding the effects of hypoxia on the levels of both soluble and insoluble α SNCA protein is essential in understanding the molecular mechanisms linking hypoxia and the initiation of the formation of α SNCA aggregates and LB formation. These findings provide evidence of a possible effect of hypoxia on the levels of the soluble monomeric form of α SNCA protein under short-term hypoxia, followed by a shift to higher order aggregations of the soluble and insoluble hexameric forms under long-term hypoxia.

9.3. Protein Expression of α SNCA in Neuronal-Like Cells Under Hypoxic Conditions

To investigate the effect of hypoxia and chemical treatments on the regulation of α SNCA protein expression in a neuronal-like cell system, further analysis of both soluble and insoluble α SNCA protein expression was studied in PC12-AC rat pheochromocytoma and SH-SY5Y human neuroblastoma cells. Findings showed the presence of two higher orders of soluble and insoluble protein forms of α SNCA in both PC12-AC and SH-SY5Y cells. Interestingly, in PC12-AC cells, only the dimeric and tetrameric forms were observed with a complete absence of monomer, in comparison to HEK293T cells. The α SNCA protein has a largely unfolded coiled structure which can self aggregate and form large multimers in the cytosol (Tsigelny et al., 2007).

Figure 30: Induction of oxidative stress and stabilization of HIF under hypoxic conditions: In the presence of 1% O₂ (hypoxia), complex III of the mitochondrial transport chain can generate superoxide ions (O₂•⁻) in the intermembrane space of the mitochondria. Superoxide is released to the cytosol, generating H₂O₂. This activates oxygen-dependent signalling pathways, through the stabilization of HIF-1α, leading to hypoxic gene expression (Klimova and Chandel, 2008).

In the case of PC12-AC cells, it is known that environmental factors cause the monomer to rapidly form multimeric structures (Stefanis et al., 2001a). There were no significant changes in the soluble dimeric and tetrameric forms of the α SNCA protein in PC12-AC between normoxic and hypoxic conditions. Since the majority of the changes at the protein level were seen in monomers in other cell lines, changes in α SNCA in PC12-AC may have been masked by rapid aggregation of the protein under long-term hypoxia. Furthermore, these findings were supported by the analysis of the insoluble tetrameric forms of α SNCA, which showed no significant changes under long-term hypoxia in comparison to its normoxic control.

Upon exposure to cobalt chloride, a significant increase of the tetrameric insoluble form of α SNCA in PC12-AC cells was observed. These findings may suggest the possible role of cobalt as an iron substitute in PHD enzymes, inducing a hypoxia-like condition. Cobalt may also produce oxidative stress and have a role in triggering covalent modifications of α SNCA, causing a rapid shift from the undetectable monomer to the higher order aggregates of the α SNCA protein (Chachami et al., 2003; Lopez-Neblina et al., 2005; Uversky et al., 2001; Wang et al., 1995). The fact that there were no observable hexameric forms of α SNCA in the urea extracts of PC12-AC cells, compared to HEK293T, suggests that the higher order aggregates were the most abundant forms in neuronal-like cells (PC12-AC and SH-SY5Y cells) and that the aggregates are highly insoluble and may not be detected with SDS-PAGE. Recent studies have used PC12 cells as a neuronal model in studying the downstream effects of either overexpressing α SNCA protein or expressing the mutant protein types (A53T mutant) and further analyzing the alterations in the cell system (Stefanis et al., 2001b). This is due to the fact that PC12

cells are known to be a sympathetic nerve-like cell line that possesses the ability to change into various phenotypes in response to nerve growth factors (which cause a promotion in differentiation of PC12, modulation of neurotransmitter release and stimulation of neuronal plasticity) (Stefanis et al., 2001a). α SNCA is also known to localize within nerve terminals of differentiated neuronal cells, such as differentiated PC12, and to be associated closely with vesicular structures (Clayton and George, 1998).

Further experiments were performed using SH-SY5Y neuroblastoma cells. SH-SY5Y is a human neuroblastoma cell line derived from bone marrow and has been used in studying human α SNCA protein (Chiba-Falek and Nussbaum, 2001; Junn and Mouradian, 2002). The soluble α SNCA protein expression was analyzed in SH-SY5Y cells under short-term hypoxia, long-term hypoxia, cobalt treatment and treatment with proteasome inhibitor. Interestingly, only the tetrameric form of α SNCA was detected in SH-SY5Y cells, with the absence of both monomeric and dimeric forms, similar to that seen in PC12-AC cells. These results show that the monomeric form of α SNCA is difficult to detect in neuronal-like cells due to its tendency to rapidly form aggregates. Furthermore, significant changes were seen in the soluble tetrameric form of α SNCA under short-term hypoxia exposure (6 hours) in SH-SY5Y cells with no changes under long-term hypoxia.

However, long-term hypoxic conditions (16 to 48 hours of hypoxia) caused a significant increase in the insoluble tetrameric protein of α SNCA in SH-SY5Y cells, compared to normoxic controls. This suggested that short-term hypoxia caused a significant increase of the tetrameric soluble form and that long-term hypoxia caused further aggregation into the insoluble form of the tetramer that can only be seen by urea

extraction. Interestingly, a significant decrease in the hexameric forms of α SNCA protein was detected under short-term hypoxia, long-term hypoxia and chemical treatments suggesting the possibility that there may be a shift of these hexameric forms into higher order aggregates that can no longer be detected by SDS-PAGE.

Thus, studying α SNCA protein expression in neuronal-like cells under hypoxia treatment was complicated due to the difficulty in detecting the monomer; the form in which the most changes occur with hypoxia in other cell lines. This is due to the presence of a highly rich environment of macromolecules within these cells that promote rapid aggregations by molecular crowding along with oxidative stress and chemicals (Cookson, 2005). Therefore, all further studies on the changes in α SNCA protein expression in response to hypoxia were carried out in HEK293T cells due to detectable changes at the monomer level.

9.4. Gene Expression of *SNCA* in HEK293T, PC12-AC, SH-SY5Y Cells Under Hypoxic Conditions

To further understand the effect of short- and long-term hypoxia, along with chemical treatments, on *SNCA* gene expression and to test its correlations to the corresponding increase at the protein level, real-time RT-PCR was performed with human *SNCA* gene-specific primers and optimal amounts of total RNA (100 ng) from HEK293T cells. *SNCA* gene expression profiles did not follow α SNCA protein expression during hypoxia and chemical treatments; *SNCA* gene expression was stable in HEK293T cells whereas protein levels increased in the soluble monomeric form under

short-term hypoxia and chemical treatments and in the insoluble hexamer form under long-term hypoxia. Similar findings were obtained from *SNCA* gene expression profiles in SH-SY5Y cells. The gene expression did not change in SH-SY5Y cells in response to hypoxia whereas protein levels increased in the soluble tetrameric form under short-term hypoxia and at the insoluble tetrameric form under long-term hypoxia. These findings suggested that hypoxia does not control the regulation of α SNCA protein expression at the level of the gene, despite the presence of putative HREs in α -synuclein promoter, but most likely at the protein level by promoting the aggregations of the soluble monomeric and dimeric forms into the insoluble tetramers, hexamers and higher order of insoluble fibrils that may not be detected with SDS-PAGE.

9.5. Knockdown of α SNCA by siRNA Molecules in HEK293T Cells

siRNA technology was utilized in studying the protein expression of both soluble and insoluble α SNCA, under siRNA knockdown and hypoxia, in HEK293T cells. Recently the development of new techniques, such as RNA interference (RNAi), have allowed researchers to use this new technology as a tool in understanding and exploring the molecular pathways and players involved in PD (Chen et al., 2008; Häbig et al., 2008; Fountaine and Wade-Martins, 2007; Lewis et al., 2008; Liu et al., 2008; Manfredsson, 2006). RNAi was first identified in plants and fungi back in 1990 as a self-defense mechanism against viruses (Argawal et al., 2003). In 1998, Fire and Mello discovered the presence of double-stranded RNA (dsRNA) as the cause of sequence-specific inhibition of protein expression in *Caenorhabditis elegans* (round worms). Recently, Fire and Mello

were the winners of 2006 Nobel Prize due to their discovery of the function of RNAi. RNAi can be produced in three ways as shown in **Figure 31** (Applied Biosystem/Ambion, Austin, TX): 1) the insertion of an artificial dsRNA (longer than 30bp), 2) the construction of DNA based expression vectors to express short hairpin RNA (shRNA) and 3) the direct synthesis of short interfering RNAs (siRNA). The components of RNAi machinery includes: a) shRNA containing the sense and antisense sequences from a target gene connected by a loop, b) dicer, a protein that processes shRNA into siRNAs and c) RNA-induced silencing complexes (RISCs) which unwind the siRNA, targeting mRNA and thus silencing the expression of functional genes (Agrawal et al., 2003; Dykxhoorn and Lieberman 2005).

In this study, synthesized siRNA molecules (20-25 nucleotides) were designed to knock down α SNCA expression in HEK293T cells. siRNA is a valuable tool in studying the effects of knocking down PD genes (Chen et al., 2008; Häbig et al., 2008; Fountaine and Wade-Martins, 2007; Lewis et al., 2008; Liu et al., 2008; Manfredsson et al., 2006). The initial siRNA knockdown results showed that the optimal amount of siRNA molecules was around 1 μ g per 1.5×10^6 cells/mL and that siRNA had a short-term effect within a 24 hour period following transfection. Also, the α SNCA knockdown was only detectable at the monomer level in comparison to the tetramer and hexamer. One explanation could be due to the fact that these later forms were part of aggregations that were already accumulated and that siRNA knockdown could only target the non-aggregated monomeric form. As an indicator of transfection efficiency, fluorescence microscopy was used to detect the successful delivery of a fluorescein conjugated, non-targeting siRNA into HEK293T cells. The fluorescent images showed that siRNA

Figure 31: Overview of the several ways to induce RNAi: Representative figure of the three methods in applying the siRNA system in cell cultures and animal models are shown. **1)** Insertion of synthesized dsRNA (longer than 30 bp) directly to the cytosol; **2)** Design of siRNA (20-25 nt) and inserting it directly to the cytosol; **3)** Construction of siRNA expression vector and inserting it to nucleus of the cells for constitutive production of siRNAs. The ultimate goal is to silence the protein expression by binding to the complementary sequence of mRNA, and inducing its cleavage by RISC and associated factors (Applied Biosystem/Ambion, Austin, TX).

molecules were successfully delivered to majority of cells (**Figure 25**). Thus, these findings directed our focus towards studying the effect of siRNA knockdown on the monomeric form of α SNCA, only in HEK293T cells, due to their high transfection efficiency and the fact that the monomeric form is only detectable in these cells.

9.6. Gene and Protein Expression of α SNCA in HEK293T Cells Under siRNA Knockdown and Hypoxia

To further investigate the effect of hypoxia on α SNCA aggregation, protein expression analysis of α SNCA soluble forms were studied following siRNA knockdown under short- and long-term hypoxia in HEK293T cells. The results showed that 65% knockdown of α SNCA, at the protein level, by siRNA was achieved. Under normoxic conditions, siRNA knockdown was successful in reducing the soluble monomeric forms of α SNCA protein down to 58% of control levels. Under short-term hypoxia (6 hours), there was a significant increase in the soluble monomeric levels of α SNCA which was consistent with the previous findings of hypoxia treatment in HEK293T cells. However, short-term siRNA knockdown (6 hours) did not significantly reduce the increase of soluble α SNCA monomer under 6 hours of hypoxia. These findings may be due to the short-term exposure of HEK293T cells to siRNA which may not be sufficient enough to cause a knockdown. Thus, the most significant effect of reducing the protein expression of α SNCA was shown at longer-term exposure to siRNA (50% reduction at 16 hours and 65% reduction at 24 hours of siRNA treatment). Interestingly, no significant changes of the soluble tetrameric form of α SNCA protein occurred under siRNA knockdown.

These findings suggested that siRNA treatment had only significant detectable effects at the level of the soluble monomeric α SNCA protein and that knockdown could suppress the effect of hypoxic conditions. Furthermore, these findings may suggest that siRNA cannot target previously aggregated forms of α SNCA directly but may prevent further aggregation under hypoxic conditions indirectly through the decrease of the monomer. Also, these findings showed the possibility that the effect of siRNA knockdown may overcome the effects of long-term hypoxia in HEK293T cells, resulting in reduced α SNCA protein expression under hypoxia and thus may reduce the hypoxic downstream effect of α SNCA aggregation. However, further experiments and testing will be required to ensure the reduction of α SNCA aggregations at the level of the hexamer and higher order of insoluble fibrils.

Further, the siRNA knockdown was tested by studying the mRNA levels profiles of *SNCA* to see if it correlates with its protein expression. The results showed a lack of observable response at the mRNA level under siRNA, which could be due to possible gene-specific aspects and variations within the RT-PCR machine such as variability in the CT values in the technical replicates (Vandesompele et al., 2002). Recent studies have shown the possibility that siRNA knockdown efficiency might not be detected at the level of the mRNA if the protein has a long half-life and thus may only be detectable at the protein level (Invitrogen, 2007; Lewis et al., 2008). Under normal conditions, a very small amount of protein may be sufficient in achieving normal cellular functions (Invitrogen, 2007). Thus, in the case of α SNCA protein, studies have shown that the half-life of α SNCA protein degradation is within the range of 8 hours and thus it is considered to have a short half-life compared to other proteins (of which some have a half-life as

long as 200 hours) (Bennett et al., 1999). Another possible hypothesis is that the cells are producing only a small amount of α SNCA transcripts and that increases in the level of the monomer, and the resulting aggregations, are controlled by environmental stressors (Cookson, 2005).

Recent study have applied the vector mediated method of siRNA to target the knockdown of α SNCA in HEK293 cells and cotransfected with plasmids overexpressing recombinant α -synuclein conjugated to Enhanced Green Fluorescent Protein (EGFP) and further measured the reduction of LBs by fluorescence microscopy and fluorescent immunocytochemistry (Chen et al., 2008). Further, recent studies have also characterized the downregulation of α SNCA protein using naked siRNA duplexes that actively reduced the endogenous *SNCA* mRNA *in vitro* and *in vivo* in the hippocampus of adult mice (Lewis et al., 2008). Other studies evaluated the effect of α -synuclein knockdown in dopaminergic neuroblastoma cells (SH-SY5Y) and the influence of *SNCA* reduction on cell death by analysis of whole genome expression using microarrays (Häbig et al., 2008). Further studies were able to test the effect of reducing α -synuclein expression in human dopaminergic neurons using siRNA molecules and results showed that suppression of α -synuclein protein reduced dopamine transport and increased survival in the presence of MPP toxin (Fountaine and Wade-Martins, 2007). Thus, the knockdown of α SNCA protein is considered to be a potential target for siRNA due to the tendency in potentially reducing the aggregations (Manfredsson et al., 2006).

9.7. Cell Viability of HEK293T Cells Under siRNA Knockdown/Hypoxic Treatments

MTT assay provides a valuable tool for achieving successful measurements of cell viability in a wide range of research (Altman et al., 1993). Various approaches to measure cell viability have been used in the past, such as the trypan blue assay. Trypan blue is based on evaluating cell membrane integrity (and thus cell death) but the assay is not sensitive, with limited accuracy and does not indicate if a cell is alive (Denizot and Lang, 1986). The MTT assay is a colorimetric dye system which works on measuring the reduction of the yellow tetrazolium salt MTT to blue-black insoluble formazan product by the mitochondria of viable (but not dead) cells (Mosmann, 1983). The assay is sensitive and the colour of product can be quantified by spectrophotometry and it is directly proportional to the number of viable cells.

The MTT assay was used to assess cell viability under siRNA knockdown of α SNCA and exposure to short and long-term hypoxia. Hypoxia treatments (6 to 24 hours) had a significant effect in reducing HEK293T cell viability by 24% compared to normoxia. This reduction in cell viability can be due to a) the build up of ROS, changing in cell metabolism and b) the reduction in ATP levels leading to initiation of cell death (Kilmova and Chandel, 2008; Lipton, 1999; Semenza, 2007). Furthermore, the α SNCA knockdown by siRNA caused a reduction in cell viability by only 10% under 24 hours hypoxia compared to controls. These findings suggest that α SNCA reduction may be detrimental to cells in response to environmental hypoxic stress (Kim and Lee, 2008;

Lücking and Brice, 2000). The role of α SNCA in either inducing apoptosis or protecting cells against apoptosis is still under research and in debate (Lücking and Brice, 2000). Studies have shown that upon serum deprivation in HEK293 cells, α SNCA protein expression increases, predicting a protective role in these cells (Kim and Lee, 2008). α -Synuclein knockout studies in mice have suggested its role in regulation of fatty metabolism as well as neurotransmission due to changes in synaptic vesicle recycling and the number of synaptic vesicles (Abeliovich et al., 2000; Kim and Lee, 2008). Also, research has shown that α SNCA can play a role in protecting cells from cytotoxic insults and stress-inducing agents by acting as molecular chaperone (Kim et al., 2000; Kim and Lee, 2008). Recent research also induced the silencing of α SNCA protein expression in MN9D (mouse dopaminergic neuronal cells) using vector mediated RNAi and examined its effect on cell proliferation by growth curves and viability by MTT assay (Liu et al., 2008). Their findings showed a reduction in cell viability of MN9D and induction of cell injury due to the loss of α SNCA suggesting a protective functional role of the protein. Nevertheless, overexpression of α SNCA and natural upregulation of its expression can cause abnormal aggregations which are known to be a major constituent of LBs and play a key role in the pathogenesis of PD (Kim and Lee, 2008). Thus, it is essential to keep the balance and maintenance of α SNCA steady state below the level where it aggregates and this is considered to be a major challenge at the cellular level (Kim and Lee, 2008).

The present study is the first study to examine the potential regulation of α SNCA, at the level of the gene and protein, by hypoxia. This thesis provides evidence supporting the hypothesis that short and long-term hypoxia may play a role in promoting α SNCA aggregation by causing the monomeric soluble forms to shift in the equilibrium that

exists between monomers and aggregates (dimers, tetramers and hexamers), in both the soluble and insoluble forms. However, there were no detectable changes in *SNCA* mRNA levels under long-term hypoxic conditions, suggesting that predicted HRE sites upstream of *SNCA* promoter might not be functional. Nevertheless, these results support our hypothesis that environmental risk factors, such as hypoxia, could control aggregations at the level of the protein. The end product of these aggregations is the formation of heavy insoluble polymers known as fibrils which are thought to be the building blocks of LBs (Cookson, 2005). Applying siRNA technology, along with the cell viability assays, the possibility of indirectly reducing α SNCA aggregation under long-term hypoxia may be possible. The studies also gave insight into aspects of α SNCA functionality by suggesting its protective role in hypoxic survival.

Although the results shown in this thesis provide good evidence of changes in α SNCA protein expression in response to hypoxia, it is not possible to say with certainty that α SNCA aggregations are regulated by short- and long-term hypoxia and that siRNA knockdown can overcome the hypoxia effects by reducing aggregations. Further experiments are required in measuring the aggregations and the amount of LB formation in dopaminergic neuronal cells upon exposure to longer periods of hypoxia. Various methods of measuring α SNCA aggregation and formation of insoluble multimeric structures can be utilized to show their response to hypoxia. Such approaches can include immune-gold labelling to probe the monomeric structures of α SNCA protein and track its presence in LBs, along with fluorescence labelling which will help to detect the dynamic shift in the equilibrium between the α SNCA conformations (Cookson, 2005, Kim and Lee, 2008). These findings have revealed the critical role of hypoxia in the expression of

α -synuclein and link the development of neurodegeneration to ischemic-associated diseases. The growing knowledge will influence our understanding of PD pathogenesis and provide us with the hallmark picture of possible approaches in treating PD patients at earlier stages, especially post-stroke.

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