Regulation of the amyloid precursor protein (APP) gene promoter by hypoxia

By

Agnieszka Bielecki, B.Sc.H.

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

Masters of Science

Biology Department
Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario
September 2006

© Copyright
2006 Agnieszka Bielecki

Library and
Archives Canada

Bibliothèque et
Archives Canada

Published Heritage
Branch

Direction du
Patrimoine de l'édition

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

ISBN: 978-0-494-18359-5

Our file Notre référence

ISBN: 978-0-494-18359-5

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

1. ABSTRACT

Alzheimer's disease is more prevalent following periods of ischemia and the accompanying hypoxia (1% O₂). The amyloid precursor protein (APP) is implicated in the pathogenesis of Alzheimer's disease. Sequence analysis of the APP promoter revealed that it contained three putative Hypoxia Response Elements (HREs) which may bind the Hypoxia Inducible Factor-1 (HIF-1) and induce hypoxic regulation of the promoter. We examined APP promoter activity and APP expression during hypoxia in PC12-AC cells. Reporter gene assays showed that APP promoter activity is activated by both hypoxia (6 to 48 hours) and over-expression of a HIF-1 subunit (HIF-1 α). Exposure of cells to hypoxia resulted in an increase in APP gene expression as well as an accumulation of both the APP and HIF-1 α protein. Electrophoretic mobility shift assays (EMSAs) indicated that HIF-1 may not bind to the APP promoter. These results suggest that hypoxia-induced APP promoter activation may be HIF-1 independent.

2. ACKNOWLEDGEMENTS

I would like to express my appreciation for the people who have supported me throughout the completion of this project. I am grateful to my supervisor, Dr. William Willmore for his consistent support and guidance. I have learned a lot from working with him; his insightful ideas and suggestions, his active attitude towards research, his vast practical and theoretical experience, his preciseness, and his kindness which will remain with me always. I would like to thank my committee members Drs. Susan Aitken, Sean Hayley and Cristofre Martin for their helpful suggestions and support, as well as Drs. Katie Gilmour and John Vierula for being on my thesis defense committee.

I would like to thank my lab mates for their help and M. Kamkar for her advice and assistance operating the luminometer. I owe much gratitude to my family and friends, especially my mother; I would not have accomplished this work without your patience, encouragement and support, thank you for believing in me.

3. TABLE OF CONTENTS

1.	ABSTRACT	ii
2.	ACKNOWLEDGEMENTS	iii
3.	TABLE OF CONTENTS	iv
4.	LIST OF FIGURES AND TABLES	vii
5.	LIST OF ABBREVIATIONS	ix
6.	INTRODUCTION	1
6.1.	Alzheimer's Disease	1
6.2.	The Amyloid β -Peptide (A β)	3
6.3.	The Amyloid Precursor Protein (APP)	7
6.4.	Regulation of APP	18
6.5.	Role of Hypoxia in Alzheimer's disease	27
6.6.	The Hypoxia Inducible Factor-1 (HIF-1)	31
6.7.	Hypothesis	42
7.	MATERIALS AND METHODS	44
7.1.	Cell Culture	44
7.2.	MTT Assay	45
7.3.	Dichlorofluorescein Assay	46
7.4.	Plasmids	47
7.4.1.	Construction of a pGL3-Basic Luciferase Reporter Vector	48

7.5.	Transient Transfections	49
7.6.	CAT and β -Galactosidase Assays	50
7.7.	Dual-Luciferase Assay	51
7.8.	Real-Time PCR	52
7.9.	Whole Cell and Nuclear Extract Preparation	55
7.10.	Western Blot Analysis	56
7.11.	Electrophoretic Mobility Shift Assay (EMSA)	57
7.12.	Statistical Analysis	59
8.	RESULTS	60
8.1.	Cell Viability under Hypoxic Conditions	60
8.2.	The APP Promoter Confers Hypoxia Inducibility to a CAT Reporter Gene	63
8.3.	The APP Promoter Confers Hypoxia Inducibility to a Luciferase Reporter Gene and Can Be Activated by Proteasome Inhibition	66
8.4.	The APP Promoter Confers Inducibility to a CAT Reporter Gene in Response to HIF-1 α	70
8.5.	Changes in DCF Fluorescence in Response to Hypoxia	73
8.6.	Changes in APP Gene Expression in Response to Hypoxia	76
8.7.	Changes in APP Protein Levels in Response to Hypoxia	77
8.8.	Changes in HIF-1 α Protein Level in Response to Hypoxia	83
8.9.	EMSA Analysis of APP Promoter DNA and Protein Interactions Under Hypoxia	86

9.	DISCUSSION	92
9.1.	PC12-AC Cells	93
9.2.	Cell Viability and ROS	94
9.3.	APP Expression	96
9.4.	Induction of APP Promoter Activity by Hypoxia and HIF-1 α	98
10.	REFERENCES	106

4. LIST OF FIGURES AND TABLES

Figure 1: The neurotoxic mechanisms of the amyloid β -peptide ($A\beta$)	4
Figure 2: Domain structure of the amyloid precursor protein (APP) and proteolytic processing of APP by α , β and γ secretases	8
Figure 3: The cellular processing and trafficking of APP	11
Figure 4: The APP Promoter	21
Figure 5: The APP Promoter sequence	25
Figure 6: Cellular responses to hypoxia	29
Figure 7: Structure of the heterodimeric transcription factor HIF-1	32
Figure 8: Stabilization of HIF-1 α under hypoxic conditions leads to HIF-1 mediated gene expression	37
Figure 9: Effect of hypoxia on PC12-AC cell viability	61
Figure 10: Effect of hypoxia on CAT activity from PC12-AC cells transfected with reporter constructs	64
Table 1: Transcriptional activation of the APP promoter in PC12-AC cells under conditions of hypoxia and proteasome inhibition	68
Figure 11: Effect of HIF-1 α over-expression on CAT activity from PC12-AC cells co-transfected with reporter and HIF-1 α constructs	71
Figure 12: Effect of hypoxia on DCF fluorescence	74
Figure 13: Real-time PCR analysis of APP gene expression in PC12-AC cells	78
Figure 14: Western immunoblot analysis of APP protein levels under hypoxic conditions	81

Figure 15: Western immunoblot analysis of HIF-1 α protein levels under conditions of hypoxia, proteasome inhibition and cobalt treatment 84

Figure 16: EMSA analysis of PC12-AC cell nuclear proteins interacting with APP promoter regions under conditions of normoxia, hypoxia and cobalt treatment 87

Figure 17: EMSA analysis of rat cortical neuronal cell nuclear proteins interacting with APP promoter regions under conditions of normoxia and hypoxia 90

5. LIST OF ABBREVIATIONS

A β : Amyloid β -Peptide

AP-1: Activator Protein-1

APP: Amyloid Precursor Protein

ARNT: Aryl Hydrocarbon Receptor Nuclear Translocator

CAT: Chloramphenicol Acetyl Transferase

CPRG: Chlorophenol Red- β -D-Galactopyranoside

DCF: Dichlorofluorescein

DM HIF-1 α : Double Mutant Hypoxia Inducible Factor-1 Alpha

β -Gal: Beta-Galactosidase

DMSO: Dimethyl Sulfoxide

EMSA: Electrophoretic Mobility Shift Assay

HIF: Hypoxia Inducible Factor

HIF-1 α : Hypoxia Inducible Factor-1 Alpha

HIF-1 β : Hypoxia Inducible Factor-1 Beta

HRE: Hypoxia Response Element

HSE: Heat Shock Element

IL: Interleukin

MAPK: Mitogen-Activated Protein Kinase

NGF: Nerve Growth Factor

NF κ B: Nuclear Factor Kappa Beta

ODDD: Oxygen Dependent Degradation Domain

P+: Construct APP(-488)pCAT2bGAL

P-: Construct pCAT2bGAL

PAS: Period/ARNT/Single-minded

PBS: Phosphate Buffered Saline

PC-12AC: Adherent Pheochromocytoma Rat Cells

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

PMSF: Phenylmethanesulfonyl Fluoride

PS: Presenilin

pVHL: Von Hippel-Lindau Tumour Suppressor Protein

ROS: Reactive Oxygen Species

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

sAPP: Soluble Secreted Form of the Amyloid Precursor Protein

SP-1: Signal Protein-1

TGF- β : Transforming Growth Factor Beta

TLC: Thin Layer Chromatography

TNF- α : Tumour Necrosis Factor Alpha

WT HIF-1 α : Wild-type Hypoxia Inducible Factor-1

6. INTRODUCTION

6.1. Alzheimer's Disease

Alzheimer's disease is an irreversible neurodegenerative disorder that targets the aging population. According to the Canadian Study of Health and Aging (CHSA) Working Group, 364,000 Canadians over 65 suffered from neurodegenerative dementia in the year of 2001 and of these, 238,000 had Alzheimer's disease (CSHA Working Group, 2000; Lindsay *et al.*, 2004). Alzheimer's disease is therefore the most common cause of dementia in Canada. Symptoms of the disease include a progressive decline of memory and language function, impaired cognitive abilities and changes in behaviour (Selkoe, 2001). These alterations are due to synaptic loss, neurotransmitter deficits and neuronal death in brain regions that are involved in learning, memory and emotional behaviours such as the entorhinal cortex, hippocampus, amygdala and the frontal, temporal, parietal and occipital association cortices (Mattson, 2004). Two types of diagnostic lesions are observed in brain regions of patients suffering from Alzheimer's disease; amyloid plaques and neurofibrillary tangles. Neurofibrillary tangles are located inside neurons and are composed of paired helical filaments of the hyper-phosphorylated form of the microtubule-associated protein Tau (Docagne *et al.*, 2004). Amyloid plaques are extracellular deposits of fibrils and aggregates of the 40-42 amino acid polypeptide referred to as the amyloid β -peptide ($A\beta$). Most of the fibrillar $A\beta$ found in plaques is the longer $A\beta_{42}$ form which is more prone to aggregation and formation of fibrils than the shorter $A\beta_{40}$ form which is more abundant in cells (Selkoe, 2001). Amyloid plaques are

associated with degenerating axons and dendrites, as well as with activated microglia and reactive astrocytes (Iversen *et al.*, 1995). There is also an accumulation of A β deposits in meningeal and cerebral blood vessels in Alzheimer's disease (Mattson, 1997; Miao *et al.*, 2005).

Aging is a major risk factor in Alzheimer's disease however the exact sequence of events that initiate this disorder remains unclear (Bazan *et al.*, 2002). Studies have shown age-related changes in plasma and brain levels of A β in transgenic mice models of Alzheimer's disease and age-related increases in plasma levels of A β in control and pre-Alzheimer's disease subjects (Lahiri *et al.*, 2003). Besides aging, both genetic and environmental factors can contribute to the onset and progression of the disease. Missense mutations identified in the human amyloid precursor protein gene (APP), the presenilin 1 gene (PS1), and the presenilin 2 gene (PS2) are known to cause early onset familial forms of Alzheimer's disease (Scheuner *et al.*, 1996). Studies have shown that these mutations increase both the levels of A β and the extracellular concentration of the longer A β 42 peptide. The A β 42 peptide is also the major species found in amyloid plaques in sporadic (non-familial) forms of Alzheimer's disease (Nunan and Small, 2000). Genetic factors account for a small percentage of Alzheimer's disease cases; instead the majority of Alzheimer's disease cases are sporadic and involve environmental factors such as traumatic head injury (Gentleman *et al.*, 1993), energy deprivation (Gabuzda *et al.*, 1994), cardiovascular disease, cerebral ischemia (Taylor *et al.*, 1999), hypoxia and oxidative stress (De la Monte *et al.*, 2000).

6.2. The Amyloid β -Peptide ($A\beta$)

$A\beta$ toxicity is thought to be the major cause of the neurodegenerative damage that occurs in the brains of Alzheimer's disease patients. Not only do mutations in various genes linked to familial cases of Alzheimer's disease lead to the overproduction of $A\beta$ but $A\beta$ is directly toxic to neurons (Behl *et al.*, 1994). The possible mechanisms whereby $A\beta$ damages and kills neurons include oxidative stress, generation of reactive oxygen species (ROS), disruption of cellular calcium homeostasis, induction of inflammatory responses, and activation of pro-apoptotic genes (Figure 1). These mechanisms are discussed below.

The brain is vulnerable to oxidative stress because it has a high rate of oxygen consumption, low levels of antioxidants and has regions that are enriched with transition metals (Tuppo and Forman, 2001). Oxidative stress results from an imbalance between the formation of ROS such as hydrogen peroxide, nitric oxide, superoxide and hydroxyl radicals, and their detoxification by antioxidants (Iversen *et al.*, 1995). Studies indicate that $A\beta$ increases ROS production in primary cortical, hippocampal and neuronal cell lines by various means. $A\beta$ can interact with iron (Fe^{2+}), copper (Cu^+) and zinc (Zn^{2+}) during the aggregation process, releasing hydrogen peroxide (Iversen *et al.*, 1995). $A\beta$ can also impair mitochondrial function, leading to decreased energy production as well as damage the electron transport chain in the mitochondria resulting in an increased production of superoxide radicals (Mattson, 2004). Mitochondria-derived superoxide radicals have been shown to contribute to the dysfunction of the endoplasmic reticulum (Mattson, 2004). Furthermore, oxidatively modified proteins, lipids and DNA, are found

Figure 1. The neurotoxic mechanisms of the amyloid β -peptide ($A\beta$): The main component of plaques in the brains of patients with Alzheimer's disease is $A\beta$. The neurotoxic actions of $A\beta$ involve the generation of reactive oxygen species (ROS), alterations in ion homeostasis, energy deprivation, inflammatory responses, and activation of signalling pathways. Interaction between $A\beta$ and the cell membrane results in ROS accumulation which cause DNA, protein and lipid peroxidation, and lead to cellular dysfunction via the inhibition of various enzyme activities and apoptotic cell death. $A\beta$ can potentiate calcium channels and disrupt mitochondrial function and energy production as well as contribute to the dysfunction of the endoplasmic reticulum. Glial cells activated by $A\beta$ can release various inflammatory mediators such as cytokines and nitric oxide which damage neighbouring cells. The secreted form of APP ($sAPP\alpha$) can counteract the excitotoxic and oxidative stress caused by $A\beta$ by activating signalling pathways leading to the opening of hyperpolarizing K^+ channels and expression of genes involved in regulating ion homeostasis (modified from Mattson, 1997).

in the brains of patients with Alzheimer's disease (Tuppo and Forman, 2001). A β is thought to interact with the cell membrane and generate ROS which then cause lipid peroxidation and protein oxidation (Suh and Checler, 2002). A β -induced oxidative stress damages the phospholipid membrane and impairs the function of membrane bound proteins such as ion-motive ATPases, glucose and glutamate transporters, and GTP-binding proteins. A β has been shown to disrupt calcium ion homeostasis leading to increased calcium influx and excitotoxic cascades (Taylor *et al.*, 1999) and to uncouple the muscarinic acetylcholine receptor from its GTP-binding protein thereby disrupting cholinergic signalling (Mattson, 1997). In addition, glucose uptake and glucose metabolism is altered by A β (Soucek, *et al.*, 2003). These dysfunctions ultimately alter signalling pathways and activate transcription factors and apoptotic pathways. A β and ROS induction may be a trigger of apoptosis in Alzheimer's disease because pro-apoptotic proteins such as p53 and Bax are associated with amyloid deposits (Mattson, 2004) and ROS activate pro-apoptotic genes (De la Monte *et al.*, 2000). Therefore, A β increases the vulnerability of neurons to oxidative, metabolic and excitotoxic insults, and triggers events that lead to cell death in Alzheimer's disease (Figure 1).

Another mechanism that may be important in the pathogenesis of Alzheimer's disease is inflammation. Inflammatory activity is elevated in brains of Alzheimer's disease patients. A β fibrils are perceived as foreign material by microglia and astrocytes which become activated as a response to neuronal injury (Mattson *et al.*, 1997). Activated glial cells have been shown to produce and secrete a variety of inflammatory mediators in the human Alzheimer's disease brain such as cytokines and neurotrophic factors. Interleukins (IL)-1, -6 and -8, tumour necrosis factor- α (TNF- α) and transforming growth

factor- β (TGF- β) levels were increased following exposure to A β (Docagne *et al.*, 2004). These molecules can damage nearby neurons and glial cells and also promote the accumulation of A β contributing to the neurodegenerative process in Alzheimer's disease (Figure 1).

6.3. The Amyloid Precursor Protein (APP)

The 4 kDa A β is derived from the much larger (110–140 kDa) APP. APP is a type I integral membrane glycoprotein with a single membrane-spanning domain, a large extracellular glycosylated N-terminal and a shorter cytoplasmic C-terminal end (Figure 2). It is a member of the mammalian APP gene family that includes APP-like proteins (APLP1 and APLP2) possessing similar molecular structures to APP. APP exists in three major forms, APP695, APP751 and APP770, named according to their amino acid number. These isoforms are encoded by the highly conserved APP gene located on human chromosome 21 (Moran *et al.*, 1995). The two longer isoforms contain an extra 56 amino acid residue coding for a Kunitz protease inhibitor (KPI), located at the extracellular part of the protein, which is absent in the smaller isoform (Abe *et al.*, 1991). APP gene transcripts are ubiquitously expressed in mammalian tissue with the kidney and the brain expressing the highest levels of APP (Vostrov *et al.*, 2002). There is differential distribution among the three isoforms. APP695 is produced mainly by neurons and is expressed at higher levels in the brain than in non-neuronal cells. APP751 and APP770 isoforms are expressed in both non-neuronal cells and neuronal cells but at somewhat lower levels in the brain.

Figure 2. Domain structure of the amyloid precursor protein (APP) and proteolytic processing of APP by α , β and γ secretases: The APP consists of a large extracellular domain, a hydrophobic transmembrane domain and a short cytoplasmic C-terminus. **A)** Depicts the structure of APP including the relative position of heparin, metal-binding, glycosylation, phosphorylation and trophic sites. Two major APP isoforms contain a Kunitz-type protease inhibitor (KPI) located in the extracellular domain. The $A\beta$ fragment includes 28 residues just outside of the membrane and 12–14 residues of the transmembrane domain (taken from Suh and Checler, 2002). **B)** Depicts the largest APP isoform comprising 770 amino acids. In the first diagram, the left arrow indicates the site at which APP is cleaved by α secretase (after residue 687) resulting in the secretion of $sAPP\alpha$ and the retention of the C-terminal fragment C83 which can be processed by γ secretase at residues 711 or 713 to release the p3 peptide. The lower diagram depicts the cleavage of APP after residue 671 by β secretase which generates $sAPP\beta$ and C99 which can be cleaved by γ secretase thereby releasing $A\beta$ (taken from Mattson, 1997).

A **B**

The cellular processing of APP is complex. Following translation on membrane-bound ribosomes, APP undergoes a series of post-translational modifications that occur during the trafficking of the protein through the secretory pathway (Schmitz *et al.*, 2002). APP is co-translationally inserted into the endoplasmic reticulum by its signal peptide where it undergoes N-glycosylation and disulfide bond formation. During its passage through the Golgi apparatus it is modified to the mature form by O-glycosylation, sulfation and phosphorylation. Only a small fraction of APP reaches the mature state; immature APP is degraded before arriving at the Golgi apparatus and does not reach the cell membrane. Mature, full-length APP molecules (holo-APP) are transported to the cell membrane via secretory vesicles. APP remains at the cell surface for a very short duration with an estimated half-life of about 20 minutes (Koo *et al.*, 1996). From the cell membrane APP is either cleaved by secretases and rapidly released into the extracellular space or internalized. Approximately 30% of cell surface APP is released (Koo *et al.*, 1996). Following internalization, a fraction of APP is recycled while the majority of APP is targeted to endosomes and lysosomes for degradation (Figure 3).

As APP passes through the secretory pathway, the protein can be proteolytically cleaved by α , β and γ secretases to produce a variety of derivatives including A β and secreted APP (sAPP) fragments (Figures 2 and 3). The major proteolytic cleavage is performed by α secretase which requires APP to be membrane bound. Both APP in secretory vesicles and at the cell surface are substrates for α secretase (Koo *et al.*, 1996). The α secretase cleaves at the extracellular domain of APP, just outside the transmembrane domain between amino acid residues 687 and 688 (based on APP770).

Figure 3. The cellular processing and trafficking of APP: APP is synthesized in the rough endoplasmic reticulum and passes through the Golgi apparatus during the maturation process. APP is then inserted into the vesicle membrane where it can be processed to generate soluble forms of APP (Pathway 1). APP can also be inserted into the cell membrane where it is cleaved by α and β secretases (Pathway 2) releasing sAPP α (Pathway 3) and sAPP β (Pathway 4) into the extracellular milieu. The C-terminal fragments of α and β secretase cleavage may be endocytosed in endosomal and lysosomal compartments and processed by γ secretase (Pathway 5). The A β fragment is released from the cell and has the potential to form A β deposits (Pathway 6) (taken from Mattson, 1997).

The cleavage generates a large, soluble N-terminal ectodomain fragment (sAPP α) that is released into the extracellular space and a C-terminal fragment (C83) that remains in the membrane. The α secretases (ADAM-10, TACE) cleave within the A β sequence, leading to the generation of the C83 fragment and eventually p3, and therefore prevent the generation of A β . This pathway was thought to be the physiological one because it would prevent amyloid deposits in Alzheimer's disease. However, a fraction of APP molecules are processed by the β secretase (BACE) which leads to the generation of A β .

APP is cleaved by β secretase in secretory vesicles or at the plasma membrane. The β secretase cuts in the extracellular domain of APP after residue 671, creating sAPP β that is secreted into the medium and a C-terminal fragment (C99) that remains membrane-bound (Selkoe, 2001). C99 contains amino acid 1 of A β as its N-terminal. The A β sequence is comprised of 28 amino acids from the extracellular domain and 12–14 amino acids from the transmembrane domain of APP. Since the β secretase cleavage site is located outside of the A β sequence this proteolytic pathway is the prerequisite for A β generation.

The C-terminal fragments generated by cleavage by α and β secretases are internalized from the cell surface and are acted upon by γ secretase in endosomal and lysosomal compartments (Gabuzda *et al.*, 1994). The γ secretase cuts within the transmembrane domain of APP at amino acid residues 711 or 713 resulting in production of a short peptide p3 (from C83) and A β 40 or A β 42 (from C99). It was formerly thought that A β formation was a pathological event; however A β is detected in both cerebrospinal fluid and plasma in healthy individuals and Alzheimer's disease patients (Scheuner *et al.*, 1996). Therefore A β production is a normal metabolic event. The majority of the A β

produced is A β 40 and only a small portion is A β 42; the most common form found in plaques. A β is rapidly secreted by neuronal and non-neuronal cell types after generation, however neuronal cells generate greater amounts of A β than other cell types. The γ secretase cleavage of the C-terminal end of A β is the final step in A β production. The identity of the γ secretase is currently unknown, but the most likely candidates are PS1 and PS2 (Lai *et al.*, 2003; Nunan and Small, 2000). Fragments other than A β liberated by γ secretase cleavage of C99 have also been shown to be neurotoxic and can translocate to the nucleus and induce the expression of apoptotic genes (Mattson, 2004). Furthermore, APP has been shown to be a substrate for caspase-8 and caspase-9 which cleave APP at the C-terminus (Lu *et al.*, 2000). Caspase cleavage generates a fragment called C31 which induces apoptosis in cultured cell lines.

The metabolic pathways of APP are regulated by a variety of factors. Cholinergic agonists (Webster *et al.*, 2004) and membrane depolarization (Mattson, 1997) stimulate the production of sAPP α in neuronal cell lines, suggesting that neuronal activity increases α secretase cleavage of APP, while inhibiting the release of A β . Phorbol esters and molecules that can activate protein kinase C (PKC) can induce α secretion of APP above basal levels (Nunan and Small, 2000). As well, nerve growth factor (NGF) can induce the release of sAPP α from cultured cell lines (Milward *et al.*, 1992). Mutations in the APP gene affect the proteolytic processing of APP. These mutations are clustered near the β and γ secretase cleavage sites and are within or adjacent to the A β sequence. Amino acid substitutions at these regions increase the release of A β and reduce sAPP α secretion, and those near the γ site specifically increase the production of A β 42 (Suh and Checler, 2002). Furthermore, energy deprivation promotes β secretase activity and leads to

decreased APP secretion (Gabuzda *et al.*, 1994) and hypoxia results in decreased α secretase cleavage thereby reducing sAPP α levels (Webster *et al.*, 2004).

Although APP metabolism has been thoroughly studied, the fate of APP synthesized in neurons is relatively unknown. APP protein expression is elevated in neurons in the mature nervous system and in growth cones of cultured cells (Mattson, 1997). These locations suggest that APP can accumulate in axons and presynaptic terminals. Indeed, Buxbaum *et al.*, (1998) demonstrated that in the rat brain APP is axonally transported to the nerve terminals. Both full-length and metabolized APP accumulates at synaptic sites of neurons and APP may be processed to A β at these sites. In addition, APP may undergo retrograde transport from the axon terminals to the cell body where it may be sorted back to the cell surface or to cellular compartments (Yamazaki *et al.*, 1995).

The functions of APP are not fully clarified. APP-knockout mice display deficiencies in locomotor activity, grip strength and post-natal growth, and exhibit reactive astrocytes (Zheng *et al.*, 1995). These mice are however viable and reproduce normally probably because APLP members homologous to APP possess similar functions. Due to its widespread tissue distribution, APP has been associated with a variety of cellular processes and events. These include regulation of neuronal survival, neurite outgrowth, cell adhesion, metal ion sequestration, and neuroprotection. Both the holo-protein and sAPP α serve one or more of these biological roles.

Neurons derived from APP-knockout mice display decreased viability and neurite outgrowth (Han *et al.*, 2005). Indeed, soluble and membrane-associated human brain APP increase neurite elongation and branching in cultured rat neuronal cell lines

(Milward *et al.*, 1992). In this study, APP antibodies blocked the effects of NGF on promoting neurite length, suggesting that APP is required for the induction of the neurotrophic actions of NGF. Furthermore, APP expression increases dramatically during neuronal differentiation and synaptogenesis in the developing rat brain (Mattson *et al.*, 1997). These findings are consistent with a role for APP as a growth factor.

APP-induced neurite outgrowth involves changes in cell adhesion. Investigators Breen *et al.*, (1991) demonstrated that APP is capable of interacting with molecules of the extracellular matrix such as proteoglycans and collagen in neuronal and fibroblast cell lines, which allows it to function as a regulator of cell-cell and cell-matrix interactions. APP antibodies inhibited cell-cell binding and cell binding to collagen and led to a severe decrease in neurite length in cells seeded on a collagen substrate. A property that is common to many glycoproteins involved in adhesion is heparin-binding. APP contains two heparin-binding domains in the extracellular portion of the protein (Figure 2A). The interaction between APP and heparin can modulate APP-induced cell growth. APP also contains a RERMS domain (named for the five amino acids within its sequence) that appears to be responsible for its growth-promoting properties through cell surface binding (Jin *et al.*, 1994). In addition to proteoglycans, APP can interact with GTP-binding proteins and may function as a receptor (Zheng *et al.*, 1995).

The KPI-containing APP isoforms appear to be serine protease inhibitors. KPI isoforms are highly expressed in platelets and have been shown to inhibit factors that are involved in blood coagulation (Storey and Cappai, 1999). Zn^{2+} can increase APP regulation of these factors. APP has metal ion binding activity for Zn^{2+} and Cu^{2+} in the extracellular domain. APP may play a role in the uptake of these metals during

internalization from the cell surface. The binding of Zn^{2+} is assumed to play mainly a structural role by altering the conformation of APP and modulating its functional properties. However, the binding of Cu^{2+} to APP results in its reduction to Cu^{1+} as well as oxidative modification of two cysteine residues of APP. An extracellular reaction may then occur between Cu^{1+} and peroxides which generates ROS and can lead to oxidative stress and neurotoxicity implicated in Alzheimer's disease.

APP plays a role in promoting neuronal survival. The APP neuroprotective site is believed to be located to amino acids 666-687 which correlates with the sAPP α molecule (Storey and Cappai, 1999). The sAPP α molecule therefore exhibits a higher degree of neuroprotection than the sAPP β form. Neurons derived from APP-deficient mice exhibit reduced metabolism and are more susceptible to glutamate-induced excitotoxicity and apoptosis (Han *et al.*, 2005). Over-expression of APP in a neuronal cell line resulted in increased resistance to glutamate toxicity (Mattson *et al.*, 1997). When pretreated with sAPP α , primary neuronal cultures and cell lines were resistant to being killed by free radicals, glutamate, and A β . These cells also showed a marked reduction in hydrogen peroxide and peroxidized lipid accumulation when exposed to oxidative insults. Furthermore, *in vivo* studies have provided evidence that sAPP α is neuroprotective against ischemic insult in the adult rat. Infusion of sAPP α into the lateral ventricles immediately following transient ischemia increased the survival of CA1 neurons of the hippocampus (Smith-Swintosky *et al.*, 1994). Protective mechanisms of APP involve effects on both ion channel function and transcription factor pathways. sAPP α activates guanylate cyclase, which in turn causes an increase in cGMP levels and activation of cGMP-dependent protein kinases. The result of this signalling pathway is K^+ channel

activation which hyperpolarizes the cell and decreases calcium influx through voltage-gated channels and NMDA receptors. In addition, sAPP α can activate mitogen-activated protein kinase (MAPK) and nuclear factor κ B (NF κ B) (Mattson, 1997). These factors have been shown to induce the expression of genes that regulate intracellular calcium levels and antioxidant enzymes and thus protect neurons from A β toxicity and counteract the neurodegenerative process in Alzheimer's disease (Figure 3).

6.4. Regulation of APP

The APP gene plays a pivotal role in Alzheimer's disease. APP gene mutations suggest that alterations in the APP gene may lead to A β deposition probably due to changes in APP processing. However, these mutations are rare and the cause of A β accumulation remains unknown (Storey and Cappai, 1999). Increased levels of APP have been observed in certain areas of the Alzheimer's disease brain and in patients with Down's syndrome who carry an extra copy of the APP gene and inevitably develop Alzheimer's disease during middle adult years (Selkoe, 2001; Lahiri and Ge, 2004). Transgenic mice that express high levels of APP isoforms containing a human APP mutation, implicated in inherited forms of Alzheimer's disease, progressively develop many of the neuropathological features of Alzheimer's disease including amyloid deposition, loss of synaptic density and gliosis (Games *et al.*, 1995). Post-mitotic neurons that over-express full-length APP isoforms degenerate severely and die within a few days (Yoshikawa *et al.*, 1992). These neurons have been shown to contain large amounts of A β and C-terminal fragments. Furthermore, transgenic mice that over-express the

APP751 isoform show age-dependent increases in A β deposits and Tau expression, particularly in the cortex and hippocampus, and they exhibit learning and memory deficits during a variety of behavioural and cognitive tasks (Moran *et al.*, 1995). These observations suggest that increased APP levels would permit increased formation of A β and could play a contributing role in amyloid deposition and development of Alzheimer's disease.

The human APP gene is 300 kB in length and contains 18 exons (Lahiri and Ge, 2004). Alternative splicing of exons 7 and 8 generates the three major APP isoforms, and exons 16 and 17 contain the A β sequence (Strooper and Annaert, 2000). The promoter of the APP gene is a necessary element in regulating changes in APP gene expression, and it has been shown to mediate cell-specific gene expression of a reporter gene in transgenic mice (Vostrov *et al.*, 2002). The APP gene promoter resembles the promoters of housekeeping genes because it lacks TATA and CCAAT elements and contains several GC boxes in the vicinity of the main transcriptional start site (+1). Transcription can be initiated from multiple sites spanning positions +1 and -4 (Quitschke *et al.*, 1996). An initiator sequence (CGTCA⁺¹GTT) is associated with the transcriptional start site and is essential for both start site selection and optimal transcriptional activity. Analysis of a series of 5'-deletions of the human APP promoter sequences from position -2832 to -96 indicated that the region -96 to +105 is an essential promoter element that is sufficient for high levels of APP expression in several cell lines (Quitschke *et al.*, 1992). Transcription factors signal protein 1 (SP-1) (Quitschke *et al.*, 1996), upstream regulatory factor (UTF) and the zinc finger protein motif CTCF (Vostrov *et al.*, 2002) recognize and bind to domains located in this region, and elimination of these binding sites reduces

transcriptional activity by 70–90%. Among the regulatory sequences of interest on the APP promoter are consensus binding sequences for the transcription factors homeobox HOX-1.3, activator proteins AP-1, AP-2, NF κ B and the heat shock element (HSE) suggesting that many mechanisms could control the regulation of the APP gene (Dewji *et al.*, 1995; Lahiri and Ge, 2004) (Figure 4).

Multiple regulatory elements have been identified in the APP promoter that respond to the stimulation of various growth factors and other reagents (Ge and Lahiri, 2002). These factors have been shown to increase APP gene expression in neuronal and non-neuronal cell lines. Increased APP gene expression occurs in response to NGF during development of the brain (Milward *et al.*, 1992) and NGF induces APP gene expression and reporter gene activity in various APP promoter constructs tested (Ge and Lahiri, 2002). IL-1, phorbol esters and retinoic acid can stimulate APP promoter activity approximately 3-fold from a –488 base pair APP promoter fragment in primary neurons (Yang *et al.*, 1998). IL-1 can increase levels of APP gene transcripts up to 6-fold in human endothelial cells and primary astrocytes (Rogers *et al.*, 1999). These factors modulate APP gene expression, possibly utilizing a distal putative AP-1 binding site located at –350 to –344 that has been established to be a responsive element to a PKC signalling pathway (Yang *et al.*, 1998). PKC can be induced by IL-1, phorbol esters and retinoic acid and has been shown to increase APP gene expression through transcriptional activation of the APP promoter (Trejo *et al.*, 1994). TGF- β stimulates APP promoter activity and APP synthesis *in vivo* and *in vitro* and promotes A β deposition in transgenic

Figure 4. The APP Promoter: Schematic diagram of the APP promoter with various transcription factors. The map of the human APP promoter is shown from position –3416 to +147. Binding sites for potential transcription factors are indicated relative to the transcriptional start site (+1). Transcription factors include activator proteins (AP-1 and AP-2), signal protein (SP-1), zinc finger protein (CTCF), upstream regulatory factor (USF), heat shock element (HSE), copper regulatory element (CRE), and the homeobox domain. The upstream regulatory element (URE) and the proximal regulatory element (PRE) are shown (adapted from Lahiri and Ge, 2004).

mice (Docagne *et al.*, 2004; Ge and Lahiri, 2002). A GC-rich domain containing a CAGA box located in the 5'–untranslated region of the APP promoter (+54 to +74) mediates TGF- β activation of APP transcriptional activity. TGF- β induces the formation of nuclear complexes containing SP-1, Smad3 and Smad4 which are capable of binding to this region and therefore participate in the regulation of APP promoter activity (Docagne *et al.*, 2004). In addition to regulation by these growth factors, APP has been associated with playing similar roles as these factors in coordinating cellular responses to brain injury.

The APP gene is induced as part of a cellular response to diverse forms of stress and injury (Gentleman *et al.*, 1993). The APP promoter contains stress responsive elements such as the HSE located at position –317 which has been shown to mediate APP gene regulation in response to stress conditions (Dewji *et al.*, 1995) and two NF κ B regulatory sequences located at –2250 to –2241 and –1837 to –1828 which may regulate APP gene expression (Yang *et al.*, 1998). The stress response is characterized by a rapid but transient change in gene expression (Dewji *et al.*, 1995). APP expression is dramatically increased following traumatic head injury in humans (Gentleman *et al.*, 1993), heat shock in cultured cell lines (Dewji *et al.*, 1995), kainic acid injection in rodents, glutamate-induced excitotoxic insults in the mammalian brain (Mattson *et al.*, 1997), NGF and serum deprivation in neuronal cell lines (Araki and Wurtman, 1998) and ischemia in the human and rodent brains (Abe *et al.*, 1991; Jendroska *et al.*, 1997).

It has been reported that increased levels of APP are part of the acute adaptive response of the brain to ischemia. Ischemic injury was shown to induce over-expression of APP in rat pups and full-term human infants (Baiden-Amissah *et al.*, 1998). KPI

containing gene isoforms of APP were induced in the rat cerebral cortex 1 to 21 days after focal ischemia caused by middle cerebral artery occlusion (Abe *et al.*, 1991). Transient global ischemia followed by reperfusion was shown to result in an increase of APP751 and APP770 isoforms in CA1 and CA3 regions of the hippocampus whereas the transcriptional level of APP695 was unaffected (Heurteaux *et al.*, 1993). Human brain infarcts were associated with increased APP expression and A β deposition (Jendroska *et al.*, 1995). Furthermore, both APP and A β have been shown to accumulate at the periphery of brain infarcts and arterial boundary zones (Jendroska *et al.*, 1995) and in surviving dystrophic neurons and axonal swellings following ischemic insult (Stephenson *et al.*, 1992). These observations suggest a clear link between ischemic insult and elevation of APP and A β levels. APP accumulation following ischemic brain damage could be considered a defense mechanism against ischemia or it could contribute to worsening ischemic damage, or it might simply represent a reaction to such injury (Taylor *et al.*, 1999). It remains unknown how ischemia might increase APP levels.

A PERL-based analysis of the human APP promoter by the Willmore laboratory identified at least three putative Hypoxia Responsive Elements (HREs) of the sequence 5'-GCGTG-3' located between positions -183 to -187, -156 to -160 and +40 to +44 relative to the transcriptional start site (Figure 5). Other HREs were found at positions -2357 to -2361 and -1889 to -1893, but these sites were considered to be too distant from the transcriptional start site to act as functional enhancers.

Figure 5. The APP Promoter sequence: Partial sequence of the human APP promoter (–488 to +210). Three Hypoxia Response Elements (HREs) representing putative binding sites for Hypoxic Inducible Factor-1 (HIF-1) are shown in red and underlined. Recognition sites for AP-1 and SP-1 are indicated. Exon 1 of APP is indicated in green. The transcriptional start site is at position +1.

-488

AAGCTTCACTCGTTCTCATTCTCTTCCAGAAACGCCTGCCCCACCTCTCCAAACCGAGAGA
 AAAAACGAAATGCGGATAAAAACGCACCCTAGCAGCAGTCCTTTATACGACACCCCCGGGA
 GGCCTGCGGGGTCGGATGATTCAAGCTCACGGGGACGAGCAGGAGCGCTCTCGACTTTTCT
 AGAGCCTCAGCGTCCTAGGACTCACCTTTCCTGATCCTGCACCGTCCCTCTCCTGGCCCC
 AGACTCTCCCTCCCCTGTTTACGAAGCCAGGTGGGCCGTGCGCCGGGGAGCGGAGGGGG
 CGCGTGGGGTGCAGGCGGCGCCAAGGGCGCGTGCACCTGTGGGCGCGGGGCGCGAGGGCCC
 CTCCCGGCGCGAGCGGGCGCAGTTCCCCGGCGGCGCCGCTAGGGGTCTCTCTCGGGTGCCG
 AGCGGGGTGGGCCGGATCAGCTGACTCGCCTGGCTCTGAGCCCCGCCCGCGCTCGGGCT
 CCGTCAGTTTCTCTCGGCAGCGGTAGGCGAGAGCACGCGCGAGGAGCGCGGGGGGCCCCG
 GGAGACGGCGGGCGGTGGGCGCGCGGGCAGAGCAAGGAAGCGGGCGGATCCCACTGGCACAGC
 AGGCGACTCGGTGCCCCCGGCGAGGTTCGGGATGCTGCCCCGTTTGGCACTGCTCTCTCTG
 CCGCCTGGACGGCTCGGGCGCTCGAGGTGGGT +210

Green = APP Exon 1

Red = HIF-1

Blue = AP-1

Brown = SP-1

AUG = START Codon (TRANSLATION)

6.5. Role of Hypoxia in Alzheimer's disease

Mammals require a steady supply of oxygen to maintain normal cellular functions. The brain is particularly vulnerable to impairments in oxygen homeostasis because neurons are highly dependent on oxygen for survival (Bazan *et al.*, 2002). The importance of oxygen is due to its role as the main electron acceptor in mitochondrial oxidative phosphorylation (Bruick, 2003). Organisms may encounter different levels of oxygen in various environments. Atmospheric (21%) oxygen is referred to as normoxia whereas a reduction in oxygen levels is known as hypoxia.

Ischemia is a restriction in blood supply to a part of the body that results in a decrease of oxygen delivery to tissues and causes tissues to become hypoxic (Taylor *et al.*, 1999). Hypoxia is a key feature of ischemia. There is a strong association between cardiovascular diseases and Alzheimer's disease. Alzheimer's disease is more prevalent following periods of cerebral ischemia induced by cardiovascular dysfunctions such as stroke or cardiac arrest (Taylor *et al.*, 1999; Chen *et al.*, 2003; Nalivaeva *et al.*, 2004). Evidence for this connection is that patients with cardiovascular disease demonstrate neuropathological features similar to those of Alzheimer's disease even when there is no clinical diagnosis of Alzheimer's disease (Nalivaeva *et al.*, 2004). Furthermore, cortical neurons exposed to transient and prolonged episodes of hypoxia display several molecular and biochemical abnormalities that occur in Alzheimer's disease including impaired mitochondrial function and membrane integrity, cell loss, increased levels of oxidative stress, ROS and DNA damage, and increased APP expression and processing resulting in accumulation of A β and C-terminal fragments (Chen *et al.*, 2003). These

findings suggest that hypoxia is an important contributor to the onset and progression of Alzheimer's disease neurodegeneration.

Cellular responses to hypoxia are complex (Figure 6). Acute hypoxia occurring over a time scale of seconds or minutes causes rapid responses such as membrane depolarization or selective inhibition of oxygen-sensitive ion channels (Del Toro *et al.*, 2003). Prolonged or chronic hypoxia with time courses of hours to days alters gene expression. These events occur after hypoxia and, because they are induced when total protein synthesis in the brain is suppressed, these events may be associated with cell death and survival after hypoxia (Kogure and Kato, 1993). Alterations can occur in the expression of several genes encoding a variety of important proteins that may be synthesized, including genes that are implicated in the stress response such as immediate-early gene products *c-fos* and *c-jun*, heat shock proteins and APP (Kogure and Kato, 1993) and genes that act as inflammatory mediators such as cyclooxygenase-2 and PS1 (Bazan *et al.*, 2002). Other genes are induced that are necessary to maintain oxygen homeostasis and survive the hypoxic episode. For instance, genes involved in switching from aerobic to anaerobic energy metabolism (glucose transporter-1, phosphofructokinase L, lactate dehydrogenase A) (Bergeron *et al.*, 1999), increasing oxygen delivery to tissues (erythropoietin, vascular endothelial growth factor, transferrin, inducible nitric oxide synthase) (Semenza, 2003) and catecholamine synthesis in electrically excitable cells like neurons (tyrosine hydroxylase) have been activated after hypoxic exposure (Taylor *et al.*, 1999). Many of these genes share a common mode of

Figure 6. Cellular responses to hypoxia: A variety of changes are induced at the cellular level following hypoxia. Transcription factors such as HIF-1 and NF κ B which mediate adaptive and stress responses are activated, leading to changes in gene expression. Production of reactive oxygen species (ROS) may increase leading to DNA and protein damage. Glial cells such as microglia are activated and mediate inflammatory responses during hypoxia. Pathways involving A β production lead to microglial activation, release of inflammatory mediators, hypoxia, ROS generation and cell death. Hypoxia may be an important contributor to the onset and progression of Alzheimer's disease (modified from Bazan *et al.*, 2002).

regulation that requires the binding of the Hypoxia-Inducible Factor-1 (HIF-1) to a specific conserved sequence in the promoter regions of genes that are regulated by hypoxia.

6.6. The Hypoxia Inducible Factor-1 (HIF-1)

The single most important transcription factor that mediates adaptive responses to reduced oxygen availability is HIF-1. HIF-1 is a heterodimeric transcription factor composed of an alpha (HIF-1 α) and a beta (HIF-1 β) subunit. HIF-1 β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) and dimerizes with the aryl hydrocarbon/dioxin receptor to mediate the transcription of genes encoding enzymes involved in detoxification (Semenza, 2000). In contrast, HIF-1 α is uniquely associated with the transcription of hypoxia-inducible genes. HIF-1 α has a very high affinity for HIF-1 β and competes with the aryl hydrocarbon receptor for the recruitment of HIF-1 β (Bergeron *et al.*, 1999). HIF-1 α and HIF-1 β are members of the basic Helix-Loop Helix (bHLH) containing PAS (named for the first proteins in which the domain was first identified Period/ARNT/Single-minded) domain family of eukaryotic transcription factors (Safran and Kaelin, 2003). The bHLH and PAS domains are located in the N-terminal half of each subunit (Figure 7). The HLH domain is involved in the dimerization of the α and β subunits while the basic domain binds to DNA. The PAS domain facilitates heterodimerization between the subunits and is necessary for DNA binding. Three

Figure 7. Structure of the heterodimeric transcription factor HIF-1: HIF-1 is composed of HIF-1 α and HIF-1 β subunits. Various HIF heterodimers comprising one of three alpha subunits (HIF-1 α , HIF-2 α and HIF-3 α) and one of three beta subunits (HIF-1 β , HIF-2 β and HIF-3 β) can be formed. Both subunits contain basic Helix-Loop Helix (bHLH) and PAS (Period/ARNT/Single-minded) domains which mediate DNA-binding and dimerization respectively. HIF- α contains two nuclear localization signals (NLS) which mediate its translocation to the nucleus, and an N-terminal (NAD) and C-terminal (CAD) transactivation domains which are necessary to recruit coactivator proteins. HIF- β contains only one NLS and CAD. PEST motifs and the oxygen dependent degradation domain (ODDD) containing the two constitutive LXXLAP sequences of HIF- α are indicated (taken from Willmore, 2004).

isoforms of the α subunit, known as HIF-1 α , HIF-2 α and HIF-3 α can interact with one of three isoforms of the β subunit, known as ARNT (HIF-1 β), ARNT2 (HIF-2 β) and ARNT3 (HIF-3 β) to form various HIF heterodimers (Semenza, 2001).

HIF-1 α and HIF-1 β are ubiquitously expressed in mammalian tissues, whereas the other subunits show a limited pattern of expression. For example, ARNT2 is expressed primarily in the brain and kidney and HIF-2 α is expressed in vascular endothelial cells, lungs, kidney and catecholamine-producing cells (electrically excitable cells such as sympathetic neurons, the Organ of Zuckerland and cardiac myocytes) (Semenza, 2001). HIF-1 α deficient mice show severe vascular defects and die during early stages of embryonic development (Semenza, 2000). In contrast, HIF-2 α deficient mice have no obvious vascular defects and die during the later stages of embryonic development due to a specific defect in catecholamine production. Deletion of either HIF-1 α or HIF-1 β genes in embryonic stem cells renders them incapable of activating the transcription of hypoxia-responsive genes and impairs physiological responses to hypoxia (Bergeron *et al.* 1999). These results demonstrate the central role of HIFs, particularly HIF-1, in cell survival during embryonic development. The remainder of the section will focus solely on HIF-1.

Over sixty genes have been identified that are directly regulated by HIF-1 (Semenza, 2003). Examples of HIF-1 target genes include erythropoietin, vascular endothelial growth factor and glucose transporter-1. The protein products of these genes play critical roles in angiogenesis, glucose and energy metabolism, cell proliferation and cell survival following low oxygen conditions. The basic regions of HIF-1 bind to the cis-core recognition DNA sequence 5'–TACGTG–3', known as the HRE, which is present in

enhancer regions of the promoters of hypoxia-inducible genes (Safran and Kaelin, 2003). The level of functional HIF-1 heterodimer in the cell is directly controlled by the amount of ambient oxygen present. HIF-1 β is constitutively expressed in cells under both normoxic and hypoxic conditions and it is not significantly affected by oxygen (Huang *et al.*, 1998). In contrast, HIF-1 α is a very unstable protein with a short-half life under normoxic conditions. HIF-1 α is rapidly ubiquitinated under normoxic conditions and degraded by the proteasome (Bergeron *et al.*, 1999). Hypoxia stabilizes HIF-1 α , and transcriptional activation of hypoxia-regulated genes by HIF-1 is therefore induced under hypoxic conditions. Interestingly, HIF-1 α can also be stabilized by oxygen-independent mechanisms. For instance, iron chelators such as desferrioxamine (Jaakkola *et al.*, 2001) and transition metal ions such as cobalt, nickel and manganese (Chun *et al.*, 2002) have been shown to stabilize HIF-1 α under normoxic conditions and induce the expression of HIF-1-regulated genes.

Stabilization of HIF-1 α by oxygen-dependent or oxygen-independent mechanisms, allows it to travel to the nucleus and dimerize with HIF-1 β , already present within the nucleus. HIF-1 α contains two nuclear localization signals (NLS) which mediate HIF-1 α translocation to the nucleus (Chun *et al.*, 2002) (Figure 7). Dimerization of the subunits results in the ability of HIF-1 to bind to HREs found in promoter regions of hypoxia-inducible genes. Although necessary, HIF-1 DNA binding is not sufficient to direct gene expression in response to hypoxia indicating that HIF-1 must interact with other proteins (Semenza, 2001). HIF-1 bound to HREs can recruit basal transcription machinery to the transcriptional start site of hypoxia-inducible genes through transcriptional activation domains (TAD) found on both HIF-1 α and HIF-1 β . HIF-1 α

contains two TADs; one is located at the C-terminus (CAD) and the other at the N-terminus (NAD) (Chun *et al.*, 2002). Although, HIF-1 β contains a TAD near the C-terminus, it does not appear to be necessary for transcriptional activation (Huang *et al.*, 1998). Transcriptional activation involves a protein that bridges a gap between HIF-1 and RNA polymerase II along with its associated factors. HIF-1 is capable of binding and recruiting coactivator proteins such as CREB-binding protein/p300, resulting in the transcriptional activation of hypoxia-inducible genes (Safran and Kaelin, 2003) (Figure 8).

The activation of HIF-1 under hypoxic conditions primarily depends on the stabilization of the HIF-1 α subunit. The oxygen sensitivity of HIF-1 α has been a subject of intense study in recent years. Huang *et al.*, (1998) identified an oxygen-dependent degradation domain (ODDD) within HIF-1 α that is responsible for its destruction under normoxic conditions. The ODDD contains two conserved oxygen regulatory sequences (LXXLAP, where L is leucine, X is any amino acid, A is alanine, and P is proline) in which the proline residues are constitutively hydroxylated under normoxic conditions by oxygen-dependent enzymes known as prolyl hydroxylases. Hydroxylation of the prolines in these regulatory motifs promotes the recognition of HIF-1 α by the von Hippel-Lindau tumour suppressor (pVHL) (Jaakkola *et al.*, 2001). pVHL neither recognizes nor binds to non-hydroxylated HIF-1 α . pVHL forms the substrate recognition subunit of the E3 ubiquitin ligase complex; a large complex responsible for ubiquitinating hydroxylated HIF-1 α (Chun *et al.*, 2002). The binding of pVHL to hydroxylated HIF-1 α results in its rapid poly-ubiquitination by the E3 ubiquitin ligase and degradation by the 26S

Figure 8. Stabilization of HIF-1 α under hypoxic conditions leads to HIF-1 mediated gene expression: In the presence of oxygen (normoxia) the proline residues of HIF-1 α are hydroxylated by oxygen-dependent prolyl hydroxylases. Hydroxylated HIF-1 α then interacts with the von Hippel-Lindau tumour suppressor protein (pVHL) and the E3 ubiquitin ligase complex, resulting in poly-ubiquitination and targeting of HIF-1 α for proteolytic degradation by the proteasome. In the absence of oxygen (hypoxia), HIF-1 α is stabilized and escapes pVHL-mediated ubiquitination and degradation, allowing HIF-1 α to translocate to the nucleus where it dimerizes with its partner protein, HIF-1 β . Dimerization allows HIF-1 to bind to HREs present in the promoter regions of hypoxia-inducible genes. HIF-1 interacts with the basal transcription machinery by recruiting CBP/p300 to activate the transcription of target genes (adapted from Willmore, 2004).

proteasome. HIF-1 α contains two PEST motifs surrounding the ODDD that are normally found in proteins which have a high probability of being targets for rapid intracellular degradation (Huang *et al.*, 1998). Deletion of the ODDD renders HIF-1 α stable under normoxic conditions. Prolyl hydroxylases require oxygen and iron as substrates (Chun *et al.*, 2002). Therefore under hypoxic conditions when the major substrate oxygen is unavailable, proline hydroxylation of HIF-1 α does not take place, preventing pVHL-mediated ubiquitination and degradation, and allowing HIF-1 α to proceed into the nucleus to dimerize with HIF-1 β and stimulate hypoxic gene expression (Figure 8).

Regulation of the transcriptional activity of HIF-1 occurs via post-translational modification of HIF-1 α . In addition to proline hydroxylation, HIF-1 α may also be regulated by asparaginyl hydroxylation and phosphorylation at multiple sites. The CAD of HIF-1 α interacts with coactivators CBP/p300 only under hypoxic conditions. Modification by hydroxylation of a conserved asparagine residue of the CAD inhibits the interaction of HIF-1 α with CBP/p300 (Safran and Kaelin, 2003). A transcriptional repressor known as factor inhibiting HIF-1 (FIH-1) that possesses asparaginyl hydroxylase activity has been shown to carry out this regulation. FIH-1 is oxygen-dependent just like prolyl hydroxylases; hence under hypoxic conditions asparagine hydroxylation of HIF-1 α does not proceed. Phosphorylation may be necessary to regulate HIF-1 activity because treatment of cells with tyrosine kinase inhibitors blocks many of the HIF-1 dependent responses to hypoxia (Bergeron *et al.*, 1999). Stimulation of MAPK, phosphatidylinositol-3-kinase and pathways normally activated by growth factors binding to receptor tyrosine kinases have been shown to regulate HIF-1 α at the translational level by increasing HIF-1 α protein expression (Semenza, 2003). Interestingly, growth factors

and cytokines such as ILs, epidermal growth factor, fibroblast growth factor and TNF- α stabilize HIF-1 α and induce HIF-1 target gene expression under normoxic conditions (Semenza, 2003). This establishes a role for HIF-1 α in inflammatory responses. ROS have been shown to regulate HIF-1 activity. Two opposing hypotheses exist to account for the mechanism of regulation. The first is that reduced oxygen tension during hypoxia would cause a decrease in the cellular levels of ROS and lead to stabilization of HIF-1 α and HIF-1 activation (Chun *et al.*, 2002). The second involves the increased ROS production in the mitochondria under hypoxic conditions. An accumulation of electrons in the respiratory compartments of the mitochondria occur with the lowered availability of oxygen (Chun *et al.*, 2002). Since hypoxia is not the complete absence of oxygen, any available oxygen is rapidly converted to ROS. There appears to be a positive correlation between ROS production and HIF-1 activation in cells containing mitochondria whereas cell depleted of mitochondria do not show this response (Chun *et al.*, 2002). In addition, HIF-1 α is up-regulated by cytokines generating ROS under normoxic conditions (Chun *et al.*, 2002).

HIF-1 plays a critical role in cardiovascular diseases. In atherosclerosis, for instance, tissues downstream of the blockage become deprived of adequate blood flow and hence oxygen (Semenza, 2001). The resulting hypoxic condition triggers an adaptive response mediated by HIF-1 which activates the transcription of vascular endothelial factor in order to stimulate new blood vessel formation (angiogenesis) in the ischemic tissue. Following middle cerebral artery occlusion, HIF-1 α induction and increased levels of genes encoding proteins involved in glucose transport and glycolysis is observed throughout the rat brain (Bergeron *et al.*, 1999). Exposure of rats to chronic hypoxia leads

to strong elevations of HIF-1 α and vascular endothelial growth factor expression (Chavez *et al.*, 2000). Hypoxic preconditioning, which involves exposure to sublethal levels of hypoxia prior to a hypoxic episode, increases HIF-1 α expression and protects newborn rats against hypoxic and ischemic injury (Bergeron *et al.*, 2000). Hypoxic preconditioning produces tolerance to ischemic and hypoxic injury (Soucek *et al.*, 2003). In addition, inducers of HIF-1 α expression and HIF-1 activity such as cobalt and desferrioxamine also afford protection to cerebral rat tissues when injected 24 hours prior to ischemic injury (Bergeron *et al.*, 2000).

HIF-1 activation is also involved in protecting cells from oxidative stress and recent studies have shown that HIF-1 may protect neurons from A β toxicity. Increased levels of HIF-1 and proteins involved in glycolysis have been observed in both A β -resistant neuronal cell lines and in surviving neurons of the frontal cortex in patients with Alzheimer's disease (Soucek *et al.*, 2003). The enhanced glycolysis in these cells is due to the activation of HIF-1 by A β , and HIF-1 protects neurons from the toxicity of high A β concentrations. In addition, metal chelators which induce HIF-1 stability and glycolysis protect cell lines from A β toxicity. HIF-1 may also be involved in regulating the activity of the PS1 and PS2 proteins implicated in Alzheimer's disease. Increased PS1 and PS2 gene levels are observed following hypoxia and HIF-1 binding regions in the PS1 gene promoter have been described (Bazan and Lukiw, 2002). These studies suggest key roles for hypoxia and HIF-1 in Alzheimer's disease.

6.7. Hypothesis

Alzheimer's disease becomes prevalent following periods of ischemia and hypoxia caused by cardiovascular disease. Various changes in gene expression are induced after hypoxia. APP is one of the few gene products that is up-regulated following ischemic and hypoxic injury (Kogure and Kato, 1993). Thus, I hypothesize that the APP gene is regulated by hypoxia. The regulation of most genes induced by hypoxia is mediated by HIF-1. This thesis investigates the role of hypoxia, specifically the involvement of HIF-1, in the regulation of APP promoter activity in mammalian neuronal cells. APP is a perfect candidate for hypoxic regulation not only for its involvement in promoting neuronal survival (sAPPs) or neuronal death (A β) following hypoxic injury, but also because the APP promoter region contains three putative HRE sites. The HRE sequence is unique to genes regulated by HIF-1 during hypoxia. To study APP promoter activity and expression during hypoxia, the APP promoter region containing three putative HREs will be ligated upstream and control the expression of two reporter genes. The transcriptional capability of the APP promoter will be estimated from the activity of the reporter genes. It is predicted that APP promoter activity will increase during hypoxic conditions. APP gene and protein expression will be analyzed during normoxic and hypoxic conditions. It is expected that APP levels will be induced following hypoxia. If HIF-1 regulates APP promoter activity during hypoxia, the HIF-1 α subunit must be expressed following hypoxic conditions. It is predicted that HIF-1 α expression will be induced under hypoxia. HIF-1 binding to the APP promoter will be determined by electrophoretic mobility shift assays (EMSA). It will be established whether the APP

promoter is regulated during hypoxia by a HIF-1-dependent mechanism or a HIF-1-independent mechanism, involving transcription factors other than HIF-1 which regulate gene expression under hypoxic conditions.

7. MATERIALS AND METHODS

7.1. Cell Culture

An adherent clone of rat adrenal medullary pheochromocytoma cells (PC12-AC; Charlier *et al.*, 2002; Bonin *et al.*, 2004) was a kind gift from S.A.L. Bennett (Dept. of Biochemistry, University of Ottawa, ON). PC12-AC cells are a sympathetic nerve-like cell line that possess the ability to change into various phenotypes in the presence of NGF. NGF-treated PC12-AC cells shift their phenotype from a proliferating, undifferentiated cell to a post-mitotic, differentiated, neurite-bearing sympathetic neuron (Charlier *et al.*, 2002). PC12-AC cells were cultured in growth medium containing RPMI-1640 medium supplemented with 10% horse serum, 5% fetal calf serum and 3% streptomycin/penicillin/antimycotic (Invitrogen, Carlsbad, CA). Cells were grown in 75 cm² flasks at an initial seeding density of 1×10^6 cells/mL and washed in 1 X phosphate buffered saline (PBS) (10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, pH 7.4; Sigma, St. Louis, MO). Confluent cultures were passed weekly by trypsinization. Prior to seeding of cells for experiments, the cells were counted and cell survival was assayed using the trypan blue exclusion method (Altman *et al.*, 1993). Briefly, 90 μ L of cells were added to an Eppendorf tube and 10 μ L of 0.4% trypan blue was added to cells. After approximately 5-10 min., cells were counted using a hemocytometer. Living cells appeared refractive and colourless while dead cells stained blue. The proportion of live to dead cells was assessed.

Cells were maintained under normoxic conditions in a humidified tissue culture incubator (21% O₂, 5% CO₂ and 37°C) (Thermo Forma, Marietta, OH). Cells treated under hypoxic conditions (1% O₂, 5% CO₂, 37°C) were placed in a triple-gas incubator (Thermo Forma, Marietta, OH) for the duration of the treatment. To mimic hypoxic conditions, cells were treated with cobalt (100 µM cobalt chloride; Sigma, St. Louis, MO) or a proteasome inhibitor (PI; 10 µM Cbz-Leu-Leu-Leu-al; Sigma, St. Louis, MO) (Lee and Goldberg, 1998) and maintained under normoxic conditions.

Cryopreserved rat cortical neuronal cells were a gift from QBM Cell Science Inc., Ottawa, Canada. The cells were thawed, suspended in Neurobasal medium supplemented with B-27, penicillin (100 Units/mL)/streptomycin (100 µg/ml) and grown in 10 cm plates. Cells were seeded to approximately 50% density and maintained under normoxic and hypoxic conditions as described above.

7.2. MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure the viability of PC12-AC cells under low oxygen conditions. (Mosmann, 1983). The principle of this assay is that the yellow tetrazolium salt MTT enters the mitochondria of living cells and is reduced by dehydrogenases of the respiratory chain to a blue-black insoluble formazan product. The formazan product is only formed in metabolically active cells and can be solubilized in dimethyl sulfoxide (DMSO). The colour is then quantified by spectrophotometry.

PC12-AC cells were seeded in 96-well plates at 1×10^6 cells/mL in growth medium for 24 hours at 21% oxygen (normoxic) before placing one third of the cultures in 1%, 2% or 4% (hypoxic) oxygen conditions for 24 hours. The MTT (Sigma, St. Louis, MO) stock solution was prepared to a final concentration of 5 mg/mL in PBS, filtered and stored at 4°C. Following the treatment period, MTT was added to each well at one tenth of the original media volume and the plates were incubated at 37°C for 1 hour. To solubilize the formazan crystals, the media was replaced with the same volume of DMSO and incubated at room temperature for about 5 min. The absorbance of the formazan was then measured in a 96-well microplate using a SpectraMax 340^{PC} microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm with background subtraction at 630 nm. Absorbance values were normalized to cells incubated in control (normoxic) conditions, which were set to a value of 100%.

7.3. Dichlorofluorescein Assay

2',7'-dihydrodichlorofluorescein diacetate (H₂DCF-DA; Molecular Probes, Burlington, ON) was used to assess ROS levels in cells during hypoxia. H₂DCF-DA is a non-fluorescent compound that readily diffuses through cell membranes of viable cells. It is hydrolyzed by intracellular esterases to H₂DCF and oxidized in the presence of reactive oxygen species (ROS) to form the fluorescent dichlorofluorescein (DCF) (Wang and Joseph, 1999).

PC12-AC cells were seeded in 96-well black plates at a density of 1×10^6 cells/mL in growth medium for 24 hours. The stock solution of H₂DCF-DA was prepared

in DMSO to a final concentration of 100 mM and stored at -80°C . The cells were rinsed twice with cold PBS and treated with 40 μM $\text{H}_2\text{DCF-DA}$ dissolved in RPMI-1640 medium without phenol red at 37°C for 1 hour. Following the removal of the media, cells were washed twice with cold PBS, placed in fresh growth media and maintained under normoxic or hypoxic conditions for 1 to 48 hours. Cellular fluorescence was measured using a Wallac 1420 VICTOR³ fluorometer (Perkin Elmer, Woodbridge, ON) with an excitation and an emission wavelength of 485 nm and 535 nm, respectively.

7.4. Plasmids

The constructs APP(–488)pCAT2bGAL and pCAT2bGAL (referred to from here on as P+ and P–, respectively) were obtained from Dr. Quitschke (Dept of Psychiatry, State University, NY) (Quitschke and Goldgaber, 1992). The 8834 bp P+ dual expression vector contains the reporter gene chloramphenicol acetyltransferase (CAT) driven by a 488 bp fragment of the human APP promoter, and a β -galactosidase gene under the control of a chicken β -actin (constitutive) promoter used as an internal control for normalization of transfection efficiency. The APP promoter fragment spans positions –488 upstream of the transcriptional start site to +100 downstream of the transcriptional start site (Quitschke and Goldgaber, 1992), and contains three putative HRE sites. The P– construct lacks the APP promoter and was used as a negative control. Apart from the above constructs, the following plasmids were also used in co-transfection experiments: pCR3.1 (empty expression vector; Invitrogen, Carlsbad, CA) and pCR3.1 containing either wild-type HIF-1 α (WT HIF-1 α) or a HIF-1 α double mutant (DM HIF-1 α) in which

the two proline residues in the ODDD which can become hydroxylated are mutated to alanine. Each of the five constructs was utilized in combinations in transfection experiments and CAT/ β -galactosidase assays. All plasmids were grown in competent *Escherichia coli* and purified using the GenElute Plasmid MiniPrep Kit (Sigma, St. Louis, MO) and Wizard Plus MidiPrep Kit (Promega, Madison, WI).

7.4.1. Construction of a pGL3-Basic Luciferase Reporter Vector

The promoter sequence of the human APP (–370 to +55) containing three putative HRE sites was cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). Briefly, the P+ construct was digested with the restriction enzyme *Sma*I (New England Biolabs, Beverly, MA) that has a double cut site within the APP promoter sequence, cleaving a blunt-ended 429 bp APP promoter fragment. The fragment was size selected on a 1% agarose gel, purified using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON) and ligated into the *Sma*I site of the pGL3-Basic luciferase reporter vector using T4 DNA Ligase (Invitrogen, Carlsbad, CA) to generate pGL3-F and pGL3-R. Plasmids were transformed into *E. coli* DH5 α competent cells (Invitrogen, Carlsbad, CA). Following subsequent isolation and purification, clones containing the desired APP promoter fragment in the correct orientation in pGL3-Basic were identified by PCR amplification and restriction enzyme analysis, and confirmed by DNA sequencing (Macrogen, Seoul, Korea). The pGL3-F construct contains the firefly luciferase gene as a reporter driven by the APP promoter. The pGL3-R construct was used as a control and contains the luciferase reporter under the control of the APP promoter fragment placed in

the reverse orientation. Apart from these constructs, pGL3-Basic (pGL3; promoterless luciferase reporter vector) and pRL-TK (*Renilla* luciferase reporter vector; used as a control for transfection efficiency; Promega, Madison, WI) were used in transfections and dual-luciferase assays.

7.5. Transient Transfections

PC12-AC cells were plated in antibiotic-free growth medium at a density of 1×10^6 cells/mL in 6 cm dishes or 6-well plates for 24 hours. Cells to be assayed for CAT/ β -galactosidase activity were transfected with constructs P+ or P- at an optimal DNA concentration of 4.0 μ g DNA/5.0 mL of cells, or co-transfected with the P+ or P- constructs (4.0 μ g) and one of each of the above-described pCR3.1 constructs (2.0 μ g) to a total DNA concentration of 6.0 μ g DNA/5.0 mL of cells in 6 cm dishes. Cells to be assayed for luciferase/*Renilla* activity were co-transfected with one of each of the pGL3 constructs (2.0 μ g) and the pRL-TK (0.005 μ g in all wells) vector at an optimal DNA concentration of approximately 2.0 μ g DNA/2.0 mL of cells in 6-well plates. Transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Briefly, 2 to 6 μ g of DNA was resuspended in serum-free Opti-MEM I (Gibco, Grand Island, NY) and mixed with Lipofectamine according to the manufacturer's protocol. Cells were incubated with the DNA/Lipofectamine solution for 6 hours, after which regular growth media was added. At this time, cells were maintained under normoxic conditions or were exposed to hypoxic treatment for 6 to 48 hours. Cells subjected to PI treatment were maintained under normoxic conditions for 24 hours. Cells were harvested

6 to 48 hours after transfection in PBS. Cells were detached from culture plates with a cell scraper and centrifuged at 1,600 X g for 5 min. The cell pellets were lysed and subsequently assayed for CAT/ β -galactosidase or luciferase/*Renilla* activities as described below.

7.6. CAT and β -Galactosidase Assays

Transfected cells were lysed in 250 mM Tris-HCl (pH 7.7) and centrifuged at 12,000 X g for 5 min. The supernatant was used for both CAT and β -galactosidase assays. β -galactosidase activity was determined using the substrate chlorophenol red- β -D-galactopyraniside (CPRG; Roche, Laval, Quebec; Quitschke and Goldgaber, 1992). Five percent of the cell extract was incubated at room temperature with CPRG and β -galactosidase buffer (0.01% Triton X-100, 1 mM MgCl₂, 10 mM KCl, 50 mM β -mercaptoethanol, and 60 mM Na₂HPO₄, pH 8) for 1 to 2 hours. Substrate conversion was determined by spectrophotometry at a wavelength of 580 nm. The β -galactosidase activity was used to normalize the CAT assay from the same sample as a measure of transfection efficiency.

Reporter CAT activity was determined using the fluorescent BODIPY FL chloramphenicol *FAST CAT* substrate (BCAM; Molecular Probes, Burlington, ON). The principle of this assay is that the substrate will be acetylated at the 1- and/or 3-hydroxyl positions of the molecule only in cell extracts that express the CAT enzyme (Hruby and Wilson, 1992). The substrate and the monoacetylated (1-AcBCAM and 3-AcBCAM) and

diacetylated (1,3-diAcBCAM) products can then be resolved by thin-layer chromatography (TLC).

Cell extracts were incubated with BCAM and acetyl CoA at 37°C for 5 hours according to the manufacturer's protocol (Molecular Probes, Burlington, ON), followed by addition of 1 mL ice-cold ethyl acetate to extract the substrate and products. After vortex mixing for 20 seconds and centrifugation at top speed for 3 min, the supernatants were recovered and dried by speed-vacuum. The residues were resuspended in 30 μ L of ethyl acetate and spotted on silica gel TLC plates. To resolve the acetylated and non-acetylated BCAM, the spotted TLC plates were developed in a mixture of chloroform:methanol (87:13). Spots were visualized under ultraviolet light and photographed with an Alpha Innotech Multi-Imager (Alpha Innotech Co., San Leandro, CA). Quantitation of spots was determined via spot densitometry using AlphaEaseFC V.3.1.2. (Alpha Innotech Co., San Leandro, CA). CAT activity was expressed as the percentage of conversion of BCAM into its total acetylated products, and normalized by β -galactosidase activity. CAT/ β -galactosidase activity of each construct was compared with that of the promoterless P– construct.

7.7. Dual-Luciferase Assay

Firefly and *Renilla* luciferase activities were measured sequentially using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, transfected cells were washed in PBS, lysed with 1 X Passive Lysis Buffer and detached with a cell scraper. The lysates were centrifuged at

12,000 X g for 15 seconds to remove cellular debris. Reporter activity was measured using a luminometer (LMaxII³⁸⁴; Molecular Devices, Sunnyvale, CA) in 20 μ L lysate in opaque 96-well microplates. Firefly and *Renilla* luciferase activities were measured over a 10 second reaction period, after an initial 2 second pre-read delay. Firefly luciferase luminescence was generated by the addition of 100 μ L of the substrate Luciferase Assay Reagent II (LAR II). The signal was then quenched and the *Renilla* luciferase signal was generated simultaneously in the same sample with the addition of 100 μ L *Renilla* luciferase reagent (Stop & Glo). Luciferase activity was expressed as relative light units normalized over the activity of the *Renilla* luciferase control reporter vector. Luciferase/*Renilla* activity of the pGL3-F construct was compared with that of the controls pGL3 and pGL3-R.

7.8. Real-Time PCR

PC12-AC cells were cultured in 10 cm plates until 50% confluency. Cells were then maintained under normoxic conditions or treated with hypoxia for 24 or 48 hours. Total RNA was extracted from cells using RNeasy kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. RNA integrity was verified by OD₂₆₀/OD₂₈₀ nm absorption ratio >1.7. One microgram of total RNA was reverse transcribed into cDNA using the Access RT-PCR System (Promega, Madison, WI) according to the manufacturer's protocol at 45°C in a 50 μ L reaction mixture containing 10 mM dNTP, 50 pmol forward and reverse primer, 25 mM MgSO₄, 5 U/ μ L AMV reverse transcriptase,

and RNase free water. Gene-specific primers were used for the amplification of the target gene APP (378 bp):

forward primer, 5'–AAGTCGTAGAAGTAGCCGAAGAGG–3'

reverse primer, 5'–GTAAACTTTGGGATGACACGCTGC–3'

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

forward primer, 5'–GCGGGAAATCGTGCGTGCATT–3'

reverse primer, 5'–GATGGAGTTGAAGGTAGTTTCGTG–3'

Primers were generated by Sigma Genosys (Oakville, ON) and optimized to an equal annealing temperature of 71°C using a gradient iCycler Thermal Cycler (Bio-Rad, Hercules, CA). PCR was performed on the cDNA to ensure that APP and β -actin products of correct molecular weight were amplified. The cDNA were pre-denatured at 95°C for 3 min, denatured at 95°C for 45 sec, annealed at gradient temperatures ranging from 65–80°C for 30 sec, and extended at 72°C for 10 min for 35 cycles. PCR products were resolved on a 2% agarose gel and visualized and analyzed with an Alpha Innotech Multi-Imager (Alpha Innotech Co., San Leandro, CA).

Real-time PCR was used to quantify the changes in APP gene expression relative to a reference gene (β -actin). Real-time PCR reactions were performed on the Roto-Gene 3000 (Corbett Research, Montreal Biotech Inc., Montreal, Quebec) using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). A 20 μ L reaction mixture was prepared to the indicated final concentration containing 10 μ L of SYBR Green Supermix (1X), 1 μ L of forward and reverse primer (250 nM each) and nuclease free water. The components were added to 1 μ g cDNA and the reaction mixture was incubated using the following protocol: denaturation at 95°C for 10 sec, amplification for 35 cycles at 95°C for 10 sec,

71°C for 30 sec and 72°C for 60 sec, and 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). The fluorescence of each sample was analyzed to determine the initial copy number of the sample using Roto-Gene 3000 software v.6.14. (Corbett Research, Montreal Biotech Inc., Montreal, Quebec). The critical threshold (CT; the point at which the fluorescence increased above the background fluorescence of the sample) was obtained based on standard curves for each gene using the 'Analysis-2 Standard Curves' and 'Calculate Automatic Threshold' options in the Roto-Gene 3000 software in which upper and lower bounds of the fluorescence curves were selected to be scanned for optimum threshold level. Standard curves were determined for each gene. A sample of APP and β -actin cDNA was amplified in a series of ten-fold serial dilutions to create the standard curves. The amplification efficiencies of the housekeeping and target genes were determined from the slopes of the standard curves according to the equation: Efficiency (E) = $10^{[-1/\text{slope}]}$. The expression of APP (target gene) was quantified according to the Pfaffl method (Pfaffl, 2001). In this mathematical model, the relative expression ratio (R) of a target gene is calculated based on E and CT deviation of a treated sample versus an untreated (control) sample, and expressed in comparison to a reference gene.

$$\text{Ratio} = \frac{(\text{E target})^{\Delta\text{CT target (untreated-treated)}}}{(\text{E reference})^{\Delta\text{CT reference (untreated-treated)}}$$

cDNA from cells exposed to normoxia and hypoxia was designated as untreated or treated samples, respectively.

7.9. Whole Cell and Nuclear Extract Preparation

Whole cell and nuclear extracts were prepared from PC12-AC cells grown in 15 cm plates. Nuclear extracts were also prepared from rat cortical neuronal cells grown in 10 cm plates. At 50% confluency, cells were exposed to normoxic or hypoxic conditions for 6 to 48 hours. Cells were harvested by washing with ice cold PBS and pelleted by centrifugation at 2,000 X g for 5 min at 4°C. For whole cell extraction, the cell pellet was lysed in buffer C [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 25% glycerol] and incubated on ice for 5 min. The cell lysate was centrifuged at 12,000 X g for 15 min and the supernatant was aliquoted and stored at -20°C. For nuclear extraction, the cell pellet was lysed in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, and 0.5 mM PMSF] on ice for 10 min. The cell suspension was centrifuged at 2,000 X g for 5 min. The pellet was resuspended in buffer A and homogenized with a glass tissue grinder. The cell lysate was centrifuged at 1,600 X g for 10 min. The supernatant (cytosolic fraction) was discarded and the pellet was centrifuged at 4,000 X g for 10 min. The supernatant was discarded and the pellet of nuclei was resuspended in buffer C and incubated at 4°C for 30 min with rotation, and then centrifuged at 13,000 X g for 15 min at 4°C. The supernatant (nuclear fraction) was frozen with liquid N₂ and stored at -80°C until use. All centrifugation steps were carried out at 4°C. The protein concentration of whole cell

lysates and nuclear extracts was 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.

7.10. Western Blot Analysis

APP and HIF-1 α protein levels were analyzed in normoxic and hypoxic PC12-AC whole cell and nuclear extracts. A different hypoxic time course was used for protein expression than gene expression as gene expression would correlate more closely with promoter induction. Protein samples of 50–100 μ g were resuspended in Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5% β -mercaptoethanol (Sigma, St. Louis, MO). The protein mixture was boiled for 3 min, briefly centrifuged at low speed to collect the denatured proteins and then loaded on a 10% (w/v) SDS-polyacrylamide gel (SDS-PAGE) in running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) to separate the proteins. The proteins were transferred from the gel to a nitrocellulose membrane (Hybond-C; 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 was carried out using a Bio-Rad (Hercules, CA) mini-gel and transfer apparatus.

The gel was stained with Coomassie Blue (Bio-Rad, Hercules, CA) to confirm the transfer of proteins. Non-specific binding of proteins 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 hour at room temperature with agitation. APP was

detected by incubation with the polyclonal rabbit antiserum CT15, which corresponds to the C-terminal residues 681–695 of APP (1:5000 dilution; a kind gift from Edward Koo University of California, San Diego, CA; Buxbaum *et al.*, 1998; Palacino *et al.*, 2000). HIF-1 α was detected with a monoclonal mouse anti-human HIF-1 α antibody (1:800 dilution; Novus Biologicals, Littleton, CO). Alpha-tubulin was used as a loading control and was detected with a mouse anti-human α -tubulin antibody (1:2000 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA). Membranes were incubated with primary antibodies for 1 hour at room temperature with agitation and then washed three times with TBST. Membranes were then incubated with goat anti-mouse (1:2000 dilution; DakoCytomation Inc., Mississauga, ON) or anti-rabbit (1:2000 dilution; Pierce, Rockford, IL) horseradish peroxidase-coupled secondary antibody for 1 hour at room temperature. Membranes were washed with TBST and proteins were visualized using the Renaissance Western Blot Chemiluminescence Reagent (New England Nuclear, Boston, MA) and by brief exposure to Kodak X-OMAT-AR film, according to the manufacturer's protocols. Protein molecular weights were estimated using prestained broad range marker standards (New England BioLabs Beverly, MA). Protein bands were quantified by densitometry (AlphaEaseFC V.3.1.2., Alpha Innotech Co., San Leandro, CA).

7.11. Electrophoretic Mobility Shift DNA Binding Assay (EMSA)

Electrophoretic mobility shift assays (EMSAs) were performed on nuclear extracts belonging to PC12-AC and rat cortical neuronal cells using the following double stranded oligonucleotides as probes: the APP promoter fragments –488 to +99 (APP),

–492 to –266 (APP1), –354 to –26 (APP2) and –45 to +166 (APP3); each containing the putative HRE site(s), and a mammalian HIF-1 binding sequence containing the human erythropoietin HRE (W18; sense strand sequence: 5'–AGCTTGCCCTACGTGCTGTCTCAG–3'). The APP promoter was isolated from the P+ plasmid by restriction endonuclease digestion and the APP promoter fragments were amplified using sequence specific primers and PCR. The sense strand of the W18 oligonucleotide was hybridized to the antisense strand by heating at a high temperature for 1 min and then slowly cooling to room temperature. The double stranded oligonucleotides (20 ng) were radiolabeled with [γ - 32 P] ATP (Amersham Biosciences, Piscataway, NJ) and T4 polynucleotide kinase (Invitrogen, Carlsbad, CA), and incubated at 37°C for 30 min and 70°C for another 30 min. Radiolabeled probes were separated from free nucleotides by centrifugation through a G-25 Sephadex column at 2,400 rpm for 4 min at 4°C, and a small fraction of the post-column sample was measured in a scintillation counter (Beckman Coulter Inc., Fullerton, CA).

Binding reactions were performed by incubating 15 μ g cell extract protein with 250,000 cpm (approximately 2.5 ng) of radiolabeled DNA probe in DNA-binding buffer [50 mM Tris (pH 7.8), 250 mM NaCl, 5 mM EDTA, 25% glycerol] with 1500 ng unlabeled mutant HIF-1 probe (M18; sense strand sequence: 5'–AGCTTGCCCTAAAAGCTGTCTCAG–3') and 75 ng poly dI-dC (Sigma, St. Louis, MO) to block nonspecific binding of proteins to radiolabeled probe. Competition experiments and supershift assays were performed with 100-fold molar excess (2000 ng) of unlabeled W18 and 1 μ L of HIF-1 α antibody (Novus Biologicals, Littleton, CO), respectively. The reactions were carried out at room temperature for 20 min and then

resolved on a 5% non-denaturing polyacrylamide gel in 1 X TBE buffer (8.9 mM Tris base, 8.9 mM boric acid, 0.25 mM EDTA) at 300 V for 2 hours at 4°C. Gels were vacuum dried at 80°C for 2 hours, and analyzed using a Cyclone PhosphorImager (Perkin Elmer, Woodbridge, ON).

7.12. Statistical Analysis

All results are expressed as mean $\pm$ S.E.M of at least three independent experiments, unless otherwise stated. Data analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons to determine the significance of values between experimental groups, utilizing a statistical software package (Origin 7.0). Means with a significant level of $p < 0.05$ were considered significant.

8. RESULTS

8.1. Cell Viability Under Hypoxic Conditions

To determine the percentage of oxygen that would expose cells to a significant level of hypoxia without affecting viability, PC12-AC cells were treated with various levels of oxygen for 24 hours and their viability was examined using the MTT assay. In this assay, the tetrazolium ring of MTT is cleaved by mitochondrial dehydrogenases to a water-insoluble formazan product, which is soluble in DMSO. Dehydrogenase activity is used as an indication of functional cell integrity (Mosmann, 1983). Since a loss of MTT reduction, detected by a decrease in the absorbance signal, is due to mitochondrial dysfunction, the MTT assay allows for a straightforward quantification of changes in cell viability.

Cell viability decreased as the concentration of oxygen decreased (Figure 9). PC12-AC cells incubated in 4% oxygen showed a small, non-significant decrease in viability $98 \pm 8\%$ compared to cells grown in 21% oxygen (normoxic control; $p=0.9$, ANOVA). Viability decreased by approximately 20% of the control value at an oxygen concentration of 2% ($p=0.1$, ANOVA) and remained the same at the lowest oxygen concentration (1%; $p=0.1$, ANOVA). The ANOVA and Tukey's tests showed that there were no significant changes in cell viability. An oxygen level of 1% was used for hypoxia in all subsequent studies.

Figure 9. Effect of hypoxia on PC12-AC cell viability: Viability of PC12-AC cells was measured under 21%, 4%, 2%, or 1% oxygen by MTT after 24 hours. Cell viability at 21% oxygen (normoxia) was used as a control and regarded as 100%. Data are presented as means $\pm$ S.E.M from three independent experiments. The ANOVA and Tukey's tests for multiple comparisons show that there were no significant changes in cell viability. 1% oxygen was utilized for hypoxic conditions for all subsequent experiments.

8.2. The APP Promoter Confers Hypoxia Inducibility to a CAT Reporter Gene

To investigate whether APP promoter activity was affected by hypoxia in PC12-AC cells, transient transfections were performed using CAT reporter gene constructs containing the APP promoter (containing three putative HREs; P+) or lacking the APP promoter (P-). APP promoter activity was determined by analysing CAT enzyme activity from each sample. The degree of conversion of a fluorescent substrate BCAM to monoacetylated (1-AcBCAM and 3-AcBCAM) and diacetylated (1,3-diAcBCAM) products, depending on the quantity of functional CAT enzyme expressed, was used as a measure of CAT activity. Substrate and product spots on TLC plates were detected by UV light and quantified by densitometry. β -galactosidase activity was measured by spectrophotometry and was used as an internal measure of transfection efficiency. APP promoter activities were presented as a ratio of CAT activities divided by β -galactosidase activities.

CAT expression was substantially induced by the APP promoter under hypoxic conditions (Figures 10A and 10B). Transfection with P+ under normoxic conditions resulted in the conversion of substrate BCAM to monoacetylated products only (1-AcBCAM and 3-AcBCAM). In contrast, transfection with P+ under hypoxic conditions (> 6 hours) resulted in the generation of both monoacetylated and diacetylated (1,3-diAcBCAM) products. The production of the monoacetylated product 1-AcBCAM was more pronounced under 12 and 24 hours of hypoxic conditions ($p < 0.01$; Tukey's test) as compared with each of the other normoxic and hypoxic time points. The appearance of

Figure 10. Effect of hypoxia on CAT activity from PC12-AC cells transfected with reporter constructs. PC12-AC cells were transfected with CAT reporter gene constructs with (P+) or without (P-) the APP promoter and maintained under normoxic (21% O₂) or hypoxic (1% O₂) conditions. **A)** Representative thin-layer chromatography (TLC) analysis of CAT activity. Activity is shown on the TLC plate as conversion of fluorescent substrate (BCAM) to monoacetylated (1-AcBCAM and 3-AcBCAM) and diacetylated (1,3-diAcBCAM) products. The appearance of the diacetylated product only occurs in hypoxic cells. UNT=untransfected sample, PC=positive control, NC=negative control, R=reference standard. **B)** Hypoxia increases activation of transcription from the APP promoter. CAT activity was normalized to β -galactosidase activity and was used as a measure of APP promoter activity. Data was presented as means $\pm$ S.E.M from three independent experiments. Statistical analysis was performed by using ANOVA with Tukey's test for multiple comparisons. Monoacetylated 1-AcBCAM product levels were significantly higher at 12 and 24 hours of hypoxia ($p < 0.01$) as compared to each of the other normoxic and hypoxic time points. Furthermore, 1,3-diAcBCAM levels increased significantly between 12 to 48 hours hypoxia ($p < 0.05$). ** $p < 0.01$ compared to normoxia; # $p < 0.05$, ### $p < 0.001$ compared to 12 hour hypoxia.

the diacetylated product in hypoxic P+ samples was indicative of a higher level of CAT enzyme activity as compared to normoxic P+ samples. The ANOVA and Tukey's tests demonstrate that there is a significant increase in 1,3-diAcBCAM levels between 12 to 48 hours of hypoxia ($p < 0.05$). Prolonged hypoxic exposure resulted in a significant increase in 1,3-diAcBCAM levels from 1.5-fold ($p = 0.03$, ANOVA; $p < 0.05$, Tukey's test) to 11.4-fold ($p = 0.0001$, ANOVA; $p < 0.001$, Tukey's test) over 24 to 48 hours hypoxia respectively, as compared with 12 hour hypoxia (Figure 10B). Neither normoxia nor hypoxia led to an induction of CAT enzyme in P- samples (Figure 10A). Cells transfected with P- displayed a conversion of BCAM to monoacetylated products only and these were of lower intensity compared to products from P+ samples. The data demonstrate that transcriptional induction of the APP promoter occurs in response to hypoxia.

8.3. The APP Promoter Confers Hypoxia Inducibility to a Luciferase Reporter Gene and Can Be Activated by Proteasome Inhibition

Due to a) the presence of putative HREs in the APP promoter, b) the possible role of HIF-1 in hypoxic APP promoter induction and c) the involvement of the proteasome in HIF-1 α degradation, the effect of proteasome inhibition (PI) on the induction of the APP promoter in transfected PC12-AC cells was examined. A 429-bp fragment of the human APP promoter containing three possible HREs was ligated upstream from a luciferase reporter gene and used to monitor APP promoter activity under hypoxia or PI treatment. Reporter constructs analysed in this study included ones containing no promoter (pGL3),

the 429 bp APP promoter fragment in the forward orientation (pGL3-F) and the same APP promoter fragment in the reverse orientation (pGL3-R). PC12-AC cells were transfected with either one of these constructs for 6 to 48 hours under normoxic or hypoxic conditions and examined for luciferase activity. APP promoter activity was determined as a ratio of firefly luciferase activity normalized to *Renilla* luciferase activity.

Measurements of luciferase activity from PC12-AC lysates indicated that the activity of the pGL3-F promoter construct significantly increased 1.4-fold in the presence of PI compared with that in the absence of PI; pGL3-F under normoxic conditions ($p=0.008$, ANOVA; $p<0.01$, Tukey's test; Table 1). Activity was induced 3.3-fold by the APP promoter under 6 hours of hypoxia compared to normoxia ($p=0.009$; ANOVA; $p<0.01$, Tukey's test). Activity decreased during 6 to 48 hours of hypoxia, but remained significantly higher relative to normoxia ($p<0.01$, Tukey's test). Placing the APP promoter in the reverse orientation almost completely eliminated promoter activity. Luciferase activity, due to the pGL3 vector alone, was less than 1% of the activity of the pGL3-F promoter construct. The ANOVA and Tukey's tests show that luciferase activity increased significantly from the pGL3-F promoter construct during hypoxia and PI treatment compared to pGL3-F under normoxic conditions and the other constructs ($p<0.01$). These findings concur with the induction of the APP promoter activity under hypoxic conditions (Figure 10B) and demonstrate that APP promoter activity is induced in response to proteasome inhibition.

Table 1. Transcriptional activation of the APP promoter in PC12-AC cells under conditions of hypoxia and proteasome inhibition: Cells were transfected with luciferase reporter constructs containing no promoter (pGL3), the APP promoter in the forward orientation (pGL3-F) or the APP promoter in the reverse orientation (pGL3-R) under normoxic (48 hours) or hypoxic (6 to 48 hours) conditions or in the presence of PI (24 hours). APP promoter activity was measured by luciferase reporter gene activity normalized by *Renilla* activity which was used as an internal control for transfection efficiency. Data was presented as means $\pm$ S.E.M from three independent experiments. Statistical analysis by ANOVA followed by Tukey's test demonstrate that luciferase activity was significantly higher in pGL3-F constructs treated with hypoxia or PI compared to pGL3-F under normoxic conditions and the other constructs (pGL3 and pGL3-R; $p < 0.01$). pGL3-F constructs displayed significant promoter activity compared to pGL3 alone and pGL3-R controls. $**p < 0.01$ compared to pGL3-F under normoxic conditions.

	Relative Luciferase Activity (ratio Luciferase/ <i>Renilla</i>)				
Plasmid	Normoxia	Hypoxia			PI
	48 hours	6 hours	24 hours	48 hours	24 hours
untransfected	1.3 ± 0.4				
pGL3	1.4 ± 0.4				
pGL3-R	38.7 ± 37.7				
pGL3-F	158.2 ± 12.8	474.8 ± 66.7 **	446.1 ± 39.2 **	252.3 ± 15.1 **	221.4 ± 2.9 **

8.4. The APP Promoter Confers Inducibility to a CAT Reporter Gene in Response to HIF-1 α

It could be argued that the observed effect of hypoxia and proteasome inhibition on the induction of APP promoter activity is dependent on HIF-1 acting through one or more of the HREs present in the APP promoter. To investigate the role of HIF-1 α in the hypoxic regulation of the APP promoter, PC12-AC cells were transfected under normoxic conditions with P+ or P- in combination with one of the following constructs: pCR3.1 mammalian expression vector, pCR3.1 expressing wildtype HIF-1 alpha subunit (WT HIF-1 α) or pCR3.1 expressing a double mutant of HIF-1 alpha (DM HIF-1 α) which is constitutively active under normoxic conditions. APP promoter activity was measured as a function of CAT activity, as previously described in section 8.2.

CAT expression was induced by the APP promoter as shown by the conversion of substrate to monoacetylated and diacetylated products in all co-transfected P+ cells under normoxia (Figure 11). The ANOVA and Tukey's tests demonstrate that there were significant differences in 1,3-diAcBCAM product levels between each of the co-transfected P+ groups ($p < 0.01$). DM HIF-1 α constructs, in which two key proline residues in HIF-1 α were mutated to prevent HIF-1 α prolyl hydroxylation, were used to stabilize HIF-1 α under normoxic conditions. Cells co-transfected with the APP promoter and DM HIF-1 α constructs showed a 1.4-fold significant increase in 1,3-diAcBCAM production ($p = 0.02$, ANOVA; $p < 0.05$, Tukey's test; Figure 11B) compared to P+ co-transfected with the pCR3.1 empty vector (control). The highest level of CAT activity

Figure 11. Effect of HIF-1 α over-expression on CAT activity from PC12-AC cells co-transfected with reporter and HIF-1 α constructs: CAT reporter gene constructs containing the APP promoter (P+) or lacking the APP promoter (P-) were co-transfected with pCR3.1, WT HIF-1 α or DM HIF-1 α constructs under normoxic (21% oxygen) conditions for 48 hours. **A)** Representative thin-layer chromatography (TLC) analysis of CAT activity. Activity is shown on the TLC plate as conversion of fluorescent substrate to monoacetylated (1-AcBCAM and 3-AcBCAM) and diacetylated (1,3-AcBCAM) products. Co-transfection of HIF-1 α constructs resulted in 1,3-diAcBCAM products that were higher in intensity compared to 1,3-diAcBCAM products resulting from pCR3.1 co-transfection. UNT=untransfected sample, PC=positive control, NC=negative control, R=reference standard. **B)** Transcriptional activation of APP promoter activity in response to HIF-1 α under normoxic conditions. APP promoter activities were measured as a ratio of CAT activities divided by β -galactosidase activities. Data was presented as means $\pm$ S.E.M from four independent experiments. Co-transfections with P- reporters resulted in minimal CAT activity. Statistical analysis by ANOVA and Tukey's tests showed that there were significant differences in 1,3-diAcBCAM product levels between groups co-transfected with P+ ($p < 0.01$). CAT activity was higher in P+ cells co-transfected with either HIF-1 α constructs, as indicated by the significant increase in 1,3-diAcBCAM levels. * $p < 0.05$, ** $p < 0.01$ compared to control (P+ and pCR3.1 co-transfections).

was observed in cells expressing P+ and WT HIF-1 α constructs in which HIF-1 α subunits were over-expressed. Recombinant WT and DM HIF-1 α are produced in such large amounts that they overwhelm the proteasome. WT HIF-1 α co-transfected with the APP promoter construct, showed a 2-fold significant increase in 1,3-diAcBCAM production ($p=0.008$, ANOVA; $p<0.01$, Tukey's test) as compared to controls. Neither normoxia nor HIF-1 α co-transfection led to an induction of CAT enzyme activity in P- transfected cells (Figure 11). These findings demonstrate that transcription from the APP promoter is enhanced in response to over-expression of HIF-1 α under normoxic conditions.

8.5. Changes in DCF Fluorescence in Response to Hypoxia

ROS are implicated in both the neurodegeneration process in Alzheimer's disease and the stabilization of HIF-1 α under normoxic conditions (Behl *et al.*, 1994; Chun *et al.*, 2002). The levels of ROS may correspond directly with the hypothesized HIF-1 induction of APP promoter controlled CAT production during hypoxia. To determine the levels of ROS produced in PC12-AC cells during hypoxia, intracellular ROS generation was monitored by H₂DCF-DA under normoxic or hypoxic conditions at time intervals between 1 and 48 hours. ROS present in the cells cause the oxidation of H₂DCF, generating the fluorescent DCF, which can be measured by fluorimetry.

PC12-AC cells demonstrated an increase in DCF fluorescence in response to prolonged hypoxia (Figure 12). Signal intensity was minimal below 6 hours and signal

Figure 12. Effect of hypoxia on DCF fluorescence: DCF fluorescence was used as a measure of ROS generation in PC12-AC cells exposed to normoxic (21% O₂) and hypoxic (1% O₂) conditions over a time course of 1 to 48 hours. Data was presented as means $\pm$ S.E.M from six independent experiments. Statistical analysis by ANOVA and Tukey's tests demonstrate that DCF fluorescence increased significantly following 6 hours of hypoxia indicating a high level of ROS induction during prolonged hypoxia. *p<0.05, **p<0.01, ***p<0.001 compared to normoxia.

intensity during hypoxia from 1 to 5 hours was not significantly different from normoxia. Under hypoxia, DCF fluorescence reached a maximum at 6 hours resulting in a 12.8-fold increase in fluorescence compared to 6 hours of normoxia ($p=0.003$, ANOVA; $p<0.01$, Tukey's test). Fluorescence decreased at 12 hours of hypoxia resulting in a 2-fold increase in signal fluorescence compared to 12 hours of normoxia ($p=0.006$, ANOVA; $p<0.001$, Tukey's test). At 24 hours, DCF fluorescence under hypoxia was not significantly different from normoxia ($p=0.2$, ANOVA), however the fluorescence during 48 hours of hypoxia significantly increased 2.3-fold above normoxic levels ($p=0.03$, ANOVA; $p<0.05$, Tukey's test). The ANOVA and Tukey's tests demonstrated that prolonged hypoxia (≥ 6 hours) significantly increased intracellular DCF fluorescence compared to normoxia. DCF fluorescence for all treated samples was significantly higher than control groups (empty well; cells + no DCF; no cells + DCF; $p<0.001$ in all cases, Tukey's test). These findings demonstrate that the highest levels of ROS in PC12-AC cells are induced following prolonged hypoxic exposure.

8.6. Changes in APP Gene Expression in Response to Hypoxia

Activation from the APP promoter suggests an increase in APP expression under hypoxic conditions. A real-time PCR approach was used to examine whether APP gene levels change during hypoxia in PC12-AC cells. Total RNA from normoxic and hypoxic PC12-AC cells was used as a template to amplify a portion of the rat APP gene by RT-PCR. The resulting cDNAs were amplified by PCR or used for real-time PCR analysis to determine a profile of APP gene expression relative to β -actin.

PCR analysis of gene products resolved via gel electrophoresis revealed a single amplicon of correct molecular weight (APP, 378 bp; β -actin, 279 bp) as well as a possible increase in APP gene levels during hypoxia compared to normoxia (Figure 13A). β -actin gene levels remained unchanged in all samples and β -actin was therefore used as an internal reference gene for real-time PCR. To confirm primer specificity during real-time PCR, a melting curve analysis was performed which resulted in single product melting temperatures (approximately 89°C for APP and 87°C for β -actin; data not shown). Figure 13B depicts the levels of APP in PC12-AC cells normalized by the amount of β -actin and expressed as fold induction over controls (normoxia). These levels were analyzed by quantitative real-time PCR and the Pfaffl method (Pfaffl, 2001). APP gene expression increased during hypoxia as compared to normoxia (Figure 13B). A 3.7- and 7.3-fold increase in APP gene levels was observed at 24 and 48 hours of hypoxia, respectively, as compared to normoxia. The ANOVA and Tukey's tests for multiple comparisons showed that there were significant changes in APP gene expression between each of the normoxic and hypoxic conditions ($p < 0.05$).

8.7. Changes in APP Protein Levels in Response to Hypoxia

To investigate whether increased APP gene expression during hypoxia correlated with increased expression of the APP protein, APP protein levels during normoxic and hypoxic conditions were analyzed in whole cell extracts from PC12-AC lysates using

Figure 13. Real-time PCR analysis of APP gene expression in PC12-AC cells: Total RNA from normoxic and hypoxic PC12-AC cells was reverse transcribed using gene specific primers for APP or β -actin. The resulting cDNAs were amplified by PCR or used for real-time PCR analysis. **A)** Representative PCR of APP and β -actin products separated by polyacrylamide gel electrophoresis. Products of the correct molecular weight were amplified (APP with a 378 bp product and β -actin with a 279 bp product). N=48 hours normoxic, H=48 hours hypoxic. **B)** Changes in APP gene expression were quantified by real-time PCR. A value of 1 was assigned to the normalized APP gene level on control (normoxic) cells. The level of APP, normalized by the amount of β -actin, in hypoxic samples is represented as fold over control. Data was presented as means $\pm$ S.E.M from six independent experiments. Statistical analysis by ANOVA and Tukey's tests demonstrated that there were significant differences in APP gene expression between each of the normoxic and hypoxic conditions ($p < 0.05$). * $p < 0.05$ compared to normoxia.

A **B**

Western immunoblotting and a specific antibody to the C-terminal residues 681–695 of APP (CT15). The CT15 antibody recognizes holo-APP and does not distinguish among the secreted forms of APP (Palacino *et al.*, 2000).

Immunoblot analysis with CT15 showed two bands of approximately 110 and 130 units in molecular weight (Figure 14A). The upper band was highly labelled by the antibody and most likely represents the holo-APP (correct molecular weight of ~120 kDa) (Buxbaum *et al.*, 1998). The lower band may represent a) an isoform of APP since its expression varies with hypoxic treatment or b) non-specific binding since CT15 is a polyclonal antibody (Buxbaum *et al.*, 1998; Palacino *et al.*, 2000). APP expression was weak under normoxic conditions. However, expression increased after 6 hours to a maximum at 24 hours of hypoxia. APP protein levels decreased from 24 to 48 hours of hypoxia. The changes in APP levels were normalized to levels of α -tubulin which was used as a loading control and detected at 55 kDa by an anti-tubulin antibody.

Densitometry revealed a general increase in APP protein levels in response to hypoxia as compared normoxia (Figure 14B). There were no significant changes in APP expression up to 12 hours of hypoxia as compared to normoxia, however APP expression increased significantly at 18 to 48 hours of hypoxia as compared to normoxia. APP expression was 7-fold higher at 18 hours of hypoxia as compared to normoxia ($p=0.03$, ANOVA; $p<0.05$, Tukey's test). At 24 hours of hypoxia, APP protein levels were 12-fold higher than normoxic levels ($p=0.004$, ANOVA; $p<0.01$, Tukey's test). APP levels were lower at 48 hours compared to 24 hours of hypoxia, but remained significantly higher

Figure 14. Western immunoblot analysis of APP protein levels under hypoxic conditions: Westerns for APP were performed using an antibody specific to APP (CT15) with whole cell extracts prepared from PC12-AC lysates after normoxic and hypoxic exposure. **A)** Representative Western blot indicates that APP holo-protein levels increased after 6 hours of hypoxia. Position of the molecular weight marker (MW) in kDa is indicated on the left. Prestained Broad Range Protein Markers (New England BioLabs) were used for molecular weight determination. α -tubulin was used as a control for loading and normalization. **B)** Densitometric analysis of changes in APP holo-protein levels after hypoxia. Data was presented as means $\pm$ S.E.M from three independent experiments. Statistical analysis by ANOVA and Tukey's tests demonstrated that there were significant differences in APP protein levels compared with each of the normoxic and hypoxic groups ($p < 0.01$). * $p < 0.05$, ** $p < 0.01$ compared to normoxia.

A **B**

than normoxic levels ($p=0.008$, ANOVA; $p<0.01$, Tukey's test). The ANOVA and Tukey's tests demonstrate that there were significant changes in APP protein expression between each of the normoxic and hypoxic conditions ($p<0.01$). These results indicate a transient induction of APP expression by hypoxia at the level of the protein that correlated with APP induction at the level of the gene until the 24 hour hypoxic time point.

8.8. Changes in HIF-1 α Protein Levels in Response to Hypoxia

If HIF-1 is implicated in the hypoxia-induced APP promoter activation in PC12-AC cells, HIF-1 α should be expressed during prolonged exposure to hypoxia. To demonstrate the stabilization of HIF-1 α under conditions of prolonged hypoxia and proteasome inhibition in PC12-AC cells, Western immunoblot analysis for HIF-1 α was performed in nuclear extracts from the same cell lysates utilized in the previous experiment.

Proteins of approximately 120 kDa representing HIF-1 α were recognized by the HIF-1 α antibody (Figure 15). Multiple bands at this molecular weight corresponded to unmodified and post-translationally modified HIF-1 α (Chavez *et al.*, 2000). The levels of HIF-1 α protein were undetectable in normoxic PC12-AC nuclear extracts, but increased dramatically in response to hypoxic conditions (6 to 48 hours). The increase in HIF-1 α protein levels was maximal at 24 hours and decreased during 48 hours of hypoxia. Proteasome inhibition dramatically increased HIF-1 α levels. Furthermore, induction of

Figure 15. Western immunoblot analysis of HIF-1 α protein levels under conditions of hypoxia, proteosome inhibition and cobalt treatment: Western blots were performed using an antibody specific to HIF-1 α (upper panel) with nuclear extracts prepared from PC12-AC lysates following normoxic and hypoxic exposure. Proteins of approximately 120 kDa were detected and represented HIF-1 α . HIF-1 α protein levels were undetectable under normoxic conditions and were induced dramatically under conditions of hypoxia, proteosome inhibition and cobalt treatment. α -Tubulin, detected at 55 kDa, was used as a control for loading and normalization. Indicated on the left are the positions of the molecular weight marker (MW) in kDa. Prestained Broad Range Protein Markers (New England BioLabs) were used for molecular weight determination.

HIF-1 α was observed with cobalt treatment (a hypoxia mimetic). Tubulin levels remained constant during treatment and were used as a loading control (detected at 55 kDa by an anti-tubulin antibody). These findings demonstrate that HIF-1 α stabilization occurs under prolonged hypoxic conditions and correlates with the induction of APP promoter/CAT and luciferase reporter activity.

8.9. EMSA Analysis of APP Promoter DNA and Protein Interactions Under Hypoxia

The Willmore laboratory identified three putative HREs corresponding to the sequence 5'-GCGTG-3', repeated in positions -183 to -187, -156 to -160 and +40 to +44 in the 5'-regulatory region of the APP promoter. To investigate whether each HRE sequence is an actual binding site for HIF-1 and whether cell-specific proteins could interact with the APP promoter region under normoxic and hypoxic conditions, DNA and protein binding was examined by the EMSA. Radiolabeled promoter regions -488 to +99 (APP), -492 to -266 (APP1), -354 to -26 (APP2) and -45 to +166 (APP3) and a mammalian HIF-1 binding sequence containing the human erythropoietin HRE (W18; 5'-AGCTTGCCCTACGTGCTGTCTCAG-3' were tested using nuclear extracts belonging to PC12-AC and rat cortical neuronal cells.

DNA-protein complexes comprising six distinct equidistant bands were detected with all radiolabeled probes in the presence of PC12-AC nuclear extracts (Figure 16).

Figure 16. EMSA analysis of PC12-AC cell nuclear proteins interacting with APP promoter regions under conditions of normoxia, hypoxia and cobalt treatment:

EMSA was performed with four different APP promoter probes or with the HIF-1 binding sequence probe (W18) and analyzed in a 5% non-denaturing polyacrylamide gel. Radiolabeled full-length APP (lanes 1-4), APP1 (not shown), APP2 (not shown), APP3 (lanes 5-8) and W18 (lanes 9-14) probes incubated in the presence of either no nuclear extracts (lanes 1, 5 and 9) or PC12-AC nuclear extracts under conditions of normoxia (lanes 2, 6 and 10), 24 hour hypoxia (lanes 3, 7 and 11), 48 hour hypoxia (lanes 4 and 12) and 24 hour cobalt (lanes 8 and 13). Major DNA-protein complexes, represented by a pattern of at least six bands, were observed with each of the probes in the presence of PC12-AC nuclear extracts. The top arrows indicate the position of the DNA-protein complex and the bottom arrow shows the position of the free probe.

Formation of the complexes was not affected by the addition of 100-fold excess of unlabeled W18 probe which failed to compete for interaction between the radiolabeled probes and DNA-binding proteins in PC12-AC extracts (data not shown). This suggests that the APP HREs do not bind HIF-1 *in vivo* under hypoxic conditions. To determine if HIF-1 bound to the APP promoter, supershift assays were performed using antibodies against HIF-1 α . No inhibition or shifting of DNA-protein complexes was observed with antibodies against HIF-1 α (data not shown) despite the fact that HIF-1 α levels were detectable by Western immunoblotting (Figure 15). These results suggest that HIF-1 may not serve as the transcriptional regulator of APP promoter activity under hypoxic conditions and that other factors may be involved.

Subsequent EMSAs using probes incubated with rat primary cortical neuronal nuclear extracts showed at least three DNA-protein complexes comprising of a single band (Figure 17). No differences were observed between normoxic and hypoxic extracts. There were more proteins from the PC12-AC cells binding to the same APP promoter probes than proteins from rat cortical neuronal cells (Figures 16 and 17). These findings demonstrate that differences in DNA-protein interactions in the APP promoter exist among primary (rat neuronal) and immortal (PC12-AC) neuronal cell lines.

Figure 17. EMSA analysis of rat cortical neuronal cell nuclear proteins interacting with APP promoter regions under conditions of normoxia and hypoxia: EMSAs were performed with four different APP promoter probes or with the HIF-1 binding sequence probe (W18) and analyzed in a 5% non-denaturing polyacrylamide gel. Radiolabeled W18 (lanes 1 and 2), full-length APP (lanes 3-5), APP1 (6-8), APP2 (9-11) and APP3 (lanes 12-14) probes incubated in the presence of either no nuclear extract (lanes 5, 8, 11 and 14) or rat cortical neuronal nuclear extract under conditions of normoxia (lanes 1, 3, 6, 9 and 12) and 24 hour hypoxia (lanes 2, 4, 7, 10 and 13). The top arrows indicate the position of the DNA-protein complex and the bottom arrows show the position of the free probe.

9. DISCUSSION

The APP gene product plays a pivotal role in Alzheimer's disease. The APP derivative, the A β peptide, is believed to be responsible for neuronal death and the resulting dementia in Alzheimer's disease. Over-expression of APP and A β are observed in the Alzheimer's disease brain and in transgenic mice models of Alzheimer's disease (Games *et al.*, 1995; Mattson, 1997). Both APP and A β also accumulate at sites of ischemic injury (Jendroska *et al.*, 1995). Abnormalities in APP gene expression may contribute to the pathology of Alzheimer's disease. It is therefore essential to examine the regulation of the APP gene promoter.

Cerebral ischemia and stroke are cardiovascular dysfunctions which have been shown to have a direct link with Alzheimer's disease (Taylor *et al.*, 1999; Nalivaeva *et al.*, 2004). Ischemia is associated with hypoxia which results in hypoxia-inducible gene expression (Kogure and Kato, 1993; Bergeron *et al.*, 1999). The present study provides evidence supporting the hypothesis that APP is regulated by hypoxia. Hypoxia has been implicated in the pathology of many diseases including ischemic damage to the brain (Semenza, 2001). Hypoxia triggers an adaptive response mechanism which involves changes at the level of gene transcription. These changes are mediated by the heterodimeric transcription factor HIF-1 to maintain oxygen homeostasis. The HIF-1 α protein and HIF-1 transcriptional activity are known to be induced in the brain as a consequence of hypoxia (Bergeron *et al.*, 1999; Chavez *et al.*, 2000). Induction of the HIF-1 α protein is detected within 1 hour of hypoxia in the brain, reaching a maximum after 5 hours, followed by a decrease to basal levels by 12 hours (Stroka *et al.*, 2001). In

contrast, in tissues such as the kidney and liver, HIF-1 α expression is highest by 2 hours of hypoxia and declines to undetectable levels by 4 hours (Stroka *et al.*, 2001). APP may be a direct target for HIF-1-induced gene expression based on the presence of three putative HREs located within its promoter. APP promoter activity is enhanced in the presence of HIF-1 α in the current study. Furthermore, we show that levels of the APP gene and its protein product are significantly induced in response to hypoxia. Increased production of APP following oxygen deprivation may be one of the key factors linking the increased incidence of Alzheimer's disease following ischemic stroke.

9.1. PC12-AC Cells

PC12-AC cells represented an ideal model system for studying the interaction between hypoxia, HIF-1 and APP promoter activity. These cells constitutively express all three major APP isoforms (695, 751 and 770; Fukuyama *et al.*, 1993) and have been documented to possess oxygen-sensing capabilities unique to cells which have evolved the ability to tolerate, hypoxia such as Type I cells of the carotid body (Spicer and Millhorn, 2003). The hypoxia response in PC12-AC cells involves rapid membrane depolarization, increased calcium influx, and subsequent neurotransmitter release, as well as activation of several signal transduction pathways (such as MAPK) and transcription factors (such as HIF-1) (Spicer and Millhorn, 2003; Gozal *et al.*, 2005). Furthermore, changes in the expression of a number of genes and proteins involved in the regulation of hypoxic adaptive responses have been observed in PC12-AC cells following an episode of hypoxia (Gozal *et al.*, 2005; Spicer and Millhorn, 2003).

9.2. Cell Viability and ROS

Measurements of cell viability provide valuable tools in many research areas and are vital to successful mammalian tissue and cell culture (Altman *et al.*, 1993). Several approaches have been used in the past such as the trypan blue exclusion assay. Trypan blue staining is a simple method used to evaluate cell membrane integrity (and thus cell death) but the assay is limited in sensitivity and accuracy since it indicates only if a cell is alive, and it has been reported to overestimate cell viability (Altman *et al.*, 1993). The MTT assay is a colorimetric dye system which measures the reduction of the yellow tetrazolium salt MTT into an insoluble blue-black coloured formazan product by the mitochondria of viable (but not dead) cells (Mosmann, 1983). The assay is quantitative and sensitive because the amount of colour produced is directly proportional to the number of viable cells.

The MTT assay was used to assess PC12-AC cell viability under different levels of hypoxia. Hypoxia induces both necrosis and apoptosis in neuronal cells and the extent of cell death is dependent on the severity and duration of the hypoxic episode (Gozal *et al.*, 2005). We wanted to determine a level of hypoxia that would induce severe hypoxia without affecting cell viability. Our results showed that neither a moderate (4%) nor a severe (1%) level of hypoxia substantially reduced cell viability following a prolonged period of hypoxia (24 hours; Figure 9). Previous studies have demonstrated that significant cell loss is detected only in PC12-AC cells that have been exposed to severe hypoxia for at least 48 hours (Gozal *et al.*, 2005). Hence our selection of a relatively shorter duration of hypoxia may account for the lower magnitude of cell loss.

Furthermore, neuronal cells appear to experience a delay in the execution of cell death after a hypoxic insult. For instance, in rat cortical neuronal cells which were also utilized in our study, significant reductions in cell viability were not detected until 48 to 72 hours after the hypoxic insult (Bossenmeyer-Pourie *et al.*, 2000; Chen *et al.*, 2003). This suggests that the adverse effect of hypoxia on cell viability may represent a delayed response and may explain why we failed to detect a significant change in cell viability after only 24 hours. It is possible that PC12-AC cells may withstand long-term exposure to hypoxic conditions without substantial loss of viability. Based on the results, we selected a level of 1% oxygen for subsequent experiments because a) it failed to cause a prominent loss in PC12-AC cell viability and b) is representative of the severe hypoxic insult associated with cerebral ischemia and stroke.

Although we failed to detect a prominent loss in PC12-AC cell viability after a severe hypoxic insult, neuronal cells subjected to hypoxia show numerous impairments. Studies have shown that a transient or prolonged episode of oxygen deprivation causes impairments in mitochondrial function and membrane integrity, DNA damage, elevated levels of ROS, activation of pro-apoptotic genes, and A β deposition in neuronal cells (Chen *et al.*, 2003). These impairments bear a striking resemblance to the cellular abnormalities that occur in Alzheimer's disease.

Increased production of ROS has been implicated in hypoxia and in neurodegenerative diseases such as Alzheimer's disease by inducing cellular damage (Bazan *et al.*, 2002; Hou *et al.*, 2003). The interaction of A β aggregates with metals such as copper and iron generates ROS which promote damage to cellular components such as lipids, DNA and proteins, and contribute to excitotoxic cascade, ultimately leading to cell

death in Alzheimer's disease (Behl *et al.*, 1994; Mattson, 1997). The production of free radicals can be conveniently measured *in vivo* through use of the cell-permeable dye DCF. H₂DCF-DA freely enters cells and becomes deacetylated by intracellular esterases. This allows the dye to interact with ROS (primarily peroxy radicals) where it becomes the fluorescent DCF. DCF is indiscriminate and can be oxidized by various ROS therefore DCF fluorescence reflects an overall oxidative stress level in cells (Wang and Joseph, 1999). Increased production of ROS has been shown during periods of hypoxia in PC12-AC cells (Hou *et al.*, 2003). Our DCF studies revealed a significant increase in ROS levels, up to 12.8-fold, after PC12-AC cells were treated with hypoxia for 6 to 48 hours (Figure 12), suggesting that ROS signalling may be involved in the hypoxic response.

9.3. APP Expression

APP accumulates at sites of ischemic brain injury in experimental animal models and APP accumulation has also been found in post-mortem samples from patients who had previously suffered an ischemic episode (Abe *et al.*, 1991; Jendroska *et al.*, 1995; Baiden-Amissah *et al.*, 1998). We utilized real-time PCR analysis to demonstrate an increase in the level of the APP gene in PC12-AC cells under 24 and 48 hour hypoxic conditions (Figure 13). A significant increase in APP gene expression was observed at 24 and 48 hours of hypoxia. APP holo-protein levels increased within 12 hours of hypoxia and were most prominent by 24 hours (Figure 14). Increased APP expression may be considered a defense mechanism against ischemia and hypoxia because the APP

catabolite, sAPP α , plays a neuroprotective role against ischemic injury. For instance, adult rats injected with sAPP α directly into the cerebral ventricles showed improved survival and function after ischemic insult (Smith-Swintosky *et al.*, 1994). However, increased APP expression following ischemia would also permit the enhanced formation of the neurotoxic A β because, as in the case of sAPP α , the induction of the pathway leading to A β production depends on the amount of APP present.

APP holo-protein expression correlated with APP gene expression until the 24 hour time point of hypoxia; APP gene levels were most prominent at 48 hours whereas protein levels decreased at 48 hours. It is likely that the decreased APP holo-protein levels at 48 hours of hypoxia are due to changes in APP metabolism rather than decreased translation. Interestingly, hypoxia causes alterations in APP metabolism and processing (Webster *et al.*, 2004; Velliquette *et al.*, 2005). Levels of ADAM 10, a protein postulated to be α secretase, are reduced under conditions of chronic hypoxia (Webster *et al.*, 2004). This would result in a decreased secretion of sAPP α . Expression of sAPP α increases in response to transient hypoxia (15 minutes) but significantly decreases following a period of chronic hypoxia (1-2% O₂; 24 hours; Nalivaeva *et al.*, 2004; Webster *et al.*, 2004). Levels of proteins involved in the amyloidogenic processing of APP such as BACE, a β secretase, and PS2, proposed to form part of the γ secretase complex, are strongly elevated following hypoxia (Lukiw *et al.*, 2002; Velliquette *et al.*, 2005). The decrease in APP holo-protein expression at 48 hours may thus be due to an increase in APP processing by the β and γ secretases. This in turn may lead to A β over-production and indeed, A β levels are elevated after both mild and severe hypoxia (Jendroska *et al.*, 1995). In addition, hypoxia promotes cleavage of APP resulting in

increased A β expression (Chen *et al.*, 2003). These observations suggest that prolonged hypoxia may not only increase APP production but also shift the balance of APP processing, thereby reducing secretory derivatives of APP which have neuroprotective properties and increasing levels of A β which have neurotoxic properties.

9.4. Induction of APP Promoter Activity by Hypoxia and HIF-1 α

The marked increase in APP gene and protein expression observed during hypoxic conditions prompted our study of the regulation of the APP gene promoter by hypoxia. The human APP promoter has been characterized (Quitschke and Goldgaber, 1992; Lahiri and Ge, 2003) and contains numerous potential recognition sequences for the binding of transcription factors such as HOX-1.3, AP-1, SP-1 and NF κ B (Figure 4). In addition, regulatory elements which increase APP gene expression have also been identified in the APP promoter. These include elements responsive to growth factors, cytokines and stress conditions (Dewji *et al.*, 1995; Yang *et al.*, 1998; Ge and Lahiri, 2002). Although it remains unknown how ischemia and hypoxia might increase APP levels, one possible mechanism would be through the induction of transcription factors such as HIF-1. The APP gene promoter contains three DNA sites of the sequence 5'-GCGTG-3' located between positions -183 to -187, -156 to -160 and +40 to +44 relative to the transcriptional start site (Figure 5), which are almost identical to the core DNA recognition sequence 5'-TACGTG-3' known as an HRE. HREs are functionally important binding sites for HIF-1 and are found in the promoter of a number of genes known to be regulated by hypoxia (Chun *et al.*, 2002).

Genetic reporters are commonly used in cell biology to study the regulation of mammalian gene transcription (Alam and Cook, 1990). Changes in the reporter expression or activity generally correlate to changes in transcriptional activity of the coupled regulated promoter. We utilized CAT and luciferase reporter gene systems to examine transcriptional activity of the APP gene promoter. Only hypoxic treatment resulted in the conversion of substrate to diacetylated product which is indicative of increased CAT reporter gene expression (Figure 10). Hypoxic treatment also increased the activity from an APP promoter/luciferase reporter up to 3.3-fold (Table 1). Our results demonstrate that hypoxia significantly enhances APP promoter activity in transiently transfected PC12-AC cells after at least 6 hours of treatment (Figure 10 and Table 1). Profiles of the time course of APP promoter induction varied between the CAT and luciferase reporter assay systems. CAT reporter assays suggested that induction from the APP promoter was strengthened during prolonged hypoxia (12 to 48 hours) and most pronounced at 48 hours (Figure 10). In contrast, luciferase reporter assays demonstrated that the highest level of APP promoter activity was observed at 6 hours of hypoxia (Table 1). The reason for the discrepancies is currently unclear, but it may be related to differences in efficiency between these two reporter assay systems (the luciferase assay possesses 100-fold greater sensitivity than can be achieved by the CAT assay) (Adam and Cook, 1990), as well as differences in the rate of degradation of these two enzymes in the mammalian system. The half-life of CAT in mammalian cells is approximately 50 hours; in contrast, luciferase has an intracellular half-life of approximately 3 hours (Thompson *et al.*, 1991). Therefore, our data obtained by the luciferase reporter assay are more likely to represent the true induction from the APP promoter as it would not be confounded by

the long-term effects of reporter stability. Up-regulation of APP promoter activity from an APP promoter/CAT or luciferase reporter suggests a role for the APP promoter in hypoxia-responsive expression.

HIF-1 is a critical component of the cellular response to low oxygen in mammalian cells. It activates transcription of a number of genes in hypoxic cells including erythropoietin, vascular endothelial growth factor and glucose transporters, whose protein products are involved in adaptive responses to hypoxia such as angiogenesis and changes in energy metabolism (Bergeron *et al.*, 1999; Chavez *et al.*, 2000). Subsequent experimentation examined the involvement of HIF-1 in the hypoxic induced increase in APP promoter activity. The proteasome regulates the level of specific proteins including certain transcription factors (Lee and Goldberg, 1998). Under normoxic conditions, HIF-1 α becomes ubiquitinated and is rapidly degraded by the proteasome (Bergeron *et al.*, 1999). PIs are commonly used to block the breakdown of short-lived proteins (Lee and Goldberg, 1998) such as HIF-1 α . In our study, the HIF-1 α protein was induced in PC12-AC cells when the proteasome was inhibited (Figure 15). Inhibition of the proteasome would stabilize HIF-1 α resulting in increased nuclear translocation, DNA-binding and transactivation by this transcription factor under normoxic conditions. The effect of proteasome inhibition on APP promoter activity was examined in PC12-AC cells. Our results demonstrate that proteasome inhibition led to a 1.4-fold induction in luciferase expression from the APP promoter construct (Table 1), thus suggesting a possible requirement for HIF-1 α in this response. Inhibition of the proteasome has also been implicated in the up-regulation of heat shock factors which bind to the HSE during conditions of stress such as heat and hypoxia (Awasthi and

Wagner, 2005). The APP promoter fragment utilized in our study contains a HSE at position -317. The role of heat shock transcription factors in regulation of the APP promoter during hypoxia requires further investigation.

Further evidence that HIF-1 could play a crucial role in the regulation of APP promoter activity comes from co-transfection studies with the APP reporter construct and mammalian vectors expressing full-length wildtype (WT) or double mutant (DM) HIF-1 α . The DM form of HIF-1 α contains an ODDD which has been rendered nonfunctional through the mutation of two key proline residues to alanine. This double mutant prevents prolyl hydroxylation of HIF-1 α and stabilizes the protein (Ivan *et al.*, 2001). Overexpression of DM and WT HIF-1 α would be expected to overwhelm the proteasome and result in HIF-1 mediated transcription under normoxic conditions. Induction of APP promoter activity was enhanced by both HIF-1 α subunits during normoxia, providing further evidence that HIF-1 may bind to the APP promoter as well as transactivate transcription from it.

We analyzed the expression of HIF-1 α in PC12-AC cells during chronic hypoxia by Western immunoblotting. Increased levels of the HIF-1 α protein were detected in the nucleus in response to hypoxia but not normoxia (Figure 15). An accumulation of HIF-1 α protein was observed at 6 hours of hypoxia which persisted for at least 48 hours. The HIF-1 α protein was also induced in the presence of cobalt and when the proteasome was inhibited, in accordance with previous studies (Jaakkola *et al.*, 2001). The HIF-1 β protein is present in PC12-AC cells under normoxic conditions and is constitutively expressed (Chavez and LaManna, 2002). The increase in HIF-1 α protein levels in PC12-AC cells mirrored increases in both ROS and APP promoter induction during prolonged hypoxia,

suggesting that HIF-1 α may be stabilized by ROS and regulate the expression of the APP gene, respectively. Increases in ROS have been shown to enhance HIF-1 DNA binding (Willmore, unpublished results; Chun *et al.*, 2002). Interestingly, A β which causes ROS to accumulate in cells activates HIF-1 and increases HIF-1 binding to DNA; implicating HIF-1 in Alzheimer's disease (Soucek *et al.*, 2003).

HIF-1 is involved in the oxygen dependent regulation of gene expression. Based on the results thus far, HIF-1 may bind to the APP promoter and regulate the APP gene under hypoxic conditions because a) putative HREs are contained within the APP promoter, b) expression from the APP promoter is induced by hypoxia and c) the accumulation of the HIF-1 α protein in the nucleus corresponds to APP promoter induction in PC12-AC cells during hypoxia. Detection of sequence specific DNA-binding proteins such as transcription factors is often performed by the EMSA. The assay is based on the observation that proteins which bind specifically to a radiolabeled DNA fragment retard the mobility of the fragment during native gel electrophoresis, resulting in discrete bands corresponding to individual DNA-protein complexes (Buratowski and Chodosh, 2001). EMSA results demonstrated that unique DNA-protein interactions occur between APP promoter regions and nuclear proteins from different cell types. The APP promoter binds nuclear proteins from PC12-AC cells and rat cortical neurons in a cell type specific manner. In PC12-AC cells, a possible increase in the signal of DNA-protein complexes was observed with nuclear extracts subjected to hypoxia versus normoxic controls (Figure 16). In contrast, there was no difference in DNA-protein interactions between hypoxic and normoxic nuclear extracts from rat cortical neurons (Figure 17) which just like PC12-AC cells express HIF-1 α during hypoxia (Chavez and LaManna, 2002). DNA-

protein complexes could not be blocked by unlabeled HIF-1 probe and an antibody specific for HIF-1 α had no effect on the DNA-protein complex in PC12-AC cells. Our results show that the HIF-1 complex may not bind to the HRE localized in the APP promoter suggesting that the APP promoter may not be regulated by hypoxia via HIF-1. The possibility exists that regulation of the APP gene is controlled by a HIF-1-independent mechanism involving factors other than HIF-1 such as AP-1, NF κ B or heat shock factors, which have been reported to regulate gene expression under hypoxia (Dewji *et al.*, 1995; Yang *et al.*, 1998; Fradette and Souich, 2003). The APP promoter fragment utilized in this study contains an AP-1 recognition site of the sequence 5'-TGATTCA-3' located between positions -355 to -349 and a HSE of the sequence 5'-CTCGACTTTTCTAG-3' at position -317 (Figures 4 and 5). The role of these factors in the hypoxic induction of the APP promoter merits further investigation.

The present study is the first to examine the potential regulation of the APP promoter by hypoxia. Although the results presented in this thesis provide evidence that the APP promoter is activated in response to hypoxia and over-expression of HIF-1 α , it is not possible to say with certainty that HIF-1 is involved in the induction of the APP gene. The hypoxic induction of the APP promoter may be HIF-1 independent. Further experiments could include a) site-directed mutagenesis of individual or all HRE sites in the APP promoter and b) deletion analysis of APP promoter fragments to assess the minimal promoter required for hypoxic induction. If hypoxic induction is through the HREs, then either mutation or deletion of these sites within the APP promoter would eliminate hypoxic transactivation from them. The Willmore laboratory has detected new proteins in nuclear extracts from PC12-AC cells under hypoxia via two-dimensional gel

electrophoresis. The proteins specific to hypoxia will be isolated from gels and identified by mass spectroscopy. Future studies are also required to clarify the role of ROS in hypoxia, HIF-1 activation and Alzheimer's disease.

Collectively, we document that hypoxia induces APP expression through the APP promoter in PC12-AC cells which may have important physiological and pathological relevance. In the developing nervous system, increases in APP have been shown to correlate with synaptogenesis (Mattson, 1997). The induction of the APP gene is also part of a cellular response to diverse forms of stress and injury such as ischemia (Dewji *et al.*, 1995). Induction of the stress response may induce resistance of neurons to cell death after hypoxia hence increased APP expression may represent an adaptive defense mechanism against hypoxia (due to sAPP α). However, the stress response may also lead to cell death and degeneration if hypoxia persists (Kogure and Kato, 1993). In our study, increases in APP promoter induction and APP gene and protein expression generally correlated with the elevation of ROS levels. Since elevated ROS levels are associated with increased A β production (Behl *et al.*, 1994; Tuppo and Forman, 2001), this suggests that over-expression of APP may be linked to increased A β production during hypoxia. Over-expression of APP would enhance the abnormal processing of APP that has been shown to occur during hypoxia (Lukiw *et al.*, 2000; Velliquette *et al.*, 2005), leading to a higher concentration of A β which may contribute to worsening ischemic or hypoxic injury and potentiating a hypoxia-induced neurodegeneration ultimately leading to the development of Alzheimer's disease. Establishment of HIF-1 in the induction of the APP gene would make HIF-1 a therapeutic target. Understanding the mechanism of hypoxic-

induced up-regulation of APP is therefore of critical importance and may lead to novel therapeutic interventions in ischemic stroke and neurodegenerative diseases.

10. REFERENCES

- Abe, K., Tanzi, R.E. and Kogure, K. 1991. Selective induction of Kunitz-type protease inhibitor domain-containing precursor protein mRNA after persistent focal ischemia in rat cerebral cortex. *Neurosci. Letters*. 125: 172-174.
- Alam, J. and Cook, J.L. 1990. Reporter genes: Application to the study of mammalian gene transcription. *Anal. Biochem.* 188: 245-254.
- Altman, S.A., Randers, L. and Rao, G. 1993. Comparison of trypan blue dye exclusion and fluorometric assays for mammalian cell viability determinations. *Biotechnol. Prog.* 9: 671-674.
- Araki, W. and Wurtman, R.J. 1998. Increased expression of amyloid precursor protein and amyloid precursor-like protein 2 during trophic factor withdrawal-induced death of neuronal PC12 cells. *Mol. Brain Res.* 56: 169-177.
- Awasthi, N. and Wagner, B.J. 2005. Upregulation of heat shock protein expression by proteasome inhibition: An antiapoptotic mechanism in the lens. *Invest. Ophthalm. Vis. Sci.* 46: 2082-2091.
- Baiden-Amissah, K., Joashi, U., Blumberg, R., Mehmett, H., Edwards, A.D. and Cox, P.M. 1998. Expression of amyloid precursor protein (β -APP) in the neonatal brain following hypoxic ischaemic injury. *Neuropathol. Appl. Neurobiol.* 24: 346-352.
- Bazan, N.G. and Lukiw, W.J. 2002. Cyclooxygenase-2 and presenilin-1 gene expression induced by interleukin-1 β and amyloid β 42 peptide is potentiated by hypoxia in primary human neural cells. *J. Biol. Chem.* 277: 30359-30367.

- Bazan, N.G., Palacios-Pelaz, R. and Lukiw, W.J. 2002. Hypoxia signalling to genes: significance in Alzheimer's disease. *Mol. Neurobiol.* 26: 283-298.
- Behl, C., Davis, J.B., Lesley, R. and Schubert, D. 1994. Hydrogen peroxide mediates amyloid β protein toxicity. *Cell.* 77: 817-827.
- Bergeron, M., Yu, A.Y., Solway, K.E., Semenza, G.L. and Sharp, F.R. 1999. Induction of hypoxia-inducible factor-1 (HIF-1) and its target genes following focal ischaemia in rat brain. *Euro. J. Neurosci.* 11: 4159-4170.
- Bergeron, M., Gidday, J.M., Yu, A.Y., Semenza, G.L., Ferriero, D.M. and Sharp, F.R. 2000. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. *Ann. Neurol.* 48: 285-296.
- Bonin, F., Ryan S.D., Migahed, L., Mo F., Lallier, J., Franks, D.J., Arai, H. and Bennett, S.A.L. 2004. Anti-apoptotic actions of the platelet-activating factor acetylhydrolase I $\alpha 2$ catalytic subunit. *J. Biol. Chem.* 279: 52425-52436.
- Bossenmeyer-Pourie, C., Koziel, V. and Daval, J.-L. 2000. Effects of hypothermia on hypoxia-inducible apoptosis in cultured neurons from developing rat forebrain: Comparison with preconditioning. *Ped. Res.* 47: 385-391.
- Breen, K.C., Bruce, M. and Anderton, B.H. 1991. Beta amyloid precursor protein mediates neuronal cell-cell and cell-surface adhesion. *J. Neurosci. Res.* 28: 90-100.
- Buratowski, S. and Chodosh, L.A. 2001. DNA-protein interactions In: *Current Protocols in Molecular Biology*. Fred M. Ausubel (ed). John Wiley & Sons, Inc., New York, New York, USA.

- Buxbaum, J.D., Thinakaran, G., Koliatsos, V., O'Callahan, J., Slunt, H.H., Price, D.L. and Sisodia, S.S. 1998. Alzheimer amyloid protein precursor in the rat hippocampus: Transport and processing through the perforant path. *J. Neurosci.* 18: 9629-9637.
- Canadian Study of Health and Aging Working Group. 2000. The incidence of dementia in Canada. The Canadian Study of Health and Aging Working Group. *Neurology.* 55: 66-73.
- Charlier, N., Leclere, N., Felderhoff, U., Heldt, J., Kietzmann, T., Obladen, M. and Gross, J. 2002. Hypoxia-induced cell death and changes in hypoxia-inducible factor-1 activity in PC12 cells upon exposure to nerve growth factor. *Mol. Brain Res.* 104: 21-30.
- Chavez, J.C., Agani, F., Pichiule, P. and LaManna, J.C. 2000. Expression of hypoxia-inducible factor-1 α in the brain of rats during chronic hypoxia. *J. Appl. Physiol.* 89: 1937-1942.
- Chavez, J.C. and LaManna, J.C. 2002. Activation of hypoxia-inducible factor-1 in the rat cerebral cortex after transient global ischemia: Potential role of insulin-like growth factor-1. *J. Neurosci.* 22: 8922-8931.
- Chen, G.-J., Xu, J., Lahousse, S.A., Caggiano, N.L. and de la Monte, S.M. 2003. Transient hypoxia causes Alzheimer-type molecular and biochemical abnormalities in cortical neurons: Potential strategies for neuroprotection. *J. Alzheimer's Dis.* 5: 209-228.
- Chun, Y.-S., Kim, M.-S. and Park, J.-W. 2002. Oxygen-dependent and -independent regulation of HIF-1 α . *J. Korean Med. Sci.* 17: 581-588.

- De la Monte, S.M., Neely, T.R., Cannon, J. and Wands, J.R. 2000. Oxidative stress and hypoxia-like injury cause Alzheimer-type molecular abnormalities in central nervous system. *Cell. Mol. Life Sci.* 57: 1471-1481.
- Del Toro, R., Levitsky, K.L., Barneo-Lopez, J. and Chiara, M.D. 2003. Induction of T-type calcium channel gene expression by chronic hypoxia. *J. Biol. Chem.* 278: 22316-22324.
- Dewji, N.N., Do, C. and Bayney, R.M. 1995. Transcriptional activity of Alzheimer's β -amyloid precursor protein gene by stress. *Mol. Brain Res.* 33: 245-253.
- Docagne, F., Gabriel, C., Lebourrier, N., Lesne, S., Hommet, Y., Plawinski, L., MacKenzie, E.T. and Vivien, D. 2004. Sp1 and Smad transcription factors co-operate to mediate TGF- β -dependent activation of amyloid- β precursor protein gene transcription. *Biochem. J.* 383: 393-399.
- Fradette, C. and Souiche P. 2003. Hypoxia-inducible factor-1 and activator protein-1 modulate the upregulation of CYP3A6 induced by hypoxia. *Br. J. Pharmacol.* 140: 1146-1154.
- Fukuyama, R., Chandrasekaran, K. and Rapoport, S.I. 1993. Nerve growth factor-induced neuronal differentiation is accompanied by differential induction and localization of the amyloid precursor protein (APP) in PC12 cells and variant PC12S cells. *Mol. Brain Res.* 17: 17-22.
- Gabuzda, D., Busciglio, J., Chen, L.B., Matsudaira, P. and Yankner, B.A. 1994. Inhibition of energy metabolism alters the processing of amyloid precursor protein and induces a potentially amyloidogenic derivative. *J. Biol. Chem.* 269: 13623-13628.

- Games, D., Adams, D., Alessandrini, R., Barbour, R., Berthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., Guido, T., Hagoplan, S., Johnson-Wodd, K., Khan, K., Lee, M., Leibowitz, P., Lieberburg, I., Little S., Masliah, E., McConlogue, L., Montoya-Zavala, M., Mucke, L., Panini, L., Pennlman, E., Power, M., Schenk, D., Soubert, P., Snyder, B., Soriano, F., Tan, H., Vitale, J., Wadsworth, S., Wolozin, B. and Zhao, J. 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F β -amyloid precursor protein. *Nature*. 373: 523-527.
- Ge, Y.-W. and Lahiri, D.K. 2002. Regulation of promoter activity of the APP gene by cytokines and growth factors. *Ann. N.Y. Acad. Sci.* 973: 463-467.
- Gentleman, S.M., Nash, M.J., Sweeting, C.J., Graham, D.I. and Roberts, G.W. 1993. β -amyloid precursor protein (β APP) as a marker for axonal injury after head injury. *Neurosci. Letters*. 160: 139-144.
- Gozal, E., Sachleben, L.R., Rane, M.J., Vega, C. and Gozal, D. 2004. Mild sustained and intermittent hypoxia induce apoptosis in PC-12 cells via different mechanisms. *Am. J. Physiol. Cell Physiol.* 288: C535-C542.
- Han, P., Dou, F., Li, F., Zhang, X., Zhang, Y.-W., Zheng, H., Lipton, S.A., Xu, H. and Liao, F.-F. 2005. Suppression of cyclin-dependent kinase 5 activation by amyloid precursor protein: A novel excitoprotective mechanism involving modulation of tau phosphorylation. *J. Neurosci.* 25: 11542-11552.
- Heurteaux, C., Bertaina, V., Widmann, C. and Lazdunski, M. 1993. K⁺ channel openers prevent global ischemia-induced expression of c-fos, c-jun, heat shock protein,

- and amyloid β -protein precursor genes and neuronal death in rat hippocampus. *Proc. Natl. Acad. Sci. USA.* 90: 9431-9435.
- Hou, R. C.-W., Huang, H.-M., Tzen, J.T.C. and Jeng, K.-C.G. 2003. Protective effects of sesamin and sesamol on hypoxic neuronal and PC12 cells. *J. Neurosci. Res.* 74: 123-133.
- Hruby, D.E. and Wilson, E.M. 1992. Use of fluorescent chloramphenicol derivative as a substrate for chloramphenicol acetyltransferase assays. *Methods Enzymol.* 216: 369-377.
- Huang, L.E., Gu, J., Schau, M. and Bunn, H.F. 1998. Regulation of hypoxia-inducible factor 1α is mediated by an O_2 -dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA.* 95: 7987-7992.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G Jr. 2001. HIF α targeted for vHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science.* 292: 464-468.
- Iversen, L. L., Mortishire-Smith, J., Pollack, S. J. and Shearman, M. S. 1995. The toxicity in vitro of β -amyloid protein. *Biochem. J.* 311: 1-16.
- Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. 2001. Targeting of HIF- 1α to the von Hippel Lindau ubiquitylation complex by O_2 -regulated prolyl hydroxylation. *Science.* 292: 468-472.

- Jendroska, K., Poewe, W., Daniel, S.E., Pluess, J., Iwerssen-Schmidt, H., Paulsen, J., Barthel, S, Schelosky, L., Cervos-Navarro, J. and DeArmond, S.J. 1995. Ischemic stress induces deposition of amyloid β immunoreactivity in human brain. *Acta Neuropathol.* 90: 461-466.
- Jin, L.W., Ninomiya, H., Roch, J.M., Schubert, D., Masliah, E., Otero, D.A. and Saitoh, T. 1994. Peptides containing the RERMS sequence of amyloid beta/A4 protein precursor bind cell surface and promote neurite extension. *J. Neurosci.* 14: 5461-5470.
- Kogure, K. and Kato, H. 1993. Altered gene expression in cerebral ischemia. *Stroke.* 24: 2121-2127.
- Koo, E.H., Squazzo, S.L., Selkoe, D.J. and Koo, C.H. 1996. Trafficking of cell-surface amyloid β -protein precursor. *J. Cell Sci.* 109: 991-998.
- Lahiri, D.K., Ghosh, C. and Ge, Y.-W. 2003. A proximal gene promoter region for the β -amyloid precursor protein provides a link between development, apoptosis, and Alzheimer's disease. *Ann. N.Y. Acad. Sci.* 1010: 643-647.
- Lahiri, D.K. and Ge, Y.-W. 2004. Role of the APP promoter in Alzheimer's disease: Cell type-specific expression of the β -amyloid precursor protein. *Ann. N.Y. Acad. Sci.* 1030: 310-316.
- Lai, M.-H., Chen, E., Crouthamel, M.-C., DiMuzio-Mower, J., Xu, M., Huan, Q., Price, E., Register, R.B., Shi, X.-P. and Donoviel, D.B. 2003. Presenilin-1 and presenilin-2 exhibit distinct yet overlapping γ secretase activities. *J. Biol. Chem.* 278: 22475-22481.

- Lee, D.H., Goldberg A.L. 1998. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol.* 8: 397-403.
- Lindsay, J., Sykes, E., McDowell, I., Verreault, R. and Laurin, D. 2004. More than the epidemiology of Alzheimer's disease: contributions of the Canadian Study of Health and Aging. *Can. J. Psych.* 49: 83-91.
- Lu, D.C., Rabizadeh, S., Chandra, S., Shayya, R.F., Ellerby, L.M, Ye, X., Salvesen, G.S., Koo, E.H. and Bredesen, D.E. 2000. A second cytotoxic proteolytic peptide derived from amyloid β -protein precursor. *Nat. Med.* 6: 397-404.
- Lukiw, W.J., Gordon, W.C., Rogaev, E.I., Thompson, H. and Bazan, N.G. 2001. Presenilin-2 (PS2) expression up-regulation in a model of retinopathy of prematurity and pathoangiogenesis. *NeuroReport* 12: 53-57.
- Mattson, M.P. 1997. Cellular actions of β -amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiol. Rev.* 77: 1081-1132.
- Mattson, M.P., Barger, S.W., Furukawa, K., Bruce, A.J., Wyss-Coray, T., Mark, R.J. and Mucke, L. 1997. Cellular signalling roles of TGF β , TNF α and APP in brain injury responses and Alzheimer's disease. *Brain Res. Rev.* 23: 47-61.
- Mattson, M.P. 2004. Pathways towards and away from Alzheimer's disease. *Nature.* 430: 631-639.
- Miao, J., Xu, F., Davis, J., Otte-Höller, I., Verbeek, M.M. and Van Nostrand, W.E.. 2005. Cerebral microvascular amyloid β protein deposition induces vascular degeneration and neuroinflammation in transgenic mice expressing human vasculotropic mutant amyloid β precursor protein. *Am. J. Path.* 167: 505-515.

- Milward, E.A., Papadopoulos, R., Fuller, S.J., Moir, R.D., Small, D., Beyreuther, K. and Masters, C.L. 1992. The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. *Neuron*. 9: 129-137.
- Moran, P.M., Higgins, L.S., Cordell, B. and Moser, P.C. 1995. Age-related learning deficits in transgenic mice expressing the 751-amino acid isoform of human β -amyloid precursor protein. *Proc. Natl. Acad. Sci. USA*. 92: 5341-5345.
- Nalivaeva, N.N., Fisk, L., Kochkina, E.G., Plesneva, S.A., Zhuravin, I.A., Babusikova, E., Dobrota, D. and Turner, A.J. 2004. Effect of hypoxia/ischemia and hypoxic preconditioning/reperfusion on expression of some amyloid-degrading enzymes. *Ann. N.Y. Acad. Sci.* 1035: 21-33.
- Nunan, J. and Small, D.H. 2000. Regulation of APP cleavage by α -, β - and γ -secretases. *FEBS Letters*. 483: 6-10.
- Palacino, J.J., Berechid, B.E., Alexander, P., Eckman, C., Younkin, S., Nye, J.S. and Wolozin, B. 2000. Regulation of amyloid precursor protein processing by presenilin 1 (PS1) and PS2 in PS1 knockout cells. *J. Biol. Chem.* 275: 215-222.
- Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic. Acid. Res.* 29: 2002-2007.
- Quitschke, W.W. and Goldgaber, D. 1992. The amyloid β -protein precursor promoter. A region essential for transcriptional activity contains a nuclear factor binding domain. *J. Biol. Chem.* 267: 17362-17368.
- Quitschke, W.W., Matthews, J.P., Krauss, R.J. and Vostrov, A.A. 1996. The initiator element and proximal upstream sequences affect transcriptional activity and start

- site selection in the amyloid β -protein precursor promoter. *J. Biol. Chem.* 271: 22231-22239.
- Rogers, J.T., Leiter, L.M., McPhee, J., Cahill, C.M., Zhan, S.-S., Potter, H. and Nilsson, L.N.G. 1999. Translation of the Alzheimer amyloid precursor protein mRNA is up-regulated by interleukin-1 through 5'-untranslated region sequences. *J. Biol. Chem.* 274: 6421-6431.
- Safran, M. and Kaelin Jr., W.G. 2003. HIF hydroxylation and the mammalian oxygen-sensing pathway. *J. Clin. Invest.* 111: 779-783.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M. and Kukull, W. 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Med.* 2: 864-870.
- Schmitz, A., Tikkanen, R., Kirfel, G. and Herzog, V. 2002. The biological role of Alzheimer amyloid precursor protein in epithelial cells. *Histochem. Cell Biol.* 117: 171-180.
- Selkoe, D.J. 2001. Alzheimer's disease: Genes, proteins, and therapy. *Physiol. Rev.* 81: 741-766.
- Semenza, G.L. 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J. Appl. Physiol.* 88: 1474-1480.
- Semenza, G.L. 2001. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol. Med.* 7: 345-350.

- Semenza, G.L. 2003. Targeting HIF-1 for cancer therapy. *Nature Rev. Cancer*. 3: 721-732.
- Smith-Swintosky, V., Pettigrew, L., Craddock, S., Culwell, A., Rydel, R. and Mattson, M.P. 1994. Secreted forms of β -amyloid precursor protein protect against ischemic brain injury. *J. Neurochem*. 63: 781-784.
- Soucek, T., Cumming, R., Dargusch, R., Maher, P. and Schubert, D. 2003. The regulation of glucose metabolism by HIF-1 mediates a neuroprotective response to amyloid beta peptide. *Neuron*. 39: 43-56.
- Spicer, Z. and Millhorn, D.E. 2003. Oxygen sensing in neuroendocrine cells and other cell types: Pheochromocytoma (PC12) cells as an experimental model. *Endo. Pathol*. 14: 277-292.
- Stephenson, D.T., Rash, K. and Clemens, J.A. 1992. Amyloid precursor protein accumulates in regions of neurodegeneration following focal cerebral ischemia in the rat. *Brain Res*. 593: 128-135.
- Storey, E. and Cappai, R. 1999. The amyloid precursor protein of Alzheimer's disease and the A β peptide. *Neuropathol. Appl. Neurobiol*. 25: 81-97.
- Stroka, D.M., Burkhardt, T., Desbaillets, I., Wenger, R.H., Neil, D.A.H, Bauer, C., Gassmann, M. and Candinas, D. 2001. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J*. 15: 2445-2453.
- Strooper, B. and Annaert, W. 2000. Proteolytic processing and cell biological functions of the amyloid precursor protein. *J. Cell Sci*. 113: 1857-1870.

- Suh, Y.-H. and Checler, F. 2002. Amyloid precursor protein, presenilins, and α -synuclein: Molecular pathogenesis and pharmacological application in Alzheimer's disease. *Pharmacol. Rev.* 54: 469-525.
- Taylor, S.C., Batten, T.F.C. and Peers, C. 1999. Hypoxic enhancement of quantal catecholamine secretion. *J. Biol. Chem.* 274: 31217-31222.
- Thompson, J.F., Hayes, L.S. and Lloyd, D.B. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene.* 103: 171-177.
- Trejo, J., Massamir, T., Deng, T., Dewji, N.N., Bayney, R.M. and Brown, J.H. 1994. A direct role for protein kinase C and the transcription factor Jun/AP-1 in the regulation of the Alzheimer's β -amyloid precursor protein gene. *J. Biol. Chem.* 269: 21682-21690.
- Tuppo, E.E. and Forman, L.J. 2001. Free radical oxidative damage and Alzheimer's disease. *J.A.O.A.* 101: S11-S15.
- Velliquette, R.A., O'Connor, T. and Vassar, R. 2005. Energy inhibition elevates β -secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *J. Neurosci.* 25: 10874-10883.
- Vostrov, A.A., Taheny, M.J. and Quitschke, W.W. 2002. A region to the N-terminal side of the CTCF zinc finger domain is essential for activating transcription from the amyloid precursor protein promoter. *J. Biol. Chem.* 277: 1619-1627.
- Wang, H. and Joseph, J.A. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med.* 27: 612-616.

- Webster, N.J., Green, K.N., Settle, V.J., Peers, C. and Vaughan, P.F.T. 2004. Altered processing of the amyloid precursor protein and decreases expression of ADAM 10 by chronic hypoxia in SH-SY5Y: no role for the stress-activated JNK and p38 signalling pathways. *Mol. Brain Res.* 130: 161-169.
- Willmore, W.G. 2004. Control of Transcription in Eukaryotic Cells. In: *Functional Metabolism: Regulation and Adaptation*. Kenneth B. Storey (ed). Wiley-Liss, Inc., Hoboken, N.J.
- Yamazaki, T., Selko, D.J. and Koo, E.H. 1995. Trafficking of cell surface β -amyloid precursor protein: Retrograde and transcytotic transport in cultured neurons. *J. Cell Biol.* 129: 431-442.
- Yang, Y., Quitschke, W.W. and Brewer, G.J. 1998. Upregulation of amyloid precursor protein gene promoter in rat primary hippocampal neurons by phorbol ester, IL-1 and retinoic acid, but not by reactive oxygen species. *Mol. Brain Res.* 60: 40-49.
- Yoshikawa, K., Alzawa, T. and Hayashi, Y. 1992. Degeneration *in vitro* of post-mitotic neurons overexpressing the Alzheimer amyloid protein precursor. *Nature.* 359: 64-67.
- Zheng, H., Jian, M., Trumbauer, M.E., Sirinathsinghji, D.J.S., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W., Stevens, K.A., Slunt, H.H., Sisodia, S.S., Chen, H.Y. and Van der Ploeg, L.H.T. 1995. β -amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. *Cell.* 81: 525-531.