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Amyloid beta resistance and the Warburg effect: Re-examining Alzheimer's disease

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

AMYLOID BETA RESISTANCE AND THE WARBURG EFFECT: RE-EXAMINING ALZHEIMER'S DISEASE

(Thesis format: Integrated Article)

by

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Abstract

Alzheimer's disease (AD) is characterized by deposition of the amyloid beta (Aβ) peptide in the brain, an event which frequently but not universally correlates with nerve cell death. Although most nerve cells die in response to Aβ, small populations of cells are able to survive by becoming resistant to Aβ toxicity. Understanding the mechanisms by which cells become resistant to Aβ may reveal novel treatments for AD. Interestingly, nerve cell lines selected for resistance against Aβ exhibit increased glucose uptake and glycolytic flux. Here I show that these metabolic changes are mediated through an upregulation of pyruvate dehydrogenase kinase 1 (PDK1) and increased lactate dehydrogenase A (LDHA) activity. These metabolic alterations in Aβ resistant nerve cells are reminiscent of the Warburg effect, also known as aerobic glycolysis, a common anti-apoptotic mechanism elicited by cancer cells. Similar to cancer cells, Aβ-resistant nerve cells exhibit reduced mitochondrial reactive oxygen species (ROS) production. Inhibiting PDK1 or LDHA expression or activity re-sensitized resistant cells to Aβ toxicity. In contrast, overexpression of either PDK1 or LDHA in sensitive cells conferred resistance to Aβ and other neurotoxins. Importantly, cells overexpressing either PDK1 or LDHA displayed repressed mitochondrial oxygen consumption with a concomitant decrease in mitochondrial ROS levels. Furthermore, these cells maintained cellular adenosine triphosphate (ATP) pools when exposed to Aβ, whereas sensitive cells became depleted of ATP. Immunoblot analysis revealed a decrease in PDK1 and LDHA levels in mouse primary cortical neurons treated with Aβ and in cortical tissue extracts from 12-month-old AD transgenic (APPswe/PS1dE9) mice. A similar decrease in PDK1 expression was observed in post-mortem brain extracts from AD patients. Treatment of cultured nerve cell lines and primary cortical neurons with CNB-001 and J147, novel neurotrophic drugs that prevent cognitive decline in AD mice, restored PDK1 and LDHA expression following Aβ treatment. Moreover, PDK1 expression was maintained in AD mice fed CNB-001. Collectively, these findings suggest that the Warburg effect plays a central role in mediating neuronal resistance to Aβ by decreasing mitochondrial activity and subsequent ROS production. Loss of this protective effect may render elderly individuals susceptible to AD.

Keywords

Alzheimer’s disease, amyloid beta, Warburg effect, aerobic glycolysis, pyruvate dehydrogenase kinase, lactate dehydrogenase, toxicity, resistance, oxidative phosphorylation, reactive oxygen species, mitochondrial membrane potential

Co-Authorship Statement

Chapter 2: **Amyloid beta resistance in nerve cell lines is mediated by the Warburg effect**

This chapter has been published in its entirety in the journal, PLoS One. I performed all the experiments, and wrote the manuscript. Though I executed all of the experiments, compiled the data and performed statistical analysis, Andrew Chien quantified the mitochondrial reactive oxygen species fluorescence. Both Andrea Pitts and Robert Arsenault helped tend to my cells throughout the project. Dave Schubert supplied all the cell lines used for the study. My supervisor Dr. Robert Cumming contributed to the experimental design and assisted in the final drafting of the manuscript.

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Chapter 3: **Overexpression of pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A in nerve cells confers resistance to amyloid beta and other toxins by decreasing mitochondrial respiration and ROS production**

This chapter has been published in the Journal of Biological Chemistry. I conceptualized all experiments and drafted the manuscript. I played a role in the execution of all experiments, data collection and statistical analysis. Tim Rappon aided in the time-lapse microscopy and the making of the videos. He also performed the O_2 consumption and ATP experiments. Shawn Albers and Daisy Wong prepared the primary cortical nerve cell cultures. R. Jane Rylette provided the primary cells and allowed for use of her facilities. Dr. Robert Cumming contributed to the experimental design and drafting of the manuscript.

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Chapter 5: **General Discussion and Conclusions**

Portions of this chapter have been taken from a review article that has been accepted for publication in the Journal of Neurodegenerative Diseases. I drafted the manuscript in its

entirety. Richard Harris helped in the making of the figures. Dr. Robert Cumming helped conceptualize and draft the manuscript.

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List of Abbreviations

α-KGDHC	α-ketoglutarate dehydrogenase complex
$\Delta\psi_m$	mitochondrial membrane potential
Aβ	amyloid beta
ABAD	alcohol dehydrogenase
Acetyl-CoA	acetyl coenzyme A
AD	Alzheimer's disease
APH-1A	anterior pharynx-defective 1A
APOE-E4	apolipoprotein E
APP	amyloid precursor protein
ATP	adensoine triphosphate
BACE	β-site APP cleaving enzyme
BBB	blood brain barrier
BNDF	brain derived neurotrophic factor
cDNA	complimentary deoxyribonucleic acid
COX	cytochrome oxidase
CNS	central nervous system
Cyt c	cytochrome c
DCA	dichloroacetic acid
DCFDA	dichlorodihydrofluorescein diacetate
DIAN	dominantly inherited Alzheimer's network
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
Drp1	dynamin-related protein 1
DS	down's syndrome
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FAD	familial Alzheimer's disease
FBS	fetal bovine Serum
FDG	fluoro-2-deoxy-D-glucose
FTDP-17	frontotemporal dementia and parkinsonism linked to chromosome 17
G6PDH	glucose 6 phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLUT	glucose transporters
GSH	glutathione
HIF-1	hypoxia inducible factor 1
HK	hexokinase
HNE	4-hydroxynonenal
HRP	horseradish peroxidase
HS	horse serum
J147	1-(2,4-dimethylphenyl)-2-(3-methoxybenzylidene) hydrazine chloride
LDHA	lactate dehydrogenase A
MAPT	microtubule associated protein tau

MCT	monocarboxylate transporters
MnSOD	manganese superoxide dismutase
MT	microtubules
MTT	3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MTR	mitotracker red CM-H2XRos
NAD	nicotinamide adenine dinucleuotide
NDAN	non-demented individuals with Alzheimer's disease neuropathology
NF-κB	nuclear factor-kappa B
NFT	neurofibrillary tangles
NGF	nerve growth factor
OMM	outer mitochondrial membrane
OXOPHOS	oxidative phosphorylation
PCA	perchloric acid
PET	positron emission tomography
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinasae
PHF	paired helical fragments
PI	propidium iodide
PIB	pittsburg compound
PK	pyruvate kinase
PMI	post-mortem interval
PMSF	phenylmethylsulfonyl fluoride
PPF	plaques per field
PPS	pentose phosphate shunt
Prx	peroxiredoxin
P/S	penicillin/streptomycin
PS1	presenilin 1
PS2	presenilin 2
PVDF	polyvinylidene difluoride
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
SCR	scramble
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin ribonucleic acid
SOD	superoxide dismutase
TCA	tricarboxylic acid cycle
TFAM	transcription factor A, mitochondrial
tg-AD	APPswe/PSEN1dE9 double transgenic mouse strain
TMRM	tetramethyl rhodamine methyl ester
WT	wild type

Chapter 1

1 Introduction and Literature Review

1.1 Alzheimer's disease

1.1.1 Alzheimer's disease overview

Alzheimer disease (AD) is one of the most common neurodegenerative disorders in the elderly and is characterized by behavioral, cognitive and memory impairment. AD is the most common form of dementia accounting for 60-80% of reported cases in the United States, where one in eight individuals over the age of 65 are suffering from some form of dementia (Corrada et al., 2008; Plassman et al., 2007). In 2009 an estimated 34.4 million individuals were affected with dementia, and an estimated $279 billion was spent worldwide for direct costs to care for patients suffering from AD and related dementias (Wimo et al., 2010). Currently, approximately 5.4 million Americans are living with AD, a number which is expected to double or triple by 2050 (Thies and Bleiler, 2011). Moreover, AD is the fifth leading cause of death in the United States (Thies and Bleiler, 2011). Clearly AD represents a critical public health issue for societies worldwide. Efforts focused on further understanding the pathology of the disease could aid in the development of effective preventions, treatments or cures which could significantly reduce the physical, emotional and financial burden of AD. Pathologically, AD is characterized by widespread nerve cell death and the accumulation of extracellular plaques and intracellular neurofibrillary tangles (NFTs) within the brain (Masters et al., 1985a,b). The frequency of plaques and tangles are often concentrated in the cortex and hippocampus, areas of the brain associated with higher cognitive functions and memory and severely affected in AD (Mann et al., 1985; Sabuncu et al., 2011). These plaques are primarily composed of amyloid-β-peptide (Aβ), a 40-42 amino acid peptide derived from the proteolytic cleavage of the amyloid precursor protein (APP) by secretase complexes, including β-secretase, Presenilin 1 (PS1), Presenilin 2 (PS2) and γ-secretase (Hardy, 1997; Masters et al., 1985b; Selkoe, 1998). The intraneuronal NFTs are composed of the microtubule (MT) associated phosphoprotein tau (Koster et al., 1989; Mandelkow and Mandelkow, 1998). In AD, tau becomes hyperphosphorylated which leads

to its aggregation resulting in destabilization of MTs and the disruption of axonal transport (Delacourte and Buee, 1997; Mandelkow et al., 1995; Trojanowski and Lee, 1995).

1.1.2 Causes of AD

The majority of AD cases are sporadic and occur at a late age (greater than 65 years), with only a small percentage of cases associated with autosomal dominant mutations. The cause or causes of AD are poorly understood, with the greatest risk factor being age. In the rare familial forms of the disease, mutations in three major genes have been identified that result in early onset AD (Levy-Lahad et al., 1995; Piaceri et al., 2013; Williamson et al., 2009). These genes encode APP, PS1 and PS2 (Levy-Lahad et al., 1995; Piaceri et al., 2013; Williamson et al., 2009). All mutations associated with AD alter the processing of APP to favor the production and accumulation of Aβ (Hardy, 1997; Williamson et al., 2009). Additionally an apolipoprotein E (APOE) allelic variant, APOE-E4, has been identified as a risk factor for the development of late-onset AD (Farrer et al., 1997; Tang et al., 1998). The APOE-E4 polymorphism is associated with increased Aβ deposition and fibril formation, and decreased Aβ clearance (Ma et al., 1994; Schmechel et al., 1993). Thus, taken together, Aβ has garnered a great deal of attention due to its likely role in the pathogenesis of AD. More recently using genome-wide association studies researchers have identified a number of allelic variants of genes associated with an increased risk for the development of late-onset AD, including CLU, PICALM, TREM, SORL1 and CR1 (Alexopoulos et al. 2011; Cruchaga et al. 2013; Guo et al. 2012; Harold et al. 2009; Jin et al. 2012; Zhang et al 2010). Further investigation into the effects of these genes is required to advance the understanding of the causes of late-onset AD. Though not associated with familial forms of the disease the accumulation of intracellular NFTs composed of tau protein has also been proposed to play a primary role in the pathogenesis of AD.

1.1.3 Tau

Tau proteins belong to the family of microtubule-associated proteins (MAPs), and are found almost exclusively in nerve cells (Schoenfeld and Obar, 1994; Tucker, 1990). Located on chromosome 17 the *tau* gene, also known as microtubule-associated protein tau (*MAPT*), expresses a primary transcript containing 16 exons which can be differentially spliced into six tau isoforms (Andreadis et al., 1992; Goedert et al., 1989; Neve et al., 1986). The six

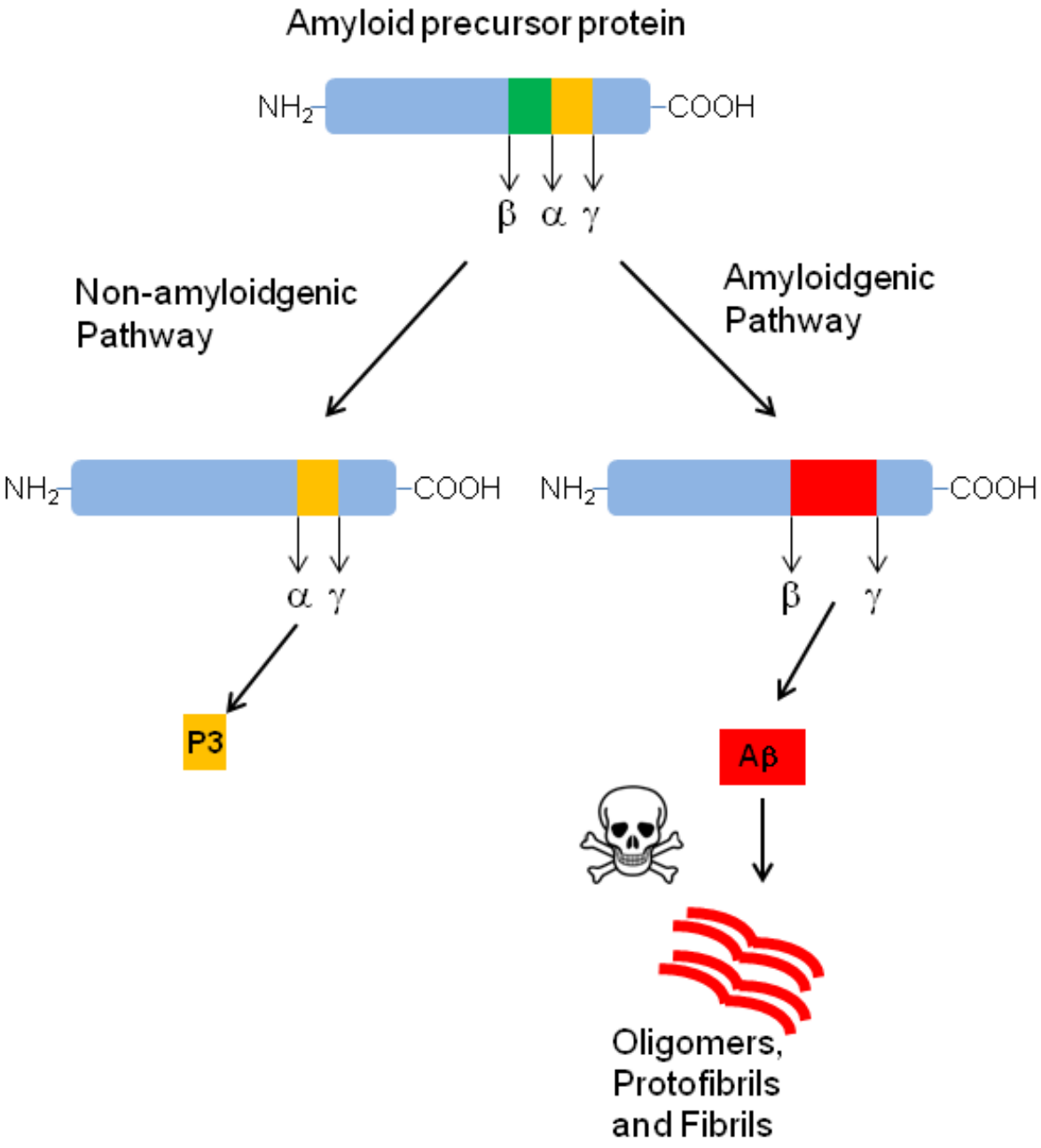
isoforms range in molecular weight from 45-65 kDa and are differentially expressed during development suggesting unique physiological roles for each isoform (Buee et al., 2000; Kosik et al., 1989). Importantly all six isoforms are expressed in the adult brain (Goedert and Jakes, 1990; Kosik et al., 1989). The mature tau proteins promote the assembly of tubulin and function to stabilize MTs through their association with this cytoskeletal component (Alonso et al., 2001; Drubin and Kirschner, 1986; Weingarten et al., 1975). The stabilization of axonal MTs plays a critical role in the transport of important proteins along axons to synapses. Tau proteins are normally regulated by their degree of phosphorylation (Lindwall and Cole, 1984). In patients with AD, tau is abnormally hyperphosphorylated affecting its ability to associate with and stabilize MTs thereby disrupting their assembly and ultimately disrupting axonal transport (Iqbal et al., 1986; Kopke et al., 1993). The hyperphosphorylation of tau promotes the aggregation of the protein into paired helical filaments (PHF) which are the major constituent of intracellular tangles found in the AD brain (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b). As a result of its dysregulation and formation of tangles in AD, hyperphosphoryated tau has been proposed to play an important role in the pathogenesis of AD (Noble et al., 2011).

1.1.4 APP and Aβ

The *APP* gene is located on chromosome 21 and encodes a receptor like integral membrane protein which is ubiquitiously expressed in cells throughout the body (De Strooper and Annaert, 2000; Kang et al., 1987). Though APP may have a role in synapse formation and transmission (Priller et al., 2006), neural plasticity (Turner et al., 2003) and iron export (Duce et al., 2010), a primary role of APP has not yet been clearly defined. APP has a short half life and is constitutively cleaved into various fragments by different secretases; namely α-secretase, β-secretase and γ-secretase. Proteolytic cleavage by α-secretase or β-secretase releases the soluble 100-120 kDa ectodomain of APP, leaving the 10-12 kDa transmembrane C-terminal domain (De Strooper and Annaert, 2000; Shoji et al., 1992). Subsequent cleavage by γ-secretase of the APP C-terminal fragment produced by α-secretase produces a non-toxic, non-aggregating P3 fragment (De Strooper and Annaert, 2000; Shoji et al., 1992). However, cleavage of the APP C-terminal fragment produced by β-secretase results in the secretion of a 4 kDa 40-42 amino acid long Aβ peptide which can be toxic to nerve cells

(Figure 1.1) (De Strooper and Annaert, 2000; Shoji et al., 1992). $A\beta_{1-40}$ is the most commonly produced peptide and is less likely to have a pathogenic effect, whereas $A\beta_{1-42}$ peptide is more fibrillogenic due to the two additional hydrophobic amino acids: isoleucine and alanine (Jarrett et al., 1993). The P3 fragment and Aβ are constitutively released by normal cells and can be detected in the cerebral spinal fluid from normal individuals and those with AD, however in the case of AD, Aβ is primarily produced and is virtually the exclusive constituent of the plaques that form in the brain (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). In normal individuals Aβ is actively metabolized, however individuals with AD have increased production of Aβ and perturbed clearance which leads to an imbalance between the production and removal of this toxic species (Hardy and Selkoe, 2002; Mawuenyega et al., 2010). Though not well understood, clinically relevant levels of Aβ deposition occurs only in the brain (Selkoe, 1998). Once released the Aβ protein undergoes conformational switching between an α-helix conformation to a β-sheet structure (Lansbury, 1999; Serpell, 2000). The β-sheet structure is tightly linked to fibril formation in which the peptide strands are aligned orthogonally to the direction of fibril growth, also known as the "cross-β" motif, which is the main constituent of neuritic plaques (Lansbury, 1999; Serpell, 2000). Small oligomeric intermediates and short protofibres consisting of 5-6 β-sheets are also present in AD and both are believed to be precursors to full length fibres (Harper et al., 1997; Stine et al., 1996; Walsh et al., 1999). An overall increase in Aβ production, as observed in AD, results in increases in Aβ oligomers, fibrils and plaques in the central nervous system (CNS). Though it is often debated whether Aβ fibrils or Aβ oligomers play a more important role in the pathogenesis of AD, both are neurotoxic and appear to be important players.

Figure 1.1 Schematic of amyloid precursor protein (APP) processing. The APP protein is a transmembrane protein that is cleaved by two distinct pathways: non-amyloidogenic (left) or amyloidgenic (right). Cleavage of APP by α-secretase followed by γ-secretase results in the production of a non-pathogenic P3 fragment. In contrast, cleavage by β-secretase followed by γ-secretase results in the generation of a pathogenic Aβ peptide which can subsequently form oligomers, protofobrils and fibrils which are neurotoxic to nerve cells.


Amyloid precursor protein
NH2
COOH
β α γ
Non-amyloidgenic Pathway
Amyloidgenic Pathway
NH2
COOH
α γ
P3
NH2
COOH
β γ
Aβ
Oligomers, Protofibrils and Fibrils

1.2 The Amyloid hypothesis

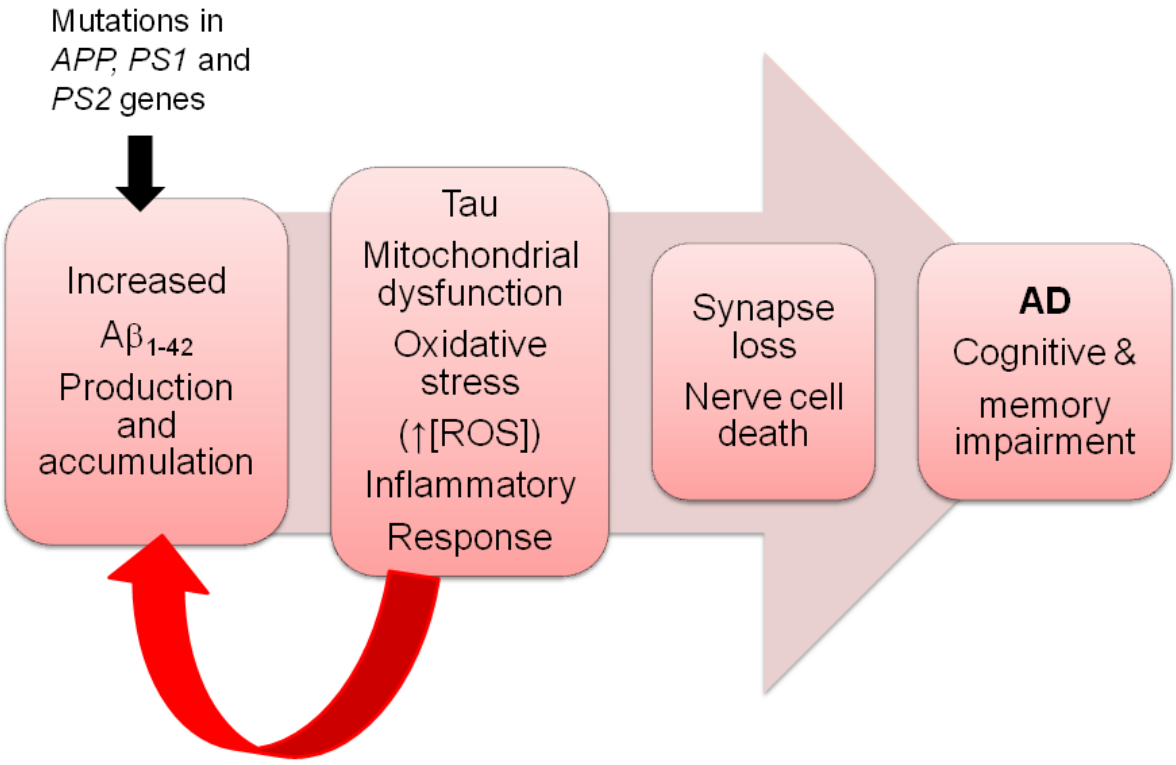
1.2.1 The Amyloid hypothesis overview

The amyloid hypothesis, first proposed over 20 years ago, suggests that Aβ deposition in the brain is the primary causative agent of AD (Hardy and Higgins, 1992; Selkoe, 1991). A few major observations formed the basis of this hypothesis. Importantly, the first genetic mutations associated with familial AD (FAD) were in the *APP* gene and were associated with the overproduction of Aβ (Goate et al., 1991; Hardy, 1992; Hendriks et al., 1992; Mullan et al., 1992). The other identified inherited mutations are in *PS1* and *PS2*, which also increase the processing of APP to favor the production of Aβ (Scheuner et al., 1996). PS1 and PS2 form a complex with γ-secretase and play a direct role in the processing of APP (Sisodia and St George-Hyslop, 2002). Thus all identified genetic mutations causing AD are associated with the increased production of Aβ. Moreover, individuals with Down's syndrome (DS) display similar neuropathological features of AD, mainly plaques, tangles and neurodegeneration, with most individuals developing dementia early in life (Head et al., 2002; Mann and Esiri, 1989; Wisniewski et al., 1985). DS is caused by an extra copy of chromosome 21, the location of the *APP* gene, therefore APP is overexpressed in people with this disorder. Though the cause of late-onset AD remains elusive, the only identified risk factor is inheritance of the *APOE-E4* allele, a genetic variant of the *APOE* gene (Corder et al., 1993). Interestingly mice lacking *APOE* crossed with transgenic mice overexpressing mutant human APP, exhibited reduced Aβ deposition compared to APP transgenic mice, suggesting that APOE affects Aβ deposition *in vivo* (Bales et al., 1997; Holtzman et al., 2000). These observations coupled with studies showing Aβ readily kills cultured nerve cells, led to the theory that Aβ is the primary factor driving AD pathogenesis (Behl et al., 1992; Mattson et al., 1992; Yankner et al., 1990).

Although hyperphosphoryated tau has also been implicated as a pathogenic protein in AD, mutations in *MAPT* have not been detected in FAD. However, mutations in *MAPT* have been associated with an autosomal dominantly inherited dementia named frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17, Pick's disease), which is characterized by severe neurodegeneration in the frontotemporal lobe and the presence of NFTs in both nerve and glial cells (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini and

Goedert, 1998). Notably, there is no Aβ deposition associated with FTDP-17 (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini and Goedert, 1998). Thus alterations in tau that lead to neurodegeneration and NFT deposition are not sufficient to promote Aβ deposition as observed in AD (Hardy and Selkoe, 2002). Therefore it has been proposed that NFTs composed of wild type (WT) tau as seen in AD, likely arise secondarily to Aβ deposition (Hardy et al., 1998). Transgenic mice expressing a human tau containing the most common FTDP-17 mutation (P301L) exhibited NFT formation in the midbrain and spinal cord, but had no NFTs in the cortex and hippocampus, areas known to be affected in AD (Lewis et al., 2000). However, crossing these transgenic mice with AD transgenic mice expressing mutant APP resulted in Aβ deposits and NFTs throughout the cortex, suggesting that Aβ influences the formation of NFTs in areas affected in AD (Lewis et al., 2001). Taken together the excess of Aβ deposition in the brains of patients with AD appears to play a principle role in the pathogenesis of AD. The proposed mechanisms by which Aβ elicits nerve cell toxicity and contributes to the pathogenic events leading to AD will be discussed in detail below. In summary, Aβ production and accumulation, as a result of genetic mutations or in most cases unknown causes, results in the hyperphosphorylation of tau, increased production of reactive oxygen species (ROS), mitochondrial dysfunction and activation of inflammatory processes. The resulting increase in oxidative stress promotes neurodegeneration leading to synaptic loss and nerve cell death which clinically manifests as cognitive deficits and memory loss which are classical symptoms of AD (Figure 1.2).

Figure 1.2 The amyloid cascade hypothesis. The amyloid cascade hypothesis contends that Aβ deposition is the primary influence driving Alzheimer's disease (AD) brain pathology. The accumulation of Aβ as a result of rare familial mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PS1*) and presenilin 2 (*PS2*) or in most cases due to unknown causes results in altered tau regulation leading to its hyperphosphorylation and aggregation into tangles within nerve cells. Moreover, Aβ directly and indirectly induces mitochondrial dysfunction and increases reactive oxygen species (ROS) levels resulting in increased oxidative stress. Aβ induced oxidative stress activates an inflammatory response which further potentiates the production of Aβ. These Aβ induced cellular changes lead to synaptic loss and widespread nerve cell loss in the brain, which is clinically manifested as cognitive deficits and memory loss typical of AD.


Mutations in
APP, *PS1* and
PS2 genes
Increased
$A\beta_{1-42}$
Production
and
accumulation
Tau
Mitochondrial
dysfunction
Oxidative
stress
(↑[ROS])
Inflammatory
Response
Synapse
loss
Nerve cell
death
AD
Cognitive &
memory
impairment

1.2.2 Oxidative stress and AD

The AD brain exhibits extensive free radical damage, due to the accumulation of ROS (Butterfield, 1997; Markesbery, 1997; Smith et al., 2000). ROS are highly reactive molecules which contain an unpaired electron on the outermost shell of oxygen. Due to their reactivity, ROS are capable of modifying macromolecules by oxidizing lipids, proteins and nucleic acids often altering their biological functions. The redox state in a cell is defined by the balance between pro-oxidant and anti-oxidant processes. Pro-oxidant processes result in the production of ROS, whereas anti-oxidant processes detoxify ROS and/or prevent their formation. ROS are formed by several different mechanisms but most notably are formed as a natural byproduct of the mitochondrial electron transport chain (ETC) due to the early offloading of electrons at Complex I or III of the ETC onto molecular oxygen during normal metabolism. In an adult, approximately 2% of molecular oxygen consumed by the respiratory chain gives rise to ROS, a number which is known to rise with age (Balaban et al., 2005). The production of ROS is balanced by the antioxidant defense system, which consists of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, the glutathione (GSH) system (GSH peroxidases and reductases), peroxiredoxins (Prxs) and thioredoxins. For example the superoxide (O_2^-) anion produced as a result of the partial reduction of oxygen in the mitochondria is quickly reduced to hydrogen peroxide (H_2O_2) by SOD which can be further degraded into water and oxygen by catalase. However, in the presence of transition metals (Fe, Cu, Cr and Co), H_2O_2 can be converted to highly reactive hydroxyl radicals (OH^-), a species for which there is no known detoxifying system (Gutteridge, 1994). These reactive hydroxyl radicals can damage almost any macromolecule, wreaking havoc on a cell when produced.

At normal physiological levels ROS function as important signal transducers which play an important role in regulating cellular function (Covarrubias et al., 2008; Marchi et al., 2012; Ufer et al., 2010). However an imbalance in the production and/or detoxification of ROS can result in oxidative stress and subsequent oxidative damage which can ultimately lead to cell death (Pratico, 2008). The brain is especially susceptible to oxidative damage due to its high rate of oxidative metabolism (it consumes 20% of oxygen while only representing 2% of body mass), low levels of antioxidants (compared to other organs), high concentrations of polyunsaturated fats and high content of transition metals (Behl, 1997; Halliwell, 1989, 1992;

Halliwell et al., 1992; Pratico, 2008). Polyunsaturated fats are particularly susceptible to oxidation by ROS resulting in lipid peroxidation or oxidative degradation of lipids in membranes which cause significant damage to the cell (Butterfield and Lauderback, 2002). Moreover, transition metals themselves are capable of catalyzing ROS formation, further contributing to oxidative stress in a cell. In AD there is an imbalance between pro- and anti-oxidant processes which results in oxidative damage (Behl, 1997; Markesbery, 1997). As a result, markers of oxidative stress have been well described in the AD brain including protein, DNA and RNA oxidation and lipid peroxidation (Butterfield, 1997; Markesbery, 1997; Smith et al., 2000). These observations have led to the oxidative stress hypothesis which posits that elevated oxidative stress results in cumulative damage over time that promotes AD pathogenesis (Markesbery, 1997; Pratico, 2008). The increase in ROS in the AD brain is believed to arise, in part, by Aβ deposition and mitochondrial dysfunction (Behl, 1997; Casley et al., 2002; Caspersen et al., 2005; Pratico, 2008). Aβ-induced ROS production and mitochondrial dysfunction will be discussed below.

1.2.3 Aβ accumulation is associated with oxidative stress

Evidence suggests that Aβ deposition both directly and indirectly induces oxidative stress in the AD brain which is likely to contribute to the peptides neurotoxicity (Behl, 1997). The overexpression of APP in M17 cells results in increased levels of cellular ROS (Wang et al., 2008). Similarly, exogenous Aβ has been shown to produce a dose dependent increase in free-radical generation (H_2O_2) and neurotoxicity *in vitro* (Behl et al., 1992; Behl et al., 1994b; Harris et al., 1995). Increased levels of H_2O_2 were also observed in the brains of AD transgenic mice, which were directly related to the level of soluble Aβ (Manczak et al., 2006). The increase in ROS elicited by Aβ exposure ultimately results in lipid peroxidation, which is prominent in the AD brain (Arlt et al., 2002; Behl et al., 1992; Subbarao et al., 1990). Importantly, exogenously applied antioxidants protect against Aβ toxicity *in vitro* (Behl et al., 1992; Behl et al., 1994b). Taken together ROS, more specifically H_2O_2, appear to be closely associated with Aβ induced neurotoxicity.

Elevated levels of H_2O_2 as observed in AD, can also activate the transcription factor nuclear factor-kappa B (NF-κB) (Schreck et al., 1991). The activation of NF-κB results in the transcription of genes involved in the inflammatory response which when activated further

potentiates the production of ROS (Schreck et al., 1991). Aβ has also been shown to induce the activity of NF-κB (Behl et al., 1994b; Kaltschmidt et al., 1997; Valerio et al., 2006). Moreover, NF-κB inhibitors prevented intraneuronal accumulation and secretion of Aβ, suggesting a possible positive feedback loop of Aβ production (Valerio et al., 2006). Further contributing to the accumulation of Aβ, H_2O_2 and 4-Hydroxynonenal (HNE), a product of lipid peroxidation, have been shown to increase the activity of β-secretase or β-site APP cleaving enzyme (BACE1) *in vitro* (Bourne et al., 2007; Tamagno et al., 2005). Additionally, NF-κB binding elements have been identified in the human *BACE1* promoter region and the overexpression of NF-κB subunit p65 up-regulates BACE1 cleavage and Aβ production (Chen et al., 2011). Thus, initial activation of NF-κB as a result of Aβ deposition and increased H_2O_2 levels is likely to induce expression of *BACE1*, leading to the increased production of Aβ and potentiating toxicity in a positive feedback loop (Chen et al., 2011). Both BACE1 and NF-κB subunit p65 levels are significantly increased in the brains of AD patients (Chen et al., 2011; Terai et al., 1996). Taken together it appears that ROS is an important mediator of Aβ induced neurotoxicity.

1.3 Aβ induced mitochondrial dysfunction

1.3.1 Mitochondria play a central role in Aβ toxicity

Mitochondria are specialized organelles that function as key regulators of cell survival and cell death. Most notably, mitochondria are the site of cellular respiration and are responsible for the production of the majority of cellular adenosine triphosphate (ATP). The impairment of mitochondrial metabolism in AD has been well documented (Casley et al., 2002; Caspersen et al., 2005; Gibson et al., 1998). Moreover, mitochondrial abnormalities are believed to be the primary source of oxidative damage in the AD brain (Beal, 2005; Hirai et al., 2001). Importantly, mitochondrial dysfunction is a common feature of different FAD mouse models (PS1, APP, APP/PS1) which occurs prior to plaque deposition and cognitive defects (Trushina et al., 2012). Moreover, functional mitochondria appear to actually be required for Aβ to elicit cellular toxicity (Cardoso et al., 2001). Treatment of NT2 cells, a human tetracarcinoma cell line capable of differentiating into neurons, with Aβ results in a dose dependent reduction in cell viability (Cardoso et al., 2001). However, NT2 cells depleted of their mitochondrial DNA (thus incapable of oxidative phosphorylation

(OXOPHOS) were unaffected by exposure to Aβ (Cardoso et al., 2001). Though often a topic of debate, several mechanisms by which Aβ induces mitochondrial dysfunction have been proposed.

1.3.2 Aβ accumulates within mitochondria and interacts with mitochondrial proteins

It is not entirely clear how Aβ perturbs mitochondrial function; however, Aβ has been shown to accumulate within mitochondria of CNS neurons in AD patients and AD transgenic mice (Hernandez-Zimbron et al., 2012; Lustbader et al., 2004; Manczak et al., 2006; Yao et al., 2011). Interestingly, Aβ is present in the mitochondria of AD transgenic mice as early as 4 months of age, prior to plaque deposition and cognitive deficits suggesting mitochondrial accumulation of Aβ is an early event in the pathogenesis of AD (Caspersen et al., 2005). The early accumulation of Aβ appears to preferentially occur in synaptic mitochondria which results in decreased respiration and increased oxidative stress (Du et al., 2010). Primary neurons from AD transgenic mice were also shown to accumulate oligomeric Aβ within their mitochondria which was accompanied by increased death in culture (Calkins et al., 2011). The mitochondrial translocase of the outer membrane (TOM) machinery is believed to mediate Aβ accumulation within the mitochondria, as Aβ has been shown to be transported into mitochondria isolated from rat liver in a TOM dependent manner (Hansson Petersen et al., 2008). Moreover, immunoelectron microscopy after import revealed Aβ was localized to mitochondrial cristae, which has also been observed in human cortical brain biopsies (Hansson Petersen et al., 2008). Once transported into the mitochondria, Aβ can directly interact with and inhibit the enzymatic activity of alcohol dehydrogenase (ABAD), a multifunctional enzyme that catalyzes the oxidation of alcohols in mitochondria (Yao et al., 2011). Disrupting the interaction between Aβ and ABAD reduces mitochondrial derived ROS and increases oxygen consumption and mitochondrial activity (Hong et al., 2007). Aβ has also been shown to bind directly to cytochrome oxidase (COX) subunit 1, a member of complex IV of the ETC, which likely accounts for the decreased activity of this enzyme in AD (Gibson et al., 1998). In addition to interacting with mitochondrial proteins involved in metabolism, Aβ was shown to interact with dynamin-related protein 1 (Drp1), a protein which plays an important role in mitochondrial fragmentation, both in the AD brain and in

primary hippocampal cells from transgenic mice carrying the FAD linked APP Swedish mutation (APPswe) (Manczak et al., 2011). Transmission electron microscopy revealed mitochondria from APPswe primary nerve cells are more fragmented and structurally damaged than WT neurons, which may be a result of the interaction between Drp1 and Aβ (Calkins et al., 2011). Therefore the transport, accumulation and interaction of Aβ with specific mitochondrial proteins leads to structural abnormalities and the disruption of mitochondrial function.

1.3.3 Aβ perturbs mitochondrial respiration and increases ROS production

Mitochondrial respiration is central to maintaining ATP levels in nerve cells. Many lines of evidence suggest that Aβ induces mitochondrial dysfunction by disrupting OXOPHOS leading to oxidative stress. Aberrations in energy metabolism including decreased mitochondrial membrane potential, ATP production and Complex IV activity have been reported as early as 3 months in AD transgenic mouse models prior to plaque formation (Hauptmann et al., 2009). The progressive accumulation of Aβ within the mitochondria of AD mice results in decreased activity of Complex III and IV of the ETC and decreased overall oxygen consumption (Caspersen et al., 2005). Similarly, isolated mitochondria from rat brain exposed to Aβ show a significant reduction in state 3 and 4 respiration (Casley et al., 2002). Additionally, the activities of key enzymes involved in respiration: COX , α-ketoglutarate dehydrogenase complex (α-KGDHC) and pyruvate dehydrogenase (PDH), were inhibited by exposure to Aβ (Casley et al., 2002). Moreover, reduced activity and expression of enzymes participating in the tricarboxylic acid cycle (TCA) cycle and ETC have been well documented in the brains of AD patients (Brooks et al., 2007; Liang et al., 2008). Treatment of SK-N-SH cells, a human neuroblastoma cell line, with Aβ results in a dose dependent decrease in mRNA levels of mitochondrial COX subunits (Liang et al., 2008). RNA levels of human mitochondrial transcription factor-1 (Transcription factor A, mitochondrial (TFAM)), a key regulator of transcription of mitochondrial genes, are also decreased following Aβ-treatment (Liang et al., 2008). These changes in mitochondrial metabolism are believed to result in increased generation of ROS which further potentiates Aβ accumulation and toxicity (Leuner et al., 2012). Mice deficient in Complex I or AD mice

treated with a complex I inhibitor (rotenone), exhibited increased levels of Aβ (Leuner et al., 2012). Thus mitochondrial ROS produced at Complex I as a result of mitochondrial dysfunction is sufficient to potentiate Aβ production (Leuner et al., 2012). Similar results were obtained when AD mice were exposed to the pesticide paraquat which resulted in oxidative damage primarily in the mitochondria, elevated Aβ levels and induced cognitive impairment, which was rescued by the overexpression of the mitochondrial antioxidant Prx3 (Chen et al., 2012). Thus mitochondrial derived ROS, as a result of dysfunctional OXOPHOS, appears to underlie cognitive impairment and elevated amyloidogenesis associated with AD.

1.4 Glucose metabolism and Aβ resistant cells

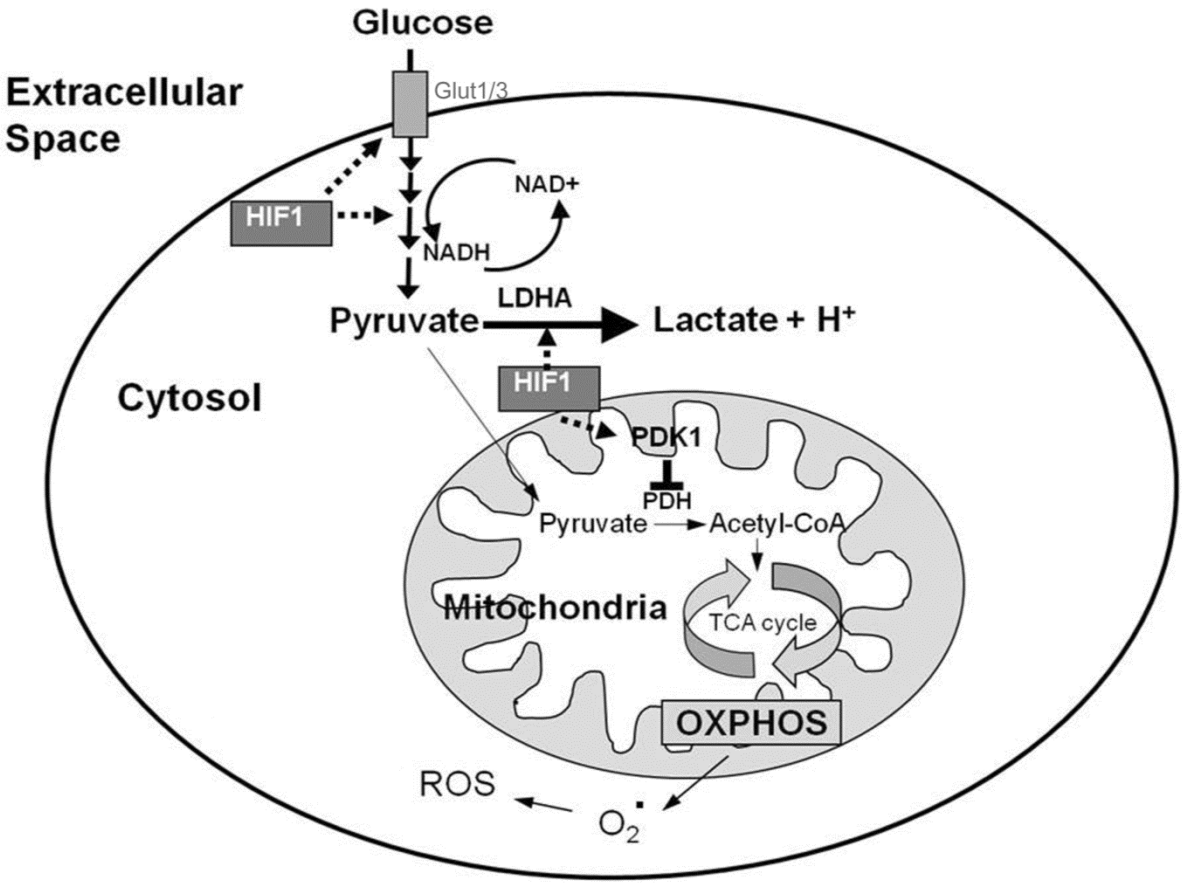
1.4.1 Glucose metabolism in nerve cells

The human brain consumes approximately 20% of the body's total energy yet only represents 2% of the total body mass, far outweighing the demand of other organs in the body. While other tissues in the body rely on a variety of energy sources, the brain is believed to primarily depend upon the oxidation of glucose to meet its energy demands. The majority of the energy produced by the oxidation of glucose is used for the maintenance and restoration of ion gradients associated with synaptic transmission, as well as uptake and recycling of neurotransmitters (Attwell and Laughlin, 2001). As an essential organ, the brain requires adequate glucose and oxygen delivery from the vasculature system, a process controlled by the precise regulation of energy supply and demand. Consequently, changes in brain activity are accompanied by changes in cerebral blood flow, a phenomenon which forms the basis of functional brain imaging technologies. Glucose is taken into nerve cells by glucose transporters (GLUTs), transmembrane proteins that facilitate the transport of glucose into the cell (Dwyer et al., 2002). Of the 12 known glucose transporters, seven have been identified in the brain: GLUT1-6 and GLUT 8 (Dwyer et al., 2002). However, GLUT 1 and 3 are the predominant glucose transporters in the brain (Dwyer et al., 2002). GLUT1 is ubiquitously expressed in the brain, with cells of the blood brain barrier (BBB), astrocytes and endothelial cells, representing major sites of expression (Boado and Pardridge, 1990; Dick et al., 1984; Gerhart et al., 1989). GLUT 3 is the predominant neuronal glucose transporter (Leino et al., 1997; Maher et al., 1992; Nagamatsu et al., 1993). Once transported into nerve cells, glucose

is converted to pyruvate by glycolysis within the cell cytosol producing a net of 2 ATP. In the presence of oxygen, pyruvate is taken into mitochondria where it is converted into acetyl coenzyme A (acetyl CoA) by the mitochondrial enzyme pyruvate dehydrogenase (PDH). Subsequently acetyl CoA can enter and drive the TCA cycle, producing the electron donors NADH and $FADH_{2,}$ which donate their electrons to the ETC ultimately producing ATP by OXOPHOS.

Eighteen times more energy is produced from mitochondrial respiration than from glycolysis (36 ATP versus 2 respectively per glucose molecule). Therefore, neurometabolism has traditionally been perceived as a process with a strict reliance on the oxidation of pyruvate in the mitochondria in order to meet the high energy needs of neurons. However, when oxygen becomes limited, cells become more dependent on glycolysis and lactate production to fuel their energy needs. In hypoxic environments, low oxygen conditions, pyruvate is reduced to lactate by lactate dehydrogenase A (LDHA) in the cell cytosol regenerating NAD^+, an important co-factor required to sustain high levels of glycolysis. The shift in metabolism from OXOPHOS to lactate production is driven by the transcription factor hypoxia inducible factor 1 (HIF-1) (Semenza, 1999). HIF-1 is a heterodimeric transcription factor made up of a HIF-1α subunit and the constitutively expressed HIF-1β subunit (Semenza, 2002). Under normoxic conditions HIF-1α is constitutively synthesized and degraded by the proteosome (Semenza, 2002). However in hypoxic microenvironments, HIF-1α is stabilized allowing for the activation of the HIF-1 transcription complex (Semenza, 2002). Importantly, HIF-1 regulates the expression of genes involved in glucose transport (*GLUT 1* and *GLUT 3*), glycolysis and lactate production (Heilig et al., 2003; Semenza, 1999; Semenza et al., 1996; Zhang et al., 1999). Additionally, HIF-1 upregulates the expression of pyruvate dehydrogenase kinase 1 (PDK1), an enzyme that phosphorylates and inhibits PDH, thereby reducing the rate of respiration (Figure 1.3) (Kim et al., 2006; Papandreou et al., 2006). Furthermore, the switch to lactate production associated with HIF-1 activation attenuates mitochondrial ROS production.

Figure 1.3 Hypoxia inducible factor 1 (HIF-1) alterations in glucose metabolism in response to hypoxia. In hypoxic microenviroments the stabilization HIF-1α and subsequent increased activity of HIF-1 stimulates increased expression of glucose transporters (Glut1/3), and glycolytic enzymes increasing the conversion of glucose to pyruvate. Additionally, HIF-1 promotes the reduction of pyruvate to lactate through the upregulation of lactate dehydrogenase A (LDHA). HIF-1 also actively suppresses the production of acetyl CoA through the mitochondria via increased expression of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inhibits pyruvate dehydrogenase (PDH) resulting in decreased flux through the tricarboxcylic acid (TCA) cycle and repressed oxidative phosphorylation (OXPHOS). The decrease in electron transport activity decreases the generation of reactive oxygen species (ROS) and allows for cell survival in absence of oxygen.


Glucose
Extracellular Space
Glut1/3
HIF1
NAD+
NADH
Pyruvate
LDHA
Lactate + H+
HIF1
Cytosol
PDK1
PDH
Pyruvate
Acetyl-CoA
Mitochondria
TCA cycle
OXPHOS
ROS
O2

1.4.2 Glucose metabolism and AD

The progressive decline in cerebral glucose utilization is known to occur with age and in AD, possibly contributing to both nerve cell loss and memory decline (Heiss et al., 1991; Mielke et al., 1998). Reductions in cerebral glucose metabolism, as measured by fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET), are a common diagnostic feature of AD (Herholz et al., 2007; Mosconi, 2005). Alterations in the expression and activity of enzymes involved in the TCA and ETC have been proposed to play a role in the observed reduction of glucose metabolism. Significant reductions, up to 70% in some areas, in the expression of genes encoding subunits of the ETC and key enzymes in the TCA cycle were observed in the cortex and hippocampus of post-mortem tissue from AD patients (Brooks et al., 2007; Liang et al., 2008). Moreover the activities of metabolic enzymes including PDH, COX and α-KGDHC, were reduced in the cerebral cortex of AD patients (Gibson et al., 1998; Kish, 1997). Reduced COX activity was also observed in the cortex of AD transgenic mice prior to the appearance of Aβ plaques (Manczak et al., 2006). In contrast, increased activity of the glycolytic enzymes pyruvate kinase (PK) and LDHA in the frontal and temporal cortex of patients with AD have been observed (Bigl et al., 1999; Soucek et al., 2003). Interestingly, young (3 month old) AD transgenic mice (APP/PS1) exhibit decreased [^{18}F]-FDG uptake when compared to their aged-matched controls (Poisnel et al., 2011). However plaque accumulation in APP/PS1 mice is associated with an age dependent increase in [^{18}F]-FDG uptake in the hippocampus and cortex, compared to control mice (Poisnel et al., 2011). Moreover, glucose uptake is significantly increased in regions associated with Aβ plaque accumulation in 12 month old AD mice when compared with the same brain regions of control mice (Poisnel et al., 2011). Collectively, studies over the last few decades have revealed a complex, and at times conflicting picture of glucose metabolism in AD.

1.4.3 Aβ resistance

As discussed above, the amyloid hypothesis posits that AD is caused primarily by Aβ deposition within the brain, which leads to mitochondrial dysfunction, increased ROS production, oxidative damage, and cell death (Hardy, 1992; Hardy and Selkoe, 2002; Selkoe, 1991). However, numerous immunohistochemical studies of brain tissue from individuals

that died without any history of dementia showed that up to 40% of the autopsied samples had significant plaque accumulation but little or no nerve cell loss (Bouras et al., 1994; Price and Morris, 1999). It has been argued that asymptomatic individuals with high plaque accumulation likely had undiagnosed mild cognitive impairment and would have eventually developed AD if they lived long enough. However, an alternative hypothesis is that these individuals may have acquired or exhibit an innate resistance mechanism to the toxic effects of Aβ. While difficult to study in patients, models of Aβ-resistance have been generated in cell culture following the continual exposure of nerve cell lines to concentrations of Aβ that would otherwise be toxic and the eventual emergence of surviving clonal nerve cell populations (Behl et al., 1994b; Sagara et al., 1996). Interestingly, these Aβ resistant nerve cells exhibit cross-resistance to a number of neurotoxins or toxic conditions including glutamate, H_2O_2, rotenone, cystine depletion and nitric oxide (Behl et al., 1994b; Dargusch and Schubert, 2002; Sagara et al., 1996). An observed cross resistance to a variety of toxins suggests a possible common resistance mechanism has been acquired by these cells to survive exposure to a variety of environmental stressors including Aβ. Analysis of these Aβ-resistant nerve cells revealed an upregulation of both anti-oxidant and glycolytic enzymes compared to the sensitive parental cells (Sagara et al., 1996; Soucek et al., 2003). Furthermore, Aβ-resistant cells exhibited increased glucose uptake and flux through the glycolytic pathway and heightened sensitivity to glucose deprivation suggesting that the altered glycolytic metabolism in these cells may mediate Aβ resistance (Soucek et al., 2003). Interestingly, the Aβ-induced changes in glucose metabolism are believed to arise due to the activation of HIF-1 (Soucek et al., 2003). Moreover, inducing HIF-1 activity in Aβ-sensitive cells by treatment with a metal chelator (mimosine) or by the expression of a non-degradable HIF-1α was sufficient to protect against Aβ toxicity (Soucek et al., 2003). Importantly the induced activity of HIF-1 was associated with increased glycolysis, and enhanced sensitivity to glucose deprivation suggesting a reliance on a high glycolytic flux is associated with resistance (Soucek et al., 2003). Taken together alterations in glucose metabolism may contribute to protection against neurotoxins including Aβ.

1.5 The Warburg effect

1.5.1 The Warburg effect (aerobic glycolysis) overview

In the early 1920's Otto Warburg made the observation that even in the presence of oxygen cancer cells predominantly produce ATP via high rates of glycolysis and lactate production. This metabolic phenotype is now referred to as a Warburg effect or aerobic glycolysis (Warburg, 1956b). Importantly, the Warburg effect appears to be a common metabolic hallmark of cancer cells and has been exploited as a clinical diagnostic tool for the detection of tumors via PET imaging following the administration of [^{18}F]-FDG (Gatenby and Gillies, 2004; Hsu and Sabatini, 2008). Due to the increased reliance on glycolysis, cancer cells must sustain high levels of glucose uptake in order to support their demanding energy needs. The increased glycolytic flux in cancer cells also renders them more sensitive to glucose deprivation (Aft et al., 2002). Initially Warburg proposed this switch in metabolism was a result of mitochondrial impairment in cancer cells however little evidence was found that indicated mitochondria in cancer cells are actually damaged (Koppenol et al., 2011; Warburg, 1956a). Furthermore, mitochondrial function is not impaired in most cancer cells (Fantin et al., 2006; Moreno-Sanchez et al., 2007; Weinhouse, 1976). A more popular hypothesis to explain aerobic glycolysis in cancer cells is that this metabolic phenotype confers a unique advantage for survival and proliferation in the tumor microenvironment (Bonnet et al., 2007; Gatenby and Gillies, 2004; Hsu and Sabatini, 2008; Koppenol et al., 2011). The initial switch from mitochondrial respiration to lactate production is believed to be triggered by a hypoxic microenvironment of a growing tumor, leading to the activation of HIF-1 (Hsu and Sabatini, 2008; Semenza, 2003, 2007, 2010). However, HIF-1α expression has been observed in a number of non-hypoxic cancer cell lines, indicating that other mechanisms of normoxic activation of HIF-1 are likely to also play an important role in inducing the Warburg effect (Akakura et al., 2001). The activation of HIF-1 shifts metabolism away from the mitochondria towards lactate production by increasing expression of enzymes such as PDK1 and LDHA (Kim et al., 2006; Papandreou et al., 2006). However, once the tumor becomes vascularized, and despite the increased availability of oxygen, cancer cells maintain high rates of lactate production and sustain HIF-1 transcriptional activity.

In non cancerous cells under normoxic conditions HIF-1α is hydroxylated at specific proline residues (Pro^{402} and Pro^{564}) by prolyl hydroxylase, which targets HIF-1α for destruction by the E3 ubiquitin ligase containing the von Hippel-Lindau tumor suppressor protein (pVHL) (Ivan et al., 2001). Importantly, normoxic stabilization of HIF-1 in cancer cells is associated with increased growth and survival of tumors, increased angiogenesis and tumor development and a poor clinical prognosis (Akakura et al., 2001; Birner et al., 2000; Kung et al., 2000; Zhong et al., 1999). Elevated HIF-1α has been detected in primary tumors of brain, breast, colon, lung, ovary and their metastases (Zhong et al., 1999). Thus it has been proposed that the activation of HIF-1 and subsequent upregulation of LDHA and PDK1 in cancer cells are key events that facilitate the Warburg effect (Semenza, 2003, 2007, 2010). The role of LDHA and PDK1 will be discussed in the following section.

Aerobic glycolysis is much less efficient than OXOPHOS in terms of ATP yield per glucose molecule, so the big question with respect to the Warburg effect in cancer cells is why highly proliferative cells which have high metabolic requirements chose a much less efficient means of producing energy? The answer appears to lie in the inhibition of apoptosis or programmed cell death. There are two well defined pathways that lead to cellular apoptosis; the 'extrinsic' and the 'intrinsic' pathway. Mitochondria are known to play a role in both pathways, but are direct regulators of the intrinsic pathway to cell death (Galluzzi et al., 2012). The intrinsic pathway involves the permeablization of the outer mitochondrial membrane (OMM) which results in the release of proapoptotic mediator cytochrome c (cyt c) into the cytoplasm which subsequently binds to Apaf-1 thereby promoting the formation of a multi complex called the apoptosome which, in turn, initiates the caspase cascade through activation of pro-caspase 9 (Galluzzi et al., 2012; Gogvadze et al., 2008). The intrinsic pathway can be initiated by a number of factors including loss of mitochondrial membrane potential, oxidative stress and changes in metabolism (Galluzzi et al., 2012; Kroemer et al., 2007; Plas and Thompson, 2002). Reduced mitochondrial metabolism in cancer cells results in the decreased production of ROS, agents which can trigger apoptosis if left unchecked (Bonnet et al., 2007; Kim et al., 2006). This has led some researchers to hypothesize that a decrease in mitochondrial respiration and the resultant decrease in mitochondrial ROS production renders cancer cells more resistant to apoptotic stimuli (Bonnet et al., 2007; Koppenol et al., 2011). Importantly, decreased ROS production is a common feature of cancer cells (Bonnet et al., 2007). The

inhibition of LDHA or PDK1, key enzymes regulating aerobic glycolysis in cancer cells, results in increased mitochondrial ROS production, arrested tumorigenesis and elevated sensitivity to chemotherapeutic agents (Bonnet et al., 2007; Michelakis et al., 2010; Michelakis et al., 2008; Zhou et al., 2010). Moreover, the proapoptotic activity of cyt c is dependent on its oxidation, and in cancer cells cyt c is maintained in a reduced state as a result of increased glycolysis and pentose phosphate shunt (PPS) activity (Vaughn and Deshmukh, 2008). Increases in ROS associated with increased ETC activity in cancer cells can result in the oxidation and activation of cyt c thereby initiating apoptosis in these cells (Vaughn and Deshmukh, 2008). Interestingly pyruvate, a major glycolytic product, scavenges H_2O_2, thus increased production of pyruvate as observed in cancer cells helps to further reduce intracellular ROS that can trigger apoptosis (Nath et al., 1995). Moreover, glycolytic enzymes including hexokinase (HK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been shown to play an active role inhibiting apoptosis (Kim and Dang, 2005). For example HK can bind to the voltage dependent anion channels (VDAC), preventing the release of cyt c from the mitochondria thereby acting as a direct inhibitor of apoptosis (Gottlob et al., 2001). Taken together aerobic glycolysis tightly regulates the redox status of a cell, which is closely linked to resistance against apoptotic stimuli.

1.5.2 LDHA

The interconversion between lactate and pyruvate is catalyzed by the enzyme LDH. There are five known LDH isoenzymes, each composed of tetramers containing different ratios of the LDHA or LDHB subunits. LDH isoenzymes made up of mostly or all LDHA subunits preferentially catalyze the reduction of pyruvate to lactate whereas those rich in LDHB subunits favor the oxidation of lactate to pyruvate. Increased LDHA expression has been well documented in a number of human breast cancer lines and contributes to a Warburg effect in these cells (Balinsky et al., 1983; Hilf et al., 1976; Koukourakis et al., 2003). The upregulation of LDHA in cancer cells occurs, in part, by the increased activity of HIF-1 which recognizes hypoxia response elements in the promoter region of *LDHA* (Semenza et al., 1996). Inhibition of LDHA activity in human lymphoma cells results in increased mitochondrial metabolism, elevated ROS levels and cell death; events which are partially counteracted with treatment of an antioxidant N-acetylcysteine (Le et al., 2010). Similarly

knockdown of LDHA expression in a variety of mammary gland tumor cell lines stimulates mitochondrial metabolism and reduces the tumorgenicity of cancer cells, which was rescued when LDHA was overexpressed in these cells (Fantin et al., 2006). Finally, LDHA inhibition reduces tumor initiation and inhibits tumor progression *in vivo* (Fantin et al., 2006; Le et al., 2010). These results suggest that LDHA is an important mediator of aerobic glycolysis and plays a key role in tumor maintenance and progression.

1.5.3 PDK1

PDH is a protein complex located in the mitochondrial matrix that controls the entry of pyruvate into the TCA cycle and is found at the interface between glycolysis and aerobic respiration. PDH is composed of three enzymes that contribute to pyruvate decarboxylation, the conversion of pyruvate to acetyl CoA. Phosphorylation of the PDHα subunit by PDK inhibits PDH activity (Roche et al., 2001). In humans there are four PDK isoforms (1-4), which are expressed in a tissue specific manner (Bowker-Kinley et al., 1998). In examining the tissue distribution of the four isozymes, PDK1, 2 and 4 were found in all regions of the brain in young and aged rats (Nakai et al., 2000). More specifically, the levels of PDK1 were higher in the cerebral cortex when compared with the other isoforms (Nakai et al., 2000). Enhanced PDK1 expression is regulated directly by HIF-1 (Kim et al., 2006; Papandreou et al., 2006). In cancer cells the inhibition of PDH through enhanced PDK1 expression contributes to the Warburg effect in a variety of different cancers, making it an important mediator of aerobic glycolysis in these cells (Bonnet et al., 2007; Fujiwara et al., 2013; Hitosugi et al., 2011; Hur et al., 2013; McFate et al., 2008). Interestingly, the inhibition of PDH is associated with normoxic stabilization of HIF-1α as knockdown of PDK1 expression eliminated the normoxic accumulation of HIF-1α (McFate et al., 2008). Similarly, treatment with dichloroacetate (DCA), a chemical inhibitor of PDK, reduced HIF-1α stabilization *in vitro* and *in vivo* (Michelakis et al., 2010). Thus PDK1 inhibition of PDH appears to play a critical role in the normoxic stabilization of HIF-1α observed in a variety of cancer cells. The overexpression of PDK1 in HIF-1α null cells attenuates mitochondrial respiration, decreases toxic ROS production and increases lactate production suggesting that PDK1 expression acts as metabolic switch that favors aerobic glycolysis (Kim et al., 2006). Knock down of PDK1 expression in cancer cells shifts metabolism towards mitochondrial respiration which

dramatically decreases the production of lactate, and attenuates tumor growth (McFate et al., 2008). Similarly, treatment of a variety of cancers *in vitro* and *in vivo* with DCA results in depolarized mitochondrial membrane potential, increased ROS and induced apoptosis (Bonnet et al., 2007; Michelakis et al., 2010). Taken together, PDK1 appears to play an important role in the survival of cancer cells by mediating a Warburg effect in these cells.

1.5.4 Aerobic glycolysis and the AD brain

Over the last few decades, key information about brain metabolism has been gathered using PET imaging. PET allows for the *in vivo* determination of the cerebral rate of glucose consumption, rate of oxygen consumption and blood flow. Traditionally, (FDG)-PET signals were believed to primarily measure glucose utilization by neurons due to the high energy demand of this cell type during activation (Sokoloff et al., 1977). However, in the mid to late 1980s, an important series of PET studies challenged this assumption by showing that cerebral glucose consumption exceeds oxygen utilization in certain regions of the human brain (Fox and Raichle, 1986; Fox et al., 1988). These early observations suggested that the metabolic needs of active neural tissue are met in a partially non-oxidative manner (Fox and Raichle, 1986; Fox et al., 1988). More recently, Marcus Raichle and colleagues, using a more refined PET analysis of 33 healthy adults, identified high rates of aerobic glycolysis in the medial and lateral parietal and prefrontal cortices; regions known to participate in cognitive control networks (Vaishnavi et al., 2010). These observations brought support to the notion that the metabolic needs of active brain tissue are met, at least partially, by aerobic glycolysis. Further support was provided by various *in vivo* ^{1}H-magnetic resonance spectroscopy (MRS) studies in healthy adults which showed activity-dependent increases in lactate levels in brain areas similar to those found in the PET studies (Lin et al., 2010; Maddock et al., 2006; Urrila et al., 2003). The question then arises as to whether the changes in glucose metabolism (oxidative versus aerobic glycolysis) are taking place in different cellular compartments of the brain and/or are dependent on the specific needs of these areas. Although a reduction in cerebral glucose metabolism is a common diagnostic feature of AD, recent evidence suggests that glucose utilization is more complex in the AD brain (Mosconi, 2005; Vlassenko et al., 2010). Studies using PET imaging, which measured both glucose consumption and oxygen utilization, revealed a strong correlation between the spatial distribution of elevated aerobic glycolysis and Aβ plaques in brain tissue from patients with

both AD, as well as normal individuals with high levels of Aβ-deposition but without clinical manifestation of the disease (Vlassenko et al., 2010). Moreover, PET studies of cognitively normal individuals have shown an age-associated decrease in FDG uptake in regions of the brain frequently affected in AD, although these studies did not determine what proportion of glucose was processed by aerobic glycolysis versus oxidative phosphorylation (Cunnane et al., 2011). Thus it appears that aerobic glycolysis plays an important role in brain metabolism and possibly AD. Understanding why cells exhibit a Warburg effect in the brains of individuals with Aβ accumulation could offer insight into why some individuals develop AD while others appear resistant to the disease.

1.6 Alzheimer's disease cell lines and mouse models

There are a number of immortalized cell culture lines that have been used to study AD. Additionally, primary nerve cells derived from various areas of the rodent brain are also widely used in the field of Alzheimer's research. In examining Aβ toxicity, these models rely on the use of exogenous synthetic Aβ. Aβ peptides containing the highly hydrophobic 29-35 amino acid residues form stable fibrils and aggregate within 24 hr following solubilization in sterilized deionized water (Pike et al., 1993). Aβ peptide mediated toxicity has been associated with its aggregation state (Pike et al., 1993). The 10 amino acid $A\beta_{25-35}$ peptide, henceforth referred to as Aβ, was used throughout this study unless otherwise stated. Though $A\beta_{1-42}$ is more toxic to cells, $A\beta_{25-35}$ readily kills cells in culture and has been widely used in both *in vitro* and *in vivo* models of AD (Pike et al., 1993; Yanker, 1996). Moreover $A\beta_{25-35}$ exhibits the same early neurotropic and late neurotoxic activities as $A\beta_{1-42}$ (Iversen et al., 1995). There are also a number of mouse models of AD. Below is a description of the cell lines and mouse model used in this study.

1.6.1 PC12 cells

PC12 cells are one of the most intensively studied cell lines in neurobiology. They were originally derived from a rat adrenal pheochromocytoma (Greene and Tischler, 1976). These cells differentiate into sympathetic-like neurons when treated with nerve growth factor (NGF) and can also synthesize and store catecholamine neurotransmitters, dopamine and norepinephrine (Greene and Tischler, 1976). Additionally, these cells are able to make

acetylcholine and form cholinergic synapses with immortalized skeletal muscle cells (Schubert et al., 1977). Importantly, PC12 cells are sensitive to $A\beta_{25-35}$ or $A\beta_{1-42}$ toxicity in culture (Behl et al., 1992; Behl et al., 1994a). Aβ resistant PC12 cells were derived by growing cells in the presence of $A\beta_{25-35}$ for 4 months (Behl et al., 1994b; Sagara et al., 1996). Subclones were then isolated and maintained in the presence of $A\beta_{25-35}$ and tested for their resistance against $A\beta_{1-42}$ (Behl et al., 1994b; Sagara et al., 1996). The study presented here made use of the resistant subclones one and seven (R1 and R7).

1.6.2 B12 cells

B12 cells are a central nervous system line, derived from nitrosoethylurea induced brain tumors in rats (Schubert et al., 1974). Similar to PC12 cells, B12 cells are sensitive to exogenously applied Aβ (25-35 and 1-42)(Behl et al., 1994a). Resistant clones were derived for the B12 cell line as described above (Behl et al., 1994b; Sagara et al., 1996). In this study, the resistant subclones two and four (R2 and R4) were examined.

1.6.3 HT22 cells

HT22 cells are an immortalized mouse hippocampal nerve cell line derived from the HT4 cell line (Morimoto and Koshland, 1990). These cells are sensitive to glutamate toxicity, but do not contain active ionotropic glutamate receptors and thus are not subject to excitotoxicity (Davis and Maher, 1994). Treatment of HT22 cells with glutamate results in a programmed cell death called oxytosis which is related to cystine depletion (Tan et al., 2001). Additionally, these cells possess functional cholinergic properties which has made them a useful *in vitro* model for elucidating mechanisms involved in cognitive defects in AD (Liu et al., 2009).

1.6.4 Primary nerve cells

Primary nerve cell cultures are isolated from different areas of the fetal brain of mice and rats and cultured in special media (Silva et al., 2006). Primary cultures have been used to investigate cell survival and cell death, neurotransmission and neuroinflammation (Silva et al., 2006). Primary cultures isolated from the cortex and hippocampus are of particular interest, as these areas of the brain are associated with extensive nerve cell death in the AD brain. Primary nerve cell cultures are a valuable model for studying neurotoxicity as a result

of their enhanced sensitivity to toxins in comparison to immortalized cell lines (Aschner and Syversen, 2004; Silva et al., 2006). Moreover, because these cells are not immortalized they more closely mimic nerve cells *in vivo*. Importantly, primary nerve cells are sensitive to Aβ toxicity in culture (Koh et al., 1990; Mattson et al., 1992; Yankner et al., 1990). Primary nerve cells taken from the cortex of fetal mice, embryonic day 14-17, were used for this study.

1.6.5 APP/PS1

Many mice models of AD are in existence and are currently used in AD research. Transgenic models of AD have become a popular choice, as these models tend to be a better representation of the human disease (Kobayashi and Chen, 2005). The amyloid hypothesis has led to the development of a variety of mouse models that are focused on amyloid accumulation (Kobayashi and Chen, 2005). A popular AD mouse model used in this study is a double transgenic model that expresses a chimeric mouse/human APP containing the Swedish mutation (Mo/HuAPP695swe) and mutant human presenilin 1 (PS1-dE9) both directed to CNS neurons. Both mutations are associated with early-onset FAD and transgenic mice carrying these mutations develop Aβ deposits throughout the cortex and hippocampus by six to seven months of age (Borchelt et al., 1997; Borchelt et al., 1996; De Strooper et al., 1995; Thinakaran et al., 1996). Aβ plaque accumulation in various brain regions of these mice is strongly associated with oxidative damage and altered mitochondrial function (Aso et al., 2012). Additionally, these mice show defects in memory and learning as early as nine months of age (Aso et al., 2012).

1.7 Research Questions

1.7.1 Summary

The Warburg effect is the phenomenon in which cells rely on aerobic glycolysis, and is a common feature of cancer cells (Warburg, 1956b). Given the inefficiency of aerobic glycolysis and lack of evidence suggesting that the mitochondria of cancer cells are damaged or dysfunctional, it has been hypothesized that this metabolic phenotype offers a special survival advantage to rapidly proliferating cancer cells. This shift in metabolism away from mitochondrial respiration and towards lactate production is mediated by HIF-1 (Semenza,

2010). Interestingly, HIF-1α has been shown to be stabilized in clonal nerve cell lines and primary cortical neurons that are resistant to Aβ toxicity (Soucek et al., 2003). Moreover, these cells exhibit increased flux through the glycolytic pathway and increased reliance on glucose uptake, which is believed to be a result of HIF-1 activation (Soucek et al., 2003). Cells selected for resistance against Aβ toxicity are also more resistant to a variety of neurotoxins, suggesting these cells exhibit a common mechanism that confers resistance to diverse environmental stressors (Behl et al., 1994b; Sagara et al., 1996). One compelling idea is Aβ resistant cells exhibit a Warburg effect, similar to cancer cells, which contributes to their resistance against Aβ toxicity and other toxins. Importantly, there exists a population of people who, despite the accumulation of plaques, fail to develop AD. Similarly, in areas of the brains of AD patients with high Aβ deposition and exhibiting significant nerve cell death, pockets of surviving neurons exist. These observations suggest that Aβ resistant cells also exist *in vivo*. Understanding the mechanisms acquired by nerve cells to evade the toxic effects of Aβ is essential to further our understanding of Aβ toxicity and resistance. Furthermore, exposing mechanisms of resistance to Aβ may uncover possible therapeutic targets for the prevention and/or treatment of AD.

1.7.2 Hypothesis and Objectives

In this study I hypothesized that Aβ-resistant cells will exhibit the Warburg effect and that this metabolic reprogramming contributes to their resistance against Aβ toxicity and other neurotoxins. Moreover, I hypothesized that the expression of key enzymes responsible for driving the Warburg effect will be down regulated in nerve cells that are sensitive to Aβ toxicity and in the brains of AD transgenic mice and individuals with AD. Compounds that activate aerobic glycolysis are hypothesized to protect nerve cells against neurotoxins including Aβ. To address my central hypothesis, the following objectives were explored:

(1) Determine if aerobic glycolysis occurs in Aβ resistant cells.

(2) Determine if mitochondrial activity is repressed in Aβ resistant cells.

(3) Determine if chemical or genetic modulation of LDHA and/or PDK1 activity in Aβ sensitive /resistant cells can alter sensitivity/resistance to Aβ and other neurotoxins.

(4) Determine if the expression pattern of LDHA and PDK1 in primary cortical neurons is altered following exposure to Aβ

(5) Determine if key Warburg effect enzymes (LDHA and PDK1) are altered in the brains of AD mice and post-mortem AD human tissue relative to control mice and unaffected individuals respectively.

(6) Examine the ability of neuroprotective compounds to regulate Warburg effect enzymes in nerve cells in the presence or absence of Aβ

1.8 References

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Chapter 2

2 Amyloid beta resistance in nerve cell lines is mediated by the Warburg effect

2.1 Introduction

2.1.1 Alzheimer's disease and amyloid resistance

Alzheimer's disease (AD) is a complex neurodegenerative condition, and is the most common form of dementia among the elderly. Currently, there is no cure for the disease and treatment options remain limited. AD is characterized at the histopathological level by widespread nerve cell death, synaptic loss and the accumulation of intracellular neurofibrillary tangles (NFT) and extracellular plaques within the brain (Selkoe, 2004). These plaques are primarily composed of amyloid β-peptide (Aβ), a 40-42 amino acid peptide derived from the proteolytic cleavage of the amyloid precursor protein (APP) (Hardy, 1997; Masters et al., 1985; Selkoe, 1998). A prevalent theory in the field is that AD is caused primarily by Aβ deposition within the brain, which leads to an increased production of reactive oxygen species (ROS), oxidative damage, mitochondrial dysfunction and cell death (Behl, 1997; Behl et al., 1992; Behl et al., 1994; Butterfield et al., 2007; Markesbery, 1997). Interestingly, some populations of cells within the brain survive by becoming resistant to Aβ toxicity. Immunohistochemical analysis of brain tissue from individuals that died without any history of dementia has revealed that up to 40% of the autopsied samples had significant plaque accumulation (Bouras et al., 1994; Price and Morris, 1999). While difficult to study *in vivo*, it is possible to examine amyloid resistance in cultured nerve cells. Clonal nerve cell lines selected for resistance to Aβ toxicity exhibit increased resistance to a wide array of neurotoxins suggesting that these cells have acquired a common resistance mechanism to survive exposure to environmental stresses (Behl et al., 1994; Dargusch and Schubert, 2002; Sagara et al., 1996). Initial studies revealed that Aβ-resistant cells upregulate several antioxidant and glycolytic enzymes (Cumming et al., 2007; Sagara et al., 1996; Soucek et al., 2003). However, further investigation into these survival mechanisms is necessary for a greater understanding of Aβ-sensitivity and resistance.

2.1.2 Aerobic glycolysis (the Warburg effect) in cancer

In normal nerve cells glucose is converted to pyruvate through a number of steps within the cytosol. In the presence of oxygen, pyruvate is converted into acetyl-Coenzyme A (acetyl-CoA), by pyruvate dehydrogenase (PDH) within the mitochondria. Acetyl-CoA is subsequently fed into the tricarboxcylic acid cycle (TCA cycle), ultimately producing ATP via oxidative phosphorylation (OXOPHOS). In an environment lacking oxygen cells must depend on glycolysis whereby pyruvate is converted into lactate by lactate dehydrogenase subunit A (LDHA). Hypoxia inducible factor 1 (HIF-1), a transcription factor induced in hypoxic microenvironments, mediates the critical cellular metabolic adaptation to hypoxia through activation of several glycolytic genes including *LDHA* (Semenza, 1999; Semenza et al., 1996). In addition to mediating the increased conversion of pyruvate to lactate, HIF-1 has recently been shown to suppress mitochondrial respiration by directly upregulating the expression of the gene encoding pyruvate dehydrogenase kinase 1 (PDK1) (Kim et al., 2006; Papandreou et al., 2006). PDK1 phosphorylates and inhibits PDH, thereby acting as a molecular switch between glycolysis and aerobic respiration to meet cellular ATP needs. Initially HIF-1 was believed to be a transcription factor involved in mediating the cellular metabolic adaptation to hypoxia, however it has more recently been shown to be active in normoxic conditions, such as vascularised cancer tissues, suggesting an addition role for the transcription factor (Semenza, 2007, 2010).

Enhanced glycolysis and increased lactate production is a common property of invasive cancers and its upregulation in cancer may result in the suppression of apoptosis (Bonnet et al., 2007; Michelakis et al., 2008). The initial upregulation of glycolysis in tumors is believed to be triggered by a hypoxic microenvironment and HIF-1 activity. However, despite increasing oxygen availability the glycolytic phenotype persists (Gatenby and Gillies, 2004; Semenza, 2010). This phenomenon has been termed the Warburg effect or aerobic glycolysis (Bonnet et al., 2007; Warburg, 1956). In addition to upregulation of glycolysis, cancer cells decrease the flux of pyruvate through the mitochondria via upregulation of PDK1, and the inhibition of PDH (Bonnet et al., 2007; Koukourakis et al., 2005; Michelakis et al., 2010). This shift in metabolism causes a drop in both mitochondrial oxygen consumption and associated ROS production (Bonnet et al., 2007). Therefore, lower levels of mitochondrial activity lead to a decrease in both ROS production and the propensity of

mitochondria to depolarize; two events that trigger apoptosis. The Warburg effect is believed to provide a selective advantage for the survival and proliferation of tumorigenic cells however it has rarely been examined in other cellular contexts (Bonnet et al., 2007; Michelakis et al., 2008).

2.1.3 Aerobic glycolysis in AD

Recent studies using PET imaging revealed a strong spatial correlation between aerobic glycolysis and Aβ deposition in the brains of AD patients (Vlassenko et al., 2010). Additionally, activities of the glycolytic enzymes pyruvate kinase (PK), and lactate LDHA, are elevated in the frontal and temporal cortex of patients with AD (Bigl et al., 1999). In contrast, the reductions of various mitochondrial enzymes involved in cellular respiration have been reported in the AD brain (Brooks et al., 2007; Gibson et al., 1998; Liang et al., 2008). Interestingly, Aβ-resistant nerve cells *in vitro* exhibit increased glucose uptake and flux through the glycolytic pathway (Soucek et al., 2003). Aβ-resistant cells are also highly sensitive to glucose deprivation suggesting that the altered glycolytic metabolism in these cells may mediate Aβ resistance. However, it is unknown if Aβ resistant cells repress mitochondrial respiration and rely primarily on glycolysis for their energy needs.

Evidence to suggest a Warburg effect may exist in AD is supported by the observation that elevated levels of HIF-1α are detected in cultured Aβ resistant cells and in the brains of AD transgenic mice compared to controls (Soucek et al., 2003). Additionally, stabilization of HIF-1α and an increase in HIF-1 activity protect cortical neurons from Aβ toxicity (Soucek et al., 2003). Potentially, HIF-1 may act in a manner similar to cancer cells by upregulating LDHA and PDK1 in Aβ resistant cells and surviving neurons of the AD brain. Elevated mitochondrial-derived ROS is strongly linked to Aβ induced death (Behl et al., 1992; Behl et al., 1994; Sagara et al., 1996). The impairment of mitochondrial metabolism in AD has been well documented and it is possible that a decrease in the flux of pyruvate through the mitochondria in Aβ resistant cells results in decreased mitochondrial respiration and ROS production similar to cancer cells (Bonnet et al., 2007; Casley et al., 2002; Caspersen et al., 2005). An increased reliance on glycolysis for production of ATP may give surviving cells an advantage in the hostile environment of the AD brain. Additionally, decreased mitochondrial activity may attenuate the release of apoptogenic factors (Bonnet et al., 2007;

Zamzami and Kroemer, 2001). Taken together, the increase in glycolysis and lactate production and/or repression of mitochondrial activity may play a role in the protection of neurons against Aβ toxicity in AD, although this hypothesis has never formally been examined.

To investigate if a Warburg effect exists in Aβ resistant cells, and if this contributes to their resistance against Aβ toxicity, two nerve like cell lines, PC12 and B12, and their Aβ resistant derivatives were selected and characterized. Here we show that nerve cell lines which are resistant to Aβ, break down glucose in a manner reminiscent of cancer cells. Western blot analysis revealed increased levels of PDK1 in Aβ resistant cells exposed to Aβ compared to parental sensitive cells. Aβ resistant cells showed higher levels of LDHA activity and also generated higher levels of lactic acid compared to sensitive cells. Chemical and genetic inactivation of either LDHA or PDK1 promoted increased cell death in Aβ-resistant cells following Aβ exposure. These findings indicate that a shift in metabolism to rely heavily on glycolysis for energy needs may provide nerve cells with a mechanism to survive Aβ accumulation within the AD brain.

2.2 Methods

2.2.1 Materials

Cell culture reagents including Dulbecco's modified Eagles medium (DMEM), penicillin/streptomycin (P/S), DMEM without phenol red and Dulbecco's phosphate buffered saline (DPBS, 1X) were purchased from Biowhittaker (Walkersville, MD, USA). Dialyzed fetal bovine serum (FBS) and horse serum (HS) were obtained from PAA Laboratories Inc. (Etobicoke, ON, Canada). OPTIMEM I (1X) and TrypLE Express (1X) were obtained from Invitrogen (Carlsbad, CA, USA). Amyloid beta (Aβ) peptide (25-35) was purchased from California peptide research (San Francisco, CA, USA). Poly-D-lysine, L-Lactic dehydrogenase solution Type II, L(+)-lactic acid~98%, β-Nicotinamide adenine dinucleotide (NAD^+), β-Nicotinamide adenine dinucleotide reduced disodium salt (NADH), sodium pyruvate, potassium hydroxide, perchloric acid, sodium oxamate, dichloroacetic acid (DCA) ≥99%, Bisbenzimide (Hoechst), Poly-D-lysine, puromycin, dimethyl formamide, 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were all purchased from

Sigma (St. Louis, MO, USA). G418 sulfate was purchased from Calbiochem (EMD Chemicals Inc., Darmstadt, Germany). Mitotracker Red CM-H2XRos (Molecular Probes) was purchased from Invitrogen (Carlsbad, CA, USA).

2.2.2 Cell culture

The PC12 and B12 immortalized nerve cell lines and their Aβ resistant derivatives were obtained from Dr. David Schubert (The Salk Institute, La Jolla, CA) and cultured as previously described (Behl et al., 1994; Sagara et al., 1996). The PC12 cell lines used in this study are a subclone of a cell line originally derived from a rat pheochromocytoma (Greene and Tischler, 1976) and were grown in DMEM supplemented with 10% FBS, 5% HS and 1% P/S. The B12 central nervous system cells are an immortalized clonal cell line derived from a nitrosoethylurea induced brain tumor in rats (Schubert et al., 1974) and were grown in DMEM supplemented with 10% FBS, and 1% P/S. The PC12 and B12 Aβ resistant cell lines were isolated following 4 months growth in the presence of Aβ and subsequent cloning (Behl et al., 1994; Sagara et al., 1996). All cells were grown in a humidified incubator at 37^{o}C and 5% CO_2. For hypoxic treatment cells were grown for 24 hr in a humidified incubator at 37^{o}C, 5% CO_2 and 1% O_2. Prior to experimentation the Aβ resistant clones were re-selected in 20 μM Aβ (25–35) for two weeks. The Aβ peptide (25-35) was dissolved in sterilized deionized water at 1 mg/ml, left overnight at room temperature to promote fibril formation and subsequently stored at -20^{o}C.

2.2.3 LDHA assay

The activity of LDHA was determined spectrophotometrically. PC12 and B12 cells were plated at an appropriate density to achieve 70-80% confluency before exposure to $A\beta_{25–35}$ (20 μM). PC12 and B12 parental samples exposed to hypoxic conditions (1% O_2) for 24 hrs were used as positive controls. Aβ –treated and untreated PC12 and B12 cells were washed twice in DPBS and harvested in lysis buffer (50 mM Tris pH 7.5, 2% SDS and 1 mM PMSF) at 24 hr and 48 hr. Following a freeze/thaw cycle, protein extracts were quantified by a Lowry Assay (Bio-Rad, Richmond, CA, USA). LDHA catalyzes the reversible reduction of pyruvate to lactate, using NADH as a co-substrate. The activity of LDHA in each sample was determined by measuring the change in absorbance as a result of the oxidation of NADH

at a wavelength of 340 nm (37^{o}C) (Shimadzu-UV 160 Spectrophotometer, Shimadzu, Kyoto, Japan) as previously described (Lamster et al., 1985). Cell lysates were added to equilibrated cuvettes containing: 460 μL dionized (DI) H_2O, 430 μL MOPS buffer (50 mM pH 7.0), 30 μL NADH (6.4 mM), 30 μL sodium pyruvate (1 M). The NADH and sodium pyruvate were dissolved in potassium phosphate buffer (50 mM pH 7.4). The reference cuvette contained an additional 30 μL of DI water and lacked pyruvate. Contents were mixed and the change in absorbance was recorded over 120 seconds. The maximum reaction rate (V_{max}) was taken between 30-90 seconds for each sample. LDHA activities were calculated from the changes in absorbance and the specific absorbance of NADH at 340 nm (6.22L/mmol for a path length of 1.0 cm) and standardized to protein concentrations.

2.2.4 Lactate assay

Lactate levels in the extracellular fluid of Aβ treated and untreated PC12 and B12 parental and resistant lines were measured enzymatically (Itoh et al., 2003). PC12 and B12 cells were plated on 35 mm dishes at an appropriate density to achieve 70-80% confluency following an overnight incubation. One day after seeding the media was aspirated, the cells were washed with DPBS and the media was replaced with OPTIMEM I media. $A\beta_{25-35}$ (20 μM) was also added to the test dishes immediately after changing the media to OPTIMEM I. Following 24 hr and 48 hr incubation lactate released into the medium was measured. One tenth volume of 70% perchloric acid (PCA) was added to the harvested media, and samples were put on ice for 10 minutes. Precipitated proteins were removed by centrifugation, and the acidified supernatant was neutralized by adding the appropriate amount of 10 M KOH to achieve pH 7.5. Lactate levels were measured by following the change in absorbance as a result of the reduction NAD^+, which is coupled to the oxidation of lactate, at a wavelength of 340 nm (37^{o}C) (Shimadzu-UV 160 Spectrophotometer, Shimadzu, Kyoto, Japan) as described previously (Itoh et al., 2003). Twenty five microliters of the sample were added to both the reference and test cuvettes containing 500 μl DI H_2O, 400 μl glycine buffer (1 M pH 9.5), 50 μl hydrazine sulfate (0.56 M pH 8.6), and 20 μl NAD^+ (1.0 M pH 6.75). Following a 5 min equilibration period at 37^{o}C, 2 μl (~2.7 units) of LDH was added to the test cuvette. The rate of the reduction of NAD^+ to NADH was recorded until completion of the reaction. Lactate concentrations were determined by comparison to a lactate standard curve.

2.2.5 Immunoblot Analysis

To assess the levels of LDHA and PDK1, treated (20 μM $A\beta_{25–35}$) and untreated PC12 and B12 cells from subconfluent cultures were washed twice in cold DPBS and harvested in a Tris extraction buffer (50 mM Tris pH 7.5, 2% SDS and 1 mM PMSF) at 24 hr and 48 hr. Protein extracts were quantified by a Lowry assay, resolved by 12% SDS PAGE and electroblotted onto PVDF membrane (Bio-Rad Richmond, CA, USA) (Cumming et al., 2007). Membranes were probed with the following antibodies: polyclonal anti-LDHA (1:1000; Cell Signaling, Danvers, MA, USA), polyclonal anti-PDK1 (1:1000; Stressgen, San Diego, CA, USA) and a monoclonal anti-β–actin (1:2000; Cell Signaling, Danvers, MA, USA) followed by incubation with an appropriate horseradish peroxidase (HRP) -conjugated secondary antibody (Bio-Rad, Richmond, CA, USA). The blots were developed using Pierce ECL western blotting substrate (Thermo Scientific, Rockford, IL, USA) and visualized with a Bio-Rad Molecular Imager (ChemiDoc XRS, Bio-Rad, Richmond, CA, USA). Densitometric analysis was performed using Image J software. Band densities were standardized against β-actin, and the ratio of LDHA/PDK1-specific bands relative to the β-actin band was determined. Relative intensity was calculated by comparing the LDHA/ or PDK1/β-actin ratio of the resistant lines to the same ratio in the parental cell line.

2.2.6 Cytotoxicity assay

Aβ induced cell cytotoxicity (cell viability) was assessed by a modified MTT assay (Behl et al., 1994; Hansen et al., 1989; Sagara et al., 1996). The MTT assay measures the reduction of the tetrazolium salt MTT to a colored formazan in living cells (Behl et al., 1994; Sagara et al., 1996). Cells were seeded ($3x10^3$ cells/well) in a 96 well microtiter plate and, following overnight incubation, $A\beta_{25-35}$ was added to the test wells at a concentration of 20 μM. Neutralized DCA (2.5 mM), an inhibitor of PDK1, or oxamate (20 mM), an inhibitor of LDHA, were also added to the appropriate test wells. Following 48 hr incubation 10 μl of MTT stock (2.5 mg/ml dissolved in DPBS) was added to each well, and plates were incubated again, for 4 hours, then 100 μl of solubilization solution (20% SDS in 50% dimethyl formamide pH 4.8) was added to each well and plates were rocked at room temperature overnight. The following day plates were read on a microplate reader (BioRad Model 3550) using 595 nm as the test wavelength (absorbance of formazan product in

solution) and 655 nm as the reference wavelength. The percent viability was calculated from the mean absorbance of the treated cells divided by the mean absorbance of the control cells and multiplied by 100%. Aβ induced cytotoxicity was also assessed by a trypan blue exclusion assay. Cells were seeded in 12-well dishes and following overnight incubation $A\beta_{25-35}$ was added to the test wells at a concentration of 20 μM. Neutralized DCA (2.5 mM) or oxamate (20 mM) were also added to the appropriate test wells. Following a 48 hr incubation cells were trypsinized, pelleted and resuspended in 200 μl of culture media to which an equal volume of trypan blue was added. Cells excluding typan blue were scored in triplicate using a hemocytometer and a light microscope. Prior to experimentation oxamate and DCA were both tested for toxicity at a range of treatment concentrations. For each inhibitor, a treatment concentration was selected that conferred little to no toxicity in cells (20 mM oxamate, 2.5 mM DCA).

2.2.7 Fluorescence microscopy

Mitochondrial ROS was visualized by the fluorescent dye Mitotracker Red CM-H2XRos (MTR). Stock MTR was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mM and stored at -20^{o}C. PC12 and B12 cells were plated ($8x10^4$) in 6 well glass bottom tissue culture dishes pretreated with polylysine (50 μg/ml for 3 hr) and incubated overnight. PC12 parental cells were plated at a higher density of $3x10^5$ cells/well. The following day cells were treated with 20 μM $A\beta_{25-35}$ for 48 hr. Following treatment with Aβ, the media was aspirated and new media was added containing MTR at a concentration of 100 nM. Plates were then incubated at 37^{o}C for 20 min, washed in DPBS containing Hoecsht stain (10 μg/ml), followed by an additional wash in DPBS and then placed in phenol red free DMEM. Cells were visualized by fluorescence microscopy (Zeiss-AxioObserver, 40X objective) and pictures were taken using a Q Imaging (Retiga 1300 monochrome 10-bit) camera and Q Capture software. Pictures were taken of three random fields of view for each experiment. MTR fluorescence was quantified with ImageJ software.

2.2.8 Derivation of PDK1 and LDHA knockdown cell lines

One resistant clone from each cell line, R7 (PC12) and R2 (B12) were selected to make stable knock down lines. All cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. Cells were plated at a density to

achieve 70-80% confluency. Cells were transfected with HuSH 29mer shRNA (Origene, Rockville, MD, USA) constructs directed at rat *Ldha* or rat *Pdk1*. The shRNA vector is cloned in the pRS plasmid under the control of the U6 promoter for mammalian cell expression. For each specific mRNA, 2 shRNA's containing expression cassettes, targeted at different parts of the mRNA were selected. The selected shRNA constructs directed against *Ldha* were: 71-TGGAATCTCAGATGTTGTGAAGGTGACAC and 72–CTTGTGCCATCAGTATCT-TAATGAAGGAC); the shRNA constructs directed against *Pdk1* were: 29-AATCACCAGGACAGCCAATACAAGTGGTT and 30-TCGGTTCTACATGAGTCGCATC-TCAATTA). A non effective shRNA construct (scrambled) in pRS plasmid was selected as a negative control. Each shRNA construct for both *Ldha* and *Pdk1* in addition to the scrambled construct (5 total) were separately transfected and selected for their resistance to 1 μg/ml puromycin. Approximately 10-12 clones were picked, expanded and screened by western blot analysis for successful knockdown.

2.2.9 Statistical Analysis

Data are presented as means ± SD resulting from a least three independent experiments. Data were analyzed statistically using either a two-way ANOVA followed by a Tukey test or a one-way ANOVA followed by a Dunnett test (VassarStats). Results were considered statistically significant at $P<0.05$.

2.3 Results

2.3.1 Increased LDHA activity and lactate production in Aβ-resistant cells

LDHA catalyzes the conversion of pyruvate to lactate and the increased activity of this enzyme in cancer cells is strongly associated with the Warburg effect (Fantin et al., 2006; Goldman et al., 1964; Le et al., 2010). To determine if a similar effect is observed in Aβ-resistant cells, we measured LDHA activity and secreted lactate levels in both Aβ-sensitive PC12 and B12 parental cells and clonal lines selected for resistance to Aβ. Interestingly, all the resistant lines displayed significantly higher levels of LDHA activity as compared to their parental counterparts, with or without exposure to Aβ (Figure 2.1 A, B, $P<0.001$).

Additionally, the activity of LDHA in both parental lines significantly decreased with increased exposure to Aβ ($P<0.001$). However, the activity of LDHA in the PC12 resistant lines, R1 and R7, appeared to decrease only slightly with exposure to Aβ, but remained significantly greater than the parental line for all treatments ($P<0.001$). Similarly, the activity of LDHA in the B12 resistant lines, R2 and R4, remained significantly greater than the parental line with or without exposure to Aβ ($P<0.001$). As a control, PC12 and B12 parental cells were exposed to hypoxic conditions (1% O_2) for 24 hr, as hypoxia is a known activator of *LDHA* (Semenza et al., 1996). Parental cells exposed to hypoxia exhibited increased LDHA activity when compared to cells cultured in control conditions ($P<0.001$). We also examined LDHA protein levels by western blot analysis using an LDHA-specific antibody. However, there was no clear trend in LDHA expression between both PC12 and B12 parental and resistant lines (data not shown). LDHA activity has been shown to be regulated by both allosteric effects and post translational modifications (Cooper et al., 1984; Cumming et al., 2004; Fritz, 1965; Yasykova et al., 2000) therefore the increased activity we observed in Aβ resistant cells may be attributed to these modifications.

LDHA catalyzes the conversion of pyruvate to lactate which is then released to the extracellular space by the monocarboxylate transporters (MCT). To determine if the elevated LDHA in Aβ resistant cells was associated with increased lactate production, the concentration of lactate in the extracellular media of cultured B12 and PC12 cells was examined. A significant increase in the concentration of lactate was detected in the extracellular media of all PC12 and B12 resistant lines when compared to their respective parental lines at 24 hr and 48 hr, with or without exposure to Aβ (20 μM) (Fig. 2.1 C, D, $P<0.001$). These findings indicated that increased LDHA activity and associated lactate production is strongly associated with Aβ-resistance.

2.3.2 PDK1 levels are elevated in Aβ-resistant cells

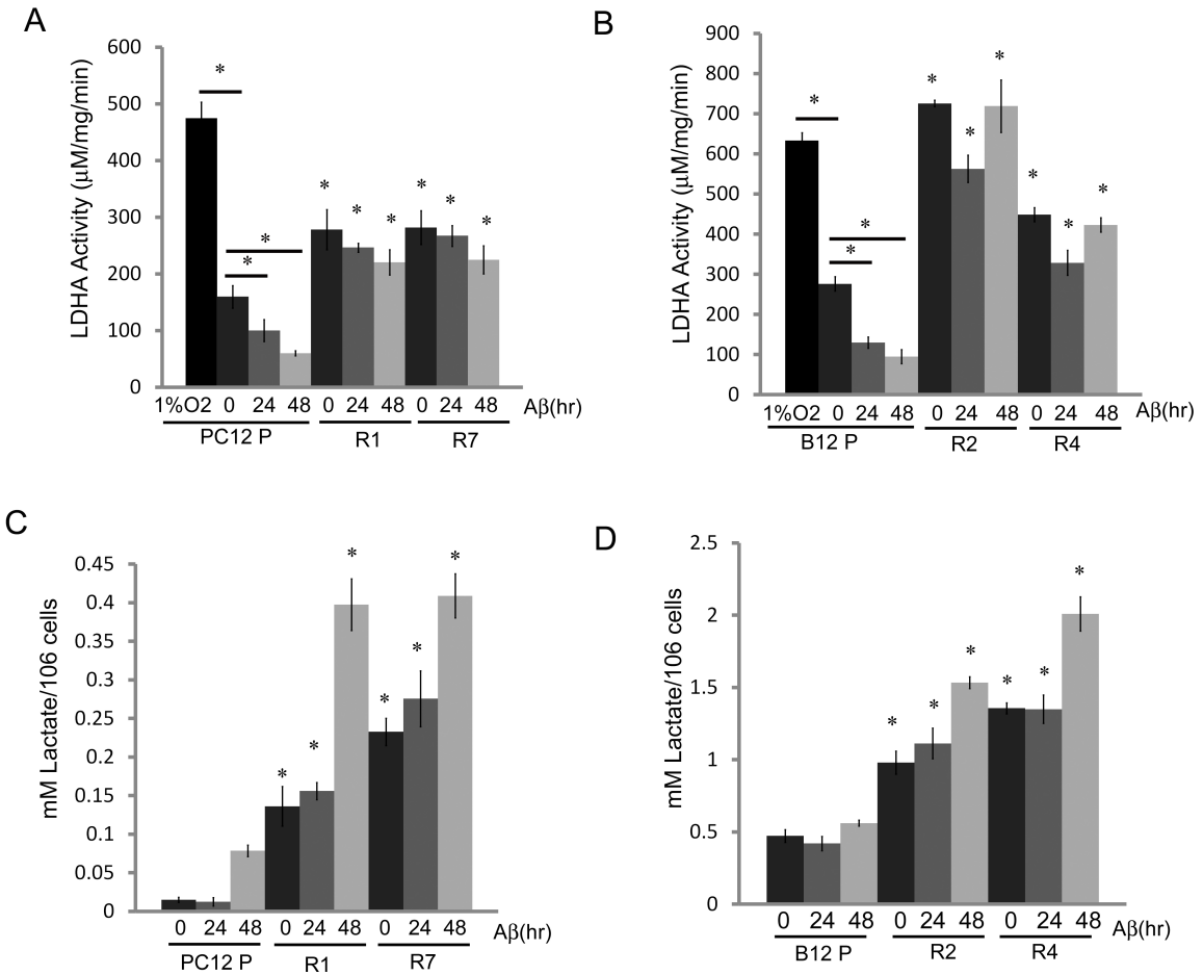
Elevated PDK1 expression in cancer cells results in reduced mitochondrial respiration and resistance to apoptosis (Bonnet et al., 2007). To determine if elevated PDK1 expression contributes to resistance to Aβ toxicity, cell extracts from sensitive and resistant cells were analyzed by Western blot analysis. Highly elevated PDK1 levels were detected in all Aβ-resistant lines relative to parental cell lines in either the presence of absence of Aβ (Figure

2.2 A and B). Interestingly, an additional band around 30 kDa was detected that was more prominent than full length PDK1 (~48 kDa) in B12 cells. This lower molecular weight band also showed the same elevated trend as full length PDK1 in the R2 and R4 lines (Figure 2.2 B). The lower molecular weight PDK1 band may represent some form of cleavage product/posttranslational modification of PDK1. In contrast, the PC12 and B12 parental cell lines exhibited a 14% and 15.6% reduction in PDK1 levels respectively following 48 hr exposure to Aβ. Thus, increased PDK1 expression strongly correlates with Aβ resistance.

2.3.3 Mitochondrial ROS production is decreased in Aβ-resistant cells

A decrease in mitochondrial-derived ROS is a key feature of the Warburg effect and plays a prominent role in resistance to apoptosis (Bonnet et al., 2007). We therefore examined mitochondrial ROS in Aβ sensitive and resistant cells using Mitotracker ROS Red (MTR); a dye that specifically incorporates in mitochondria and only fluoresces in the presence of ROS (Bonnet et al., 2007). A significant decrease in mitochondrial ROS, as measured by mean MTR fluorescence, was observed in PC12 and B12 resistant cells compared to their parental counterparts with or without exposure to Aβ (20 μM) (Figure 2.3 A, B, $P<0.001$). In contrast, there was a pronounced increase in mitochondrial ROS in both PC12 and B12 parental lines following 48 hr exposure to Aβ ($P<0.01$). Thus Aβ-resistant cells appear to have an altered metabolism resulting in significantly less mitochondrial-derived ROS under both basal and stressed conditions.

Figure 2.1 LDHA activity and lactate levels are elevated in Aβ-resistant cells. A) LDHA activity was significantly greater in PC12 Aβ resistant cells lines, R1 and R7, as compared to the parental line (PC12 P) under similar conditions both in the absence (0) and presence (24 hr, 48 hr) of Aβ (20 μM; *P<0.001). LDHA activity significantly decreased in PC12 P cells exposed to Aβ (*P<0.001). PC12 P cells exposed to 24 hr hypoxia (1% O_2) exhibited significantly greater LDHA activity when compared to untreated parental cells (*P<0.001). **B)** LDHA activity was also significantly greater in B12 Aβ resistant cells lines, R2 and R4, as compared to the parental line (B12 P) cultured in the same conditions (*P<0.001). LDHA activity significantly decreased in B12 P cells treated with Aβ (*P<0.001). B12 P cells exposed to 24 hr hypoxia (1% O_2) also exhibited greater LDHA activity when compared to untreated parental cells (*P<0.001). **(C)** Extracellular lactate was significantly elevated in PC12 Aβ-resistant lines, R1 and R7 and **(D)** B12 Aβ resistant lines, R2 and R4, when compared to their respective parental cells cultured under similar conditions (*P<0.001). Data represent the mean ± SD of three independent experiments. Parental cells exposed to hypoxia were compared to parental cells cultured under control conditions by a one tailed T-test. All other data were analyzed by a two-way ANOVA followed by a Tukey test.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R2
R4

Figure 2.2 PDK1 is upregulated in Aβ resistant cells. A) Immunoblot analysis revealed PDK1 levels were significantly elevated in both PC12 resistant lines, R1 and R7, when compared to the parental cell line (P) (*P<0.001). These elevated levels were maintained following 24 and 48 hr Aβ treatment (*20 μM). **B)** PDK1 levels were also significantly greater in both B12 resistant lines, R2 and R4, when compared to the parental line (** P<0.01). These increased levels were also maintained with Aβ treatment. In the B12 cell lines an additional band of approximately 30 kDa was observed that was more prominent than full length PDK1 (~48 kDa). This additional band also showed the same elevated trend as full length PDK1 in the R2 and R4 lines when compared to the parental under similar conditions. The smaller band may represent a cleavage product of PDK1. Densitometric analysis of full length PDK1 band densities relative to actin are found in the lower panel. Relative intensity was calculated by comparing the PDK1/actin ratio of the resistant lines to the same ratio in the parental cell line. Data represent the mean ± SD of three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.

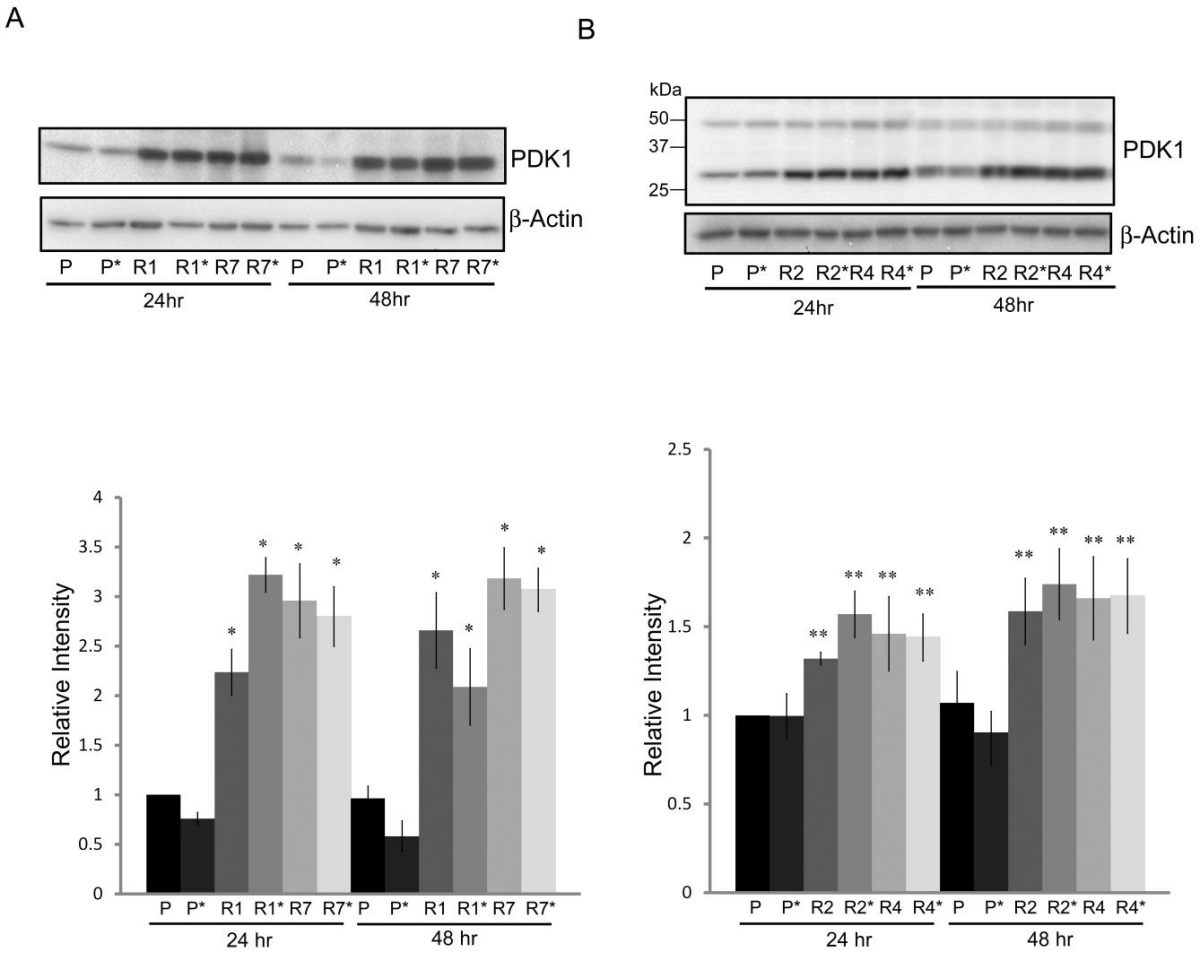
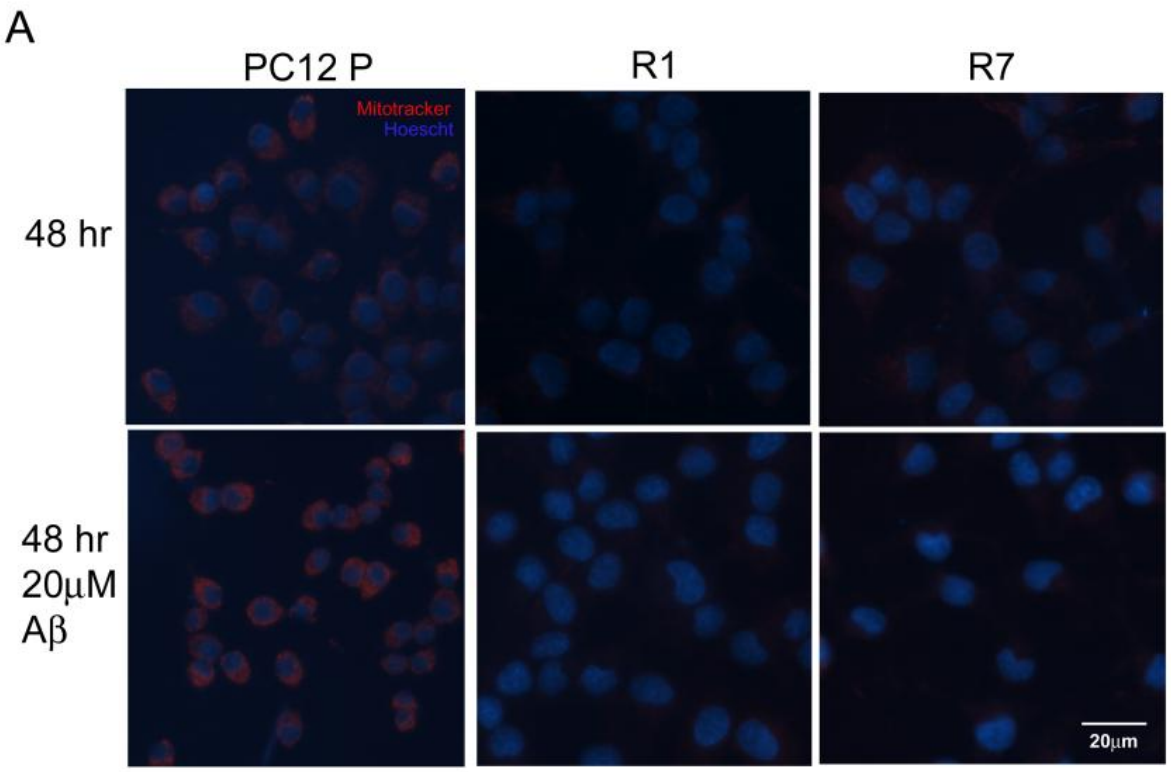
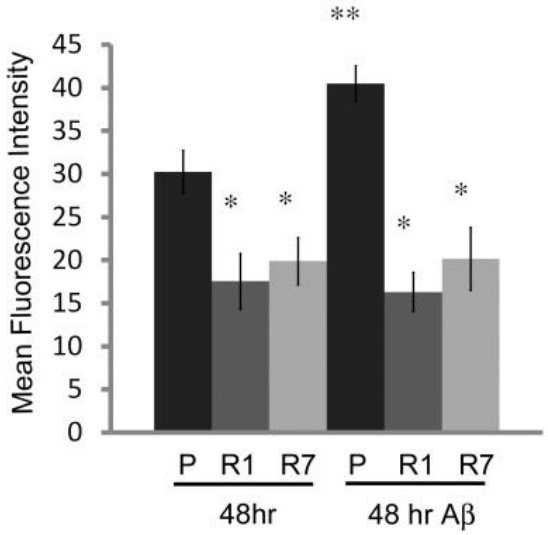
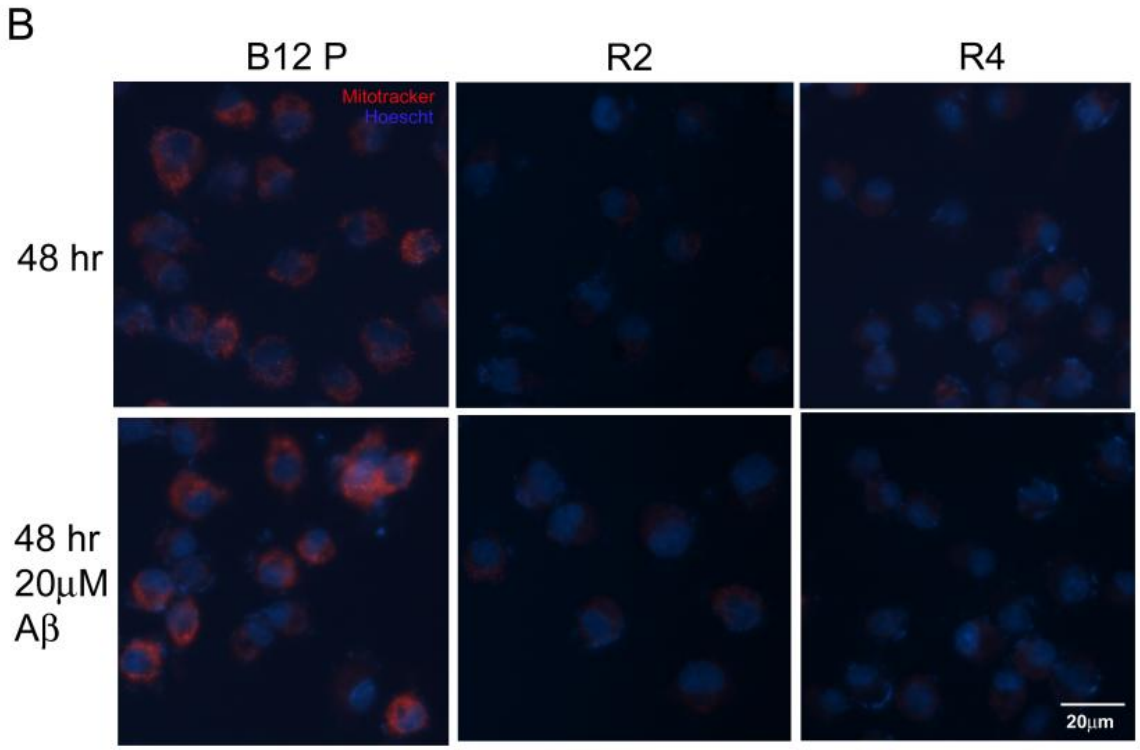
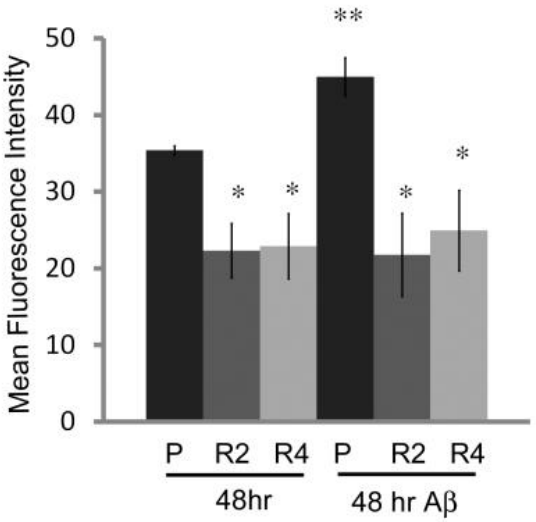

A
PDK1
β-Actin
P P* R1 R1* R7 R7* P P* R1 R1* R7 R7*
24hr
48hr
Relative Intensity
P P* R1 R1* R7 R7* P P* R1 R1* R7 R7*
24 hr
48 hr
B
kDa
50
37
25
PDK1
β-Actin
P P* R2 R2*R4 R4* P P* R2 R2*R4 R4*
24hr
48hr
Relative Intensity
P P* R2 R2* R4 R4* P P* R2 R2* R4 R4*
24 hr
48 hr

Figure 2.3 Decreased mitochondrial reactive oxygen species in Aβ resistant cells. A) PC12 Aβ resistant lines R1 and R7 exhibited a significant reduction in mitochondrial reactive oxygen species (ROS) compared to the parental cell line (PC12 P) under normal culture conditions (*P<0.001). This decrease in ROS was maintained with 48 hr Aβ (20 μM) exposure. Conversely, mitochondrial ROS significantly increased in the parental line when exposed to Aβ (**P<0.01). **B)** The B12 resistant lines, R2 and R4 also exhibited decreased mitochondrial ROS when compared to parental cells under similar conditions (*P<0.001). A similar increase in mitochondrial ROS in B12 parental cells (B12 P) was also observed following treatment with Aβ for 48 hr (**P<0.01). Cells were stained with MitoTracker Red (100 nM) and nuclei were stained with Hoescht (10 μg/ml) and visualized by fluorescence microscopy at 400X magnification and quantified with ImageJ software. Pictures were taken from 3 random fields of view for each treatment. Data represent the mean ± SD of three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.


A
PC12 P
R1
R7
Mitotracker
Hoescht
48 hr
48 hr
20μM
Aβ
20μm


Mean Fluorescence Intensity
45
40
35
30
25
20
15
10
5
0
**
*
*
*
*
P R1 R7 P R1 R7
48hr
48 hr Aβ

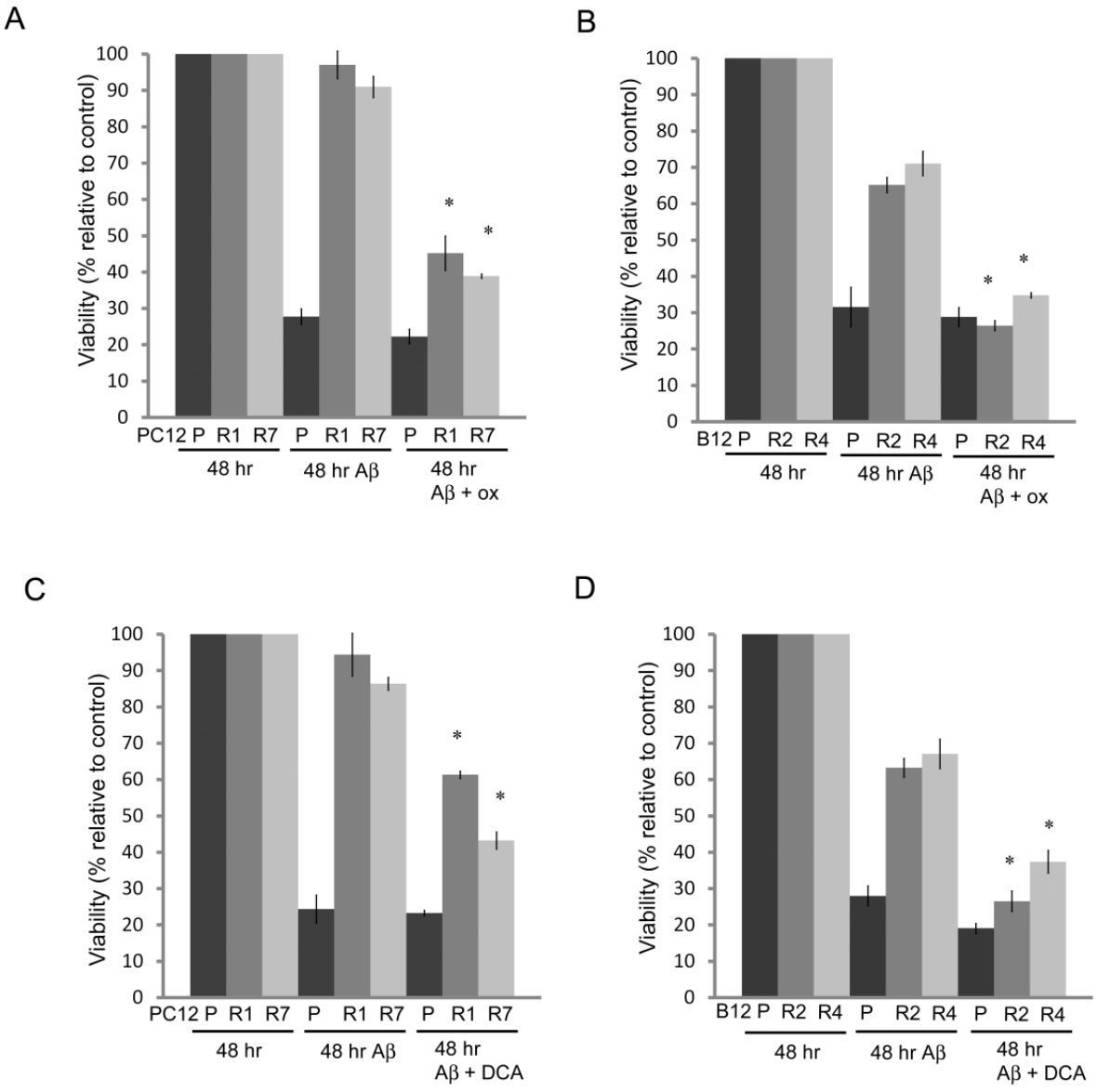

B
B12 P
R2
R4
Mitotracker
Hoescht
48 hr
48 hr
20μM
Aβ
20μm


Mean Fluorescence Intensity
50
40
30
20
10
0
**
*
*
*
*
*
P
R2
R4
P
R2
R4
48hr
48 hr Aβ

2.3.4 Chemical inhibition of LDHA or PDK1 restores sensitivity to Aβ in resistant cells

Inhibition of LDHA or PDK1 using the chemical inhibitors oxamate and dichloroacetate (DCA) respectively, has been shown to counter the Warburg effect and apoptosis resistance in cancer cells (Bonnet et al., 2007; Michelakis et al., 2010; Zhou et al., 2010). When the PC12 and B12 resistant cell lines were treated with either chemical inhibitor, oxamate (20 mM) or DCA (2.5 mM), and Aβ (20 μM) there was a significant decrease in cell viability when compared to Aβ treatment alone (Figure 2.4 A-D, $P<0.01$). Interestingly, exposure of the PC12 and B12 parental lines to either oxamate or DCA did not appear to potentiate Aβ toxicity. To ensure that oxamate and DCA did not interfere with the reduction of MTT and subsequent formazan formation, these experiments were repeated using a trypan-blue exclusion viability assay and similar results were observed (data not shown). These findings indicate that chemical inhibition of either LDHA or PDK1 can resensitize Aβ-resistant cells to Aβ.

Figure 2.4 Chemically inhibiting LDHA or PDK1 decreases cell viability in Aβ resistant cells. A significant decrease in cell viability in both PC12 **(A)** and B12 **(B)** resistant lines was observed after 48 hr concomitant exposure to Aβ (20 μM) and 20 mM oxamate (ox), a chemical inhibitor of LDHA (*P<0.01). Similarly, 48 hr Aβ exposure significantly decreased cell viability in both PC12 **(C)** and B12 **(D)** resistant lines when cells were co-treated with 2.5 mM dichloroacetate (DCA), a chemical inhibitor of PDK1 (*P<0.01). Interestingly, the cell viability of the parental lines does not appear to decrease with treatment of either inhibitor and Aβ. Cell viability was determined by the reduction of the tetrazolium salt MTT. Data represent the mean ± SD of three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.

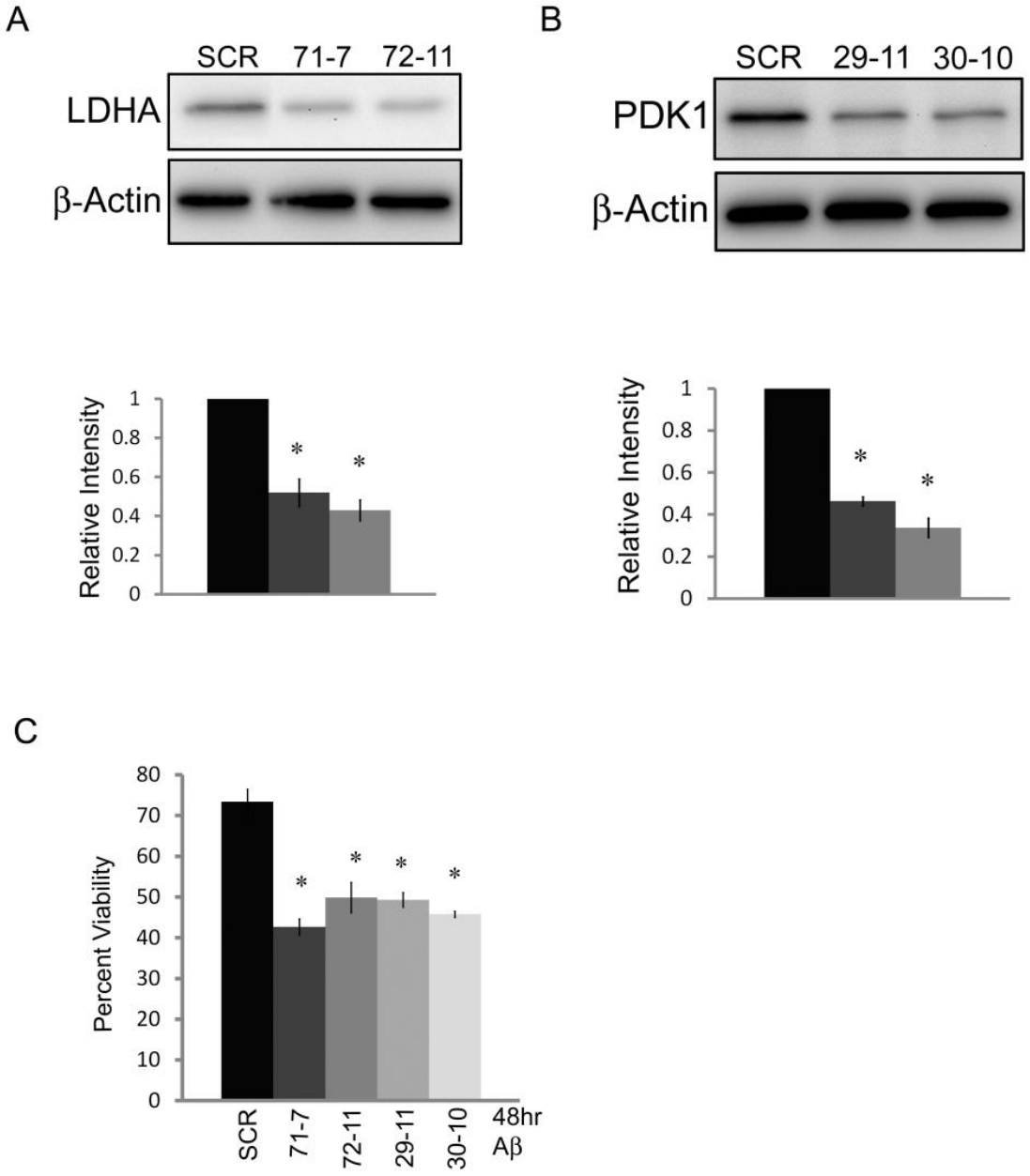

A
Viability (% relative to control)
100
90
80
70
60
50
40
30
20
10
0
PC12 P R1 R7 P R1 R7 P R1 R7
48 hr
48 hr Aβ
48 hr
Aβ + ox
B
Viability (% relative to control)
B12 P R2 R4 P R2 R4 P R2 R4
48 hr
48 hr Aβ
48 hr
Aβ + ox
C
Viability (% relative to control)
PC12 P R1 R7 P R1 R7 P R1 R7
48 hr
48 hr Aβ
48 hr
Aβ + DCA
D
Viability (% relative to control)
B12 P R2 R4 P R2 R4 P R2 R4
48 hr
48 hr Aβ
48 hr
Aβ + DCA

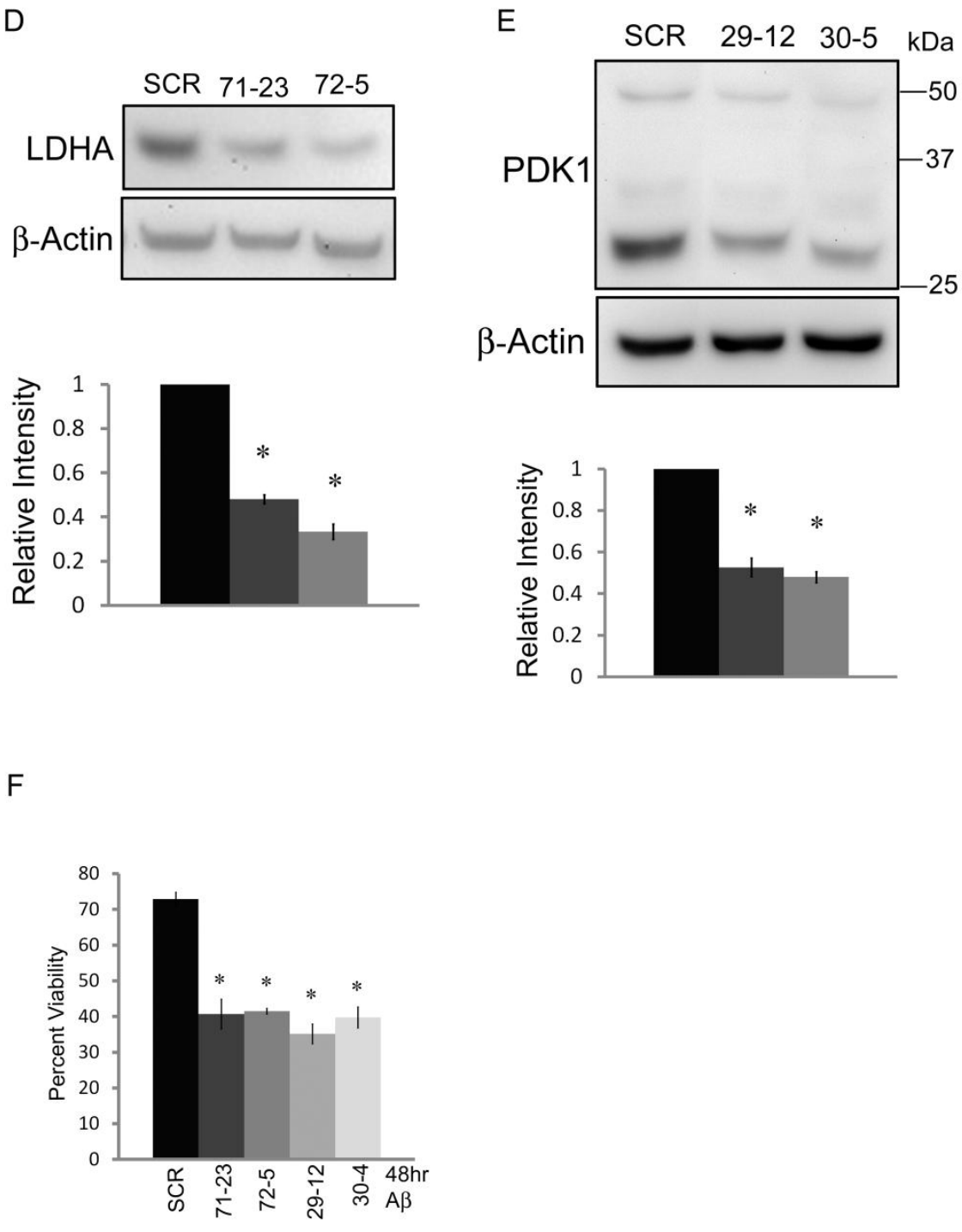
2.3.5 Attenuated LDHA and PDK1 expression reverses Aβ resistance

Because chemical inhibitors such as oxamate and DCA could potentially have off-target effects, we sought to determine if specific inhibition of LDHA and PDK1 expression by shRNA–mediated knockdown could also render resistant cells sensitive to Aβ. Immunoblot analysis confirmed that PC12 R7 and B12 R2 cell lines stably transfected with shRNA vectors containing sequences directed at rat *Ldha* or *Pdk1* transcripts exhibited decreased expression of the targeted mRNAs compared to cells transfected with a control shRNA containing a non-specific/scrambled (scram) sequence (Figure 2.5). Densitometric analysis revealed a 52% and 62% reduction in LDHA levels in the R7 knockdown lines 71-7 and 72-11 respectively, and a 65% and 72% reduction in PDK1 levels in the R7 knockdown lines 29-11 and 30-10 respectively when compared to the R7 control line scram-10 (Figure 2.5 A, B). By knocking down either LDHA or PDK1 expression in the R7 Aβ resistant cell line, we observed a significant decrease in cell viability, following exposure to 48 hr Aβ (20 μM) when compared to the control (Figure 2.5 C, $P<0.01$). Reduction of LDHA or PDK1 expression in R7 cells resulted in a 40 to 50% reduction in viability compared to the control cell lines (Figure 2.5 C).

Western blot analysis revealed there was a 50% and 62% reduction in LDHA levels in the R2 knockdown lines 71-23 and 72-5 respectively, and a 48% and 49% reduction in PDK1 levels in the R2 knockdown lines 29-12 and 30-4 respectively, when compared to R2 line scram 5 (Figure 2.5 D, E). Exposure of the R2 LDHA or PDK1 knockdown clones to 48 hr Aβ (20 μM) resulted in a significant reduction in cell viability (between 43 to 52%) when compared to the control (Figure 2.5 F, $P<0.01$). Thus, specifically targeting either LDHA or PDK1 expression in Aβ resistant cells results in re-sensitization to Aβ and increased death.

Figure 2.5 Attenuated LDHA or PDK1 expression increases sensitivity of resistant cell lines to Aβ. A) Immunoblot analysis of PC12 R7 resistant cells stably transfected with LDHA-specific shRNA vectors revealed two clonal cell lines (clones 71-7 and 72-11) exhibited a significant decrease in LDHA protein levels when compared to an R7 cell line transfected with a non-specific shRNA (SCR) (*P<0.01). **B)** Immunoblot analysis also confirmed a significant decrease in PDK1 protein in two PDK-1 shRNA stably transfected R7 cell lines, (clones 29-1 and 30-10) when compared to the control cell line (*P<0.01). **C)** A significant decrease in the cell viability of the R7 clones with attenuated expression of either LDHA or PDK1 was observed when cells were exposed to Aβ (20 μM) for 48 hr when compared to the control (*P<0.01). **D)** Immunoblots of R2 cell lines (clones 71-23 and 72-5) confirming significantly decreased LDHA expression when compared to a control cell line (SCR) (*P<0.01). **E)** R2 clonal cell lines, (clones 29-12 and 30-4) stably expressing PDK-1 shRNA showed a significant decrease in the PDK1 full length protein (~48 kDa) as well as a decrease in the proposed PDK1 cleavage product (~30 kDa) when compared to the control (*P<0.01). **F)** Both R2 LDHA and PDK1 knockdown cell lines exposed to 48 hr Aβ (20 μM) treatment showed a significant decrease in cell viability when compared to R2 control (* P<0.01). Densitometric analysis of LDHA and PDK1 band densities relative to actin are found below the corresponding blot. Relative intensity was calculated by comparing the LDHA/actin or PDK1/actin ratio of the resistant lines to the same ratio in the scrambled shRNA cell line. Data represent the average ± SD of three independent experiments. Data were analyzed by a one-way ANOVA followed by a Dunnett test.


A
SCR
71-7
72-11
LDHA
β-Actin
Relative Intensity
1
0.8
0.6
0.4
0.2
0
*
*
B
SCR
29-11
30-10
PDK1
β-Actin
Relative Intensity
1
0.8
0.6
0.4
0.2
0
*
*
C
Percent Viability
80
70
60
50
40
30
20
10
0
*
*
*
*
SCR
71-7
72-11
29-11
30-10
48hr
Aβ


D
SCR
71-23
72-5
LDHA
β-Actin
Relative Intensity
1
0.8
0.6
0.4
0.2
0
*
*
E
SCR
29-12
30-5
kDa
50
37
25
PDK1
β-Actin
Relative Intensity
1
0.8
0.6
0.4
0.2
0
*
*
F
Percent Viability
80
70
60
50
40
30
20
10
0
*
*
*
*
SCR
71-23
72-5
29-12
30-4
48hr Aβ

2.4 Discussion

Understanding how nerve cells become resistant to Aβ toxicity is central to understanding how some nerve cells within the AD brain are able to survive while large numbers of cells die. It had been previously shown that Aβ resistant cells, through increased HIF-1 activity, modulate their cell metabolism to increase glucose uptake and glycolysis in the presence of Aβ (Soucek et al., 2003). Additionally, HIF-1 activation in Aβ sensitive cells is sufficient for neuroprotection against Aβ (Soucek et al., 2003). However, this earlier study did not precisely define how HIF-1 functions to protect cells from Aβ toxicity. One compelling idea is that HIF-1 driven alteration in metabolism in Aβ resistant cells confers a selective advantage for survival in the hostile environment of the AD brain (Schubert, 2005; Soucek et al., 2003). Increased HIF-1 activity has been shown to enhance transcription of both *LDHA* and *PDK1* (Kim et al., 2006; Semenza et al., 1996). In this study we observed that Aβ resistant cells have increased LDHA activity and PDK1 expression, both in the absence or presence of Aβ, and these events correlate with decreased mitochondrial derived ROS.

The question arises as to why decreased mitochondrial respiration in Aβ resistant cells would be advantageous? A previous study showed that cells depleted of mitochondrial DNA, lacking critical catalytic subunits of the respiratory chain and incapable of mitochondrial respiration, were unaffected by Aβ exposure (Cardoso et al., 2001). Mitochondria are the major site of ROS production, which is believed to be a major factor leading to Aβ induced death (Behl et al., 1994). This is supported by the observation that exogenously applied antioxidants protect primary CNS cultures and clonal lines from Aβ toxicity (Behl et al., 1994). In addition, overexpression of the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD) in AD transgenic mice increases resistance to Aβ and attenuates the AD phenotype (Dumont et al., 2009). These studies suggest that functional mitochondria are required for Aβ to elicit a toxic effect.

In support of these findings, the data in this study reveal that Aβ resistant nerve cell lines exhibit metabolic reprogramming and decreased mitochondrial ROS which contribute to their resistance against Aβ toxicity. Interestingly, these low levels of ROS were maintained even after exposure to Aβ. In contrast, the parental sensitive lines showed elevated mitochondrial

ROS with exposure to Aβ. The increase in LDHA activity, lactate production and PDK1 levels, and decrease in mitochondrial ROS strongly indicate that a Warburg effect exists in Aβ resistant cells. Interestingly chemical or genetic manipulation of either LDHA or PDK1 resulted in increased sensitivity to Aβ in all resistant cell lines. These observations indicate that both enzymes, LDHA and PDK1, contribute to resistance to Aβ. However, inhibiting or knocking down either enzyme resulted in similar levels of sensitivity to Aβ suggesting that both enzymes appear to play an equal and possibly redundant role in the resistance pathway. Knocking down or chemically inhibiting LDHA or PDK in various cancers shifts metabolism from glycolysis and lactate production to mitochondrial respiration, with an associated increase in mitochondrial ROS production and induction of apoptosis (Bonnet et al., 2007; Fantin et al., 2006; Le et al., 2010; Michelakis et al., 2010). Furthermore, inhibition of PDK1 in cancer cells *in vitro* and *in vivo* results in a reduction of phosphorylated PDH and HIF-1α levels (McFate et al., 2008). Moreover, inhibition of PDK1 expression has been shown to decrease lactate levels and HIF-1α expression and reduce the malignant phenotype of cancer cells (McFate et al., 2008). Therefore an increase in PDK1 and lactate production may aid in sustaining the Warburg effect through a positive feedback mechanism. We propose that HIF-1 activation of the glycolytic genes, *LDHA* and *PDK1* functions to suppress apoptosis in Aβ resistant cells by decreasing mitochondrial respiration and ROS production.

In the past, aerobic glycolysis within the brain has been given little attention, despite early observations suggesting that a basal level of aerobic glycolysis occurs in specific areas of the brain (Boyle et al., 1994; Raichle et al., 1970). In a healthy adult, levels of aerobic glycolysis can range from ~10-15% of the glucose utilized by the human brain (Boyle et al., 1994; Powers et al., 2007; Raichle et al., 1970). More recently, PET imaging revealed a significant increase in aerobic glycolysis in specific areas of the brain (Vaishnavi et al., 2010). Specifically, the levels of aerobic glycolysis are elevated in the medial and lateral parietal and prefrontal cortices (Vaishnavi et al., 2010). Interestingly, PET imaging studies using a radiotracer with a high affinity to Aβ plaques, revealed a strong correlation between the spatial distribution of aerobic glycolysis and Aβ plaques in both patients with AD, as well as cognitively normal patients with high levels of Aβ deposition but without clinical manifestation of the disease (Vlassenko et al., 2010). Additionally, the spatial distribution of Aβ deposition and increased aerobic glycolysis closely mirrors the distribution of aerobic

glycolysis in the normal healthy brain (Vaishnavi et al., 2010; Vlassenko et al., 2010). Thus one could conclude that areas of increased aerobic glycolysis within the brain are more susceptible to Aβ accumulation. However, the opposing viewpoint is that an increase in aerobic glycolysis is an innate protective response that limits the toxicity of Aβ. Areas within the normal brain that show increased aerobic glycolysis highly overlap with the 'default mode network'- brain regions that are most active when an individual is awake but not engaged in a specific task (Vaishnavi et al., 2010; Vlassenko et al., 2010). We propose that these areas of the brain are the most susceptible to insult in AD and thus exhibit a Warburg effect as a protective mechanism or a built in resistance mechanism that can be further activated in the presence of high levels of Aβ.

Although a Warburg effect aids in the survival against Aβ toxicity, it may have a few detrimental effects. First, a reliance on glycolysis, an inefficient means of producing ATP, increases the demand for glucose in these cells. Thus a small decrease in glucose availability may render these cells incapable of producing sufficient energy to sustain their function and lead to cell death. Secondly, an increase in lactate production may result in decreased glutathione levels, the major antioxidant in the brain responsible for detoxifying ROS (Lewerenz et al., 2010). Lactate inhibits the enzymatic steps of glutathione synthesis, thus an increase in lactate would likely result in a decrease in glutathione synthesis and levels, which could result in a decreased tolerance to ROS (Lewerenz et al., 2010). However, intracellular lactate levels were not significantly different between cells suggesting that lactate is efficiently exported from the cell (data not shown). Lastly, HIF-1 mediated increase in glycolysis may result in an increase in Aβ production and deposition within the brain. HIF-1 has been shown to transcriptionally regulate both the β-site APP cleaving enzyme (β-secretase/BACE1) and an essential enzyme involved in the presenilin/γ-secretase complex anterior pharynx-defective 1A (APH-1A)(Wang et al., 2006; Zhang et al., 2007). Hypoxia or overexpression of HIF-1α increases the BACE1 mRNA and protein levels in mouse neuroblastoma N2a cells (Zhang et al., 2007). Similarly, treatment of HeLa cells stably expressing the human APP Swedish mutation with $NiCl_2$ (a chemical mimic of hypoxia) results in an increase in APH-1A mRNA and protein expression accompanied by an increased secretion of Aβ (Wang et al., 2006). These findings have quelled enthusiasm for the treatment of AD using metal chelators to enhance HIF1α activity. However, our findings

suggest that enhancement of PDK1 activity alone may offer a better neuroprotective strategy as it is unlikely to affect APP processing. Further studies will need to be conducted to examine this hypothesis.

2.5 Conclusion

We have shown for the first time a Warburg effect exists in Aβ resistant nerve cell lines, and contributes to resistance against Aβ toxicity. These findings suggest that the Warburg effect may act as a common resistance mechanism in a variety of cell types in response to diverse environmental stresses. However the physiological relevance of these findings in an *in vivo* AD model remains unknown. This shift in cell metabolism to rely heavily on glycolysis and lactate production for energy needs may provide nerve cells with a mechanism to overcome the pro-oxidant conditions elicited by Aβ exposure. Chemical means of modulating this pathway may be of therapeutic interest in the treatment of AD. Moreover, characterization of the mechanisms by which glycolysis is upregulated in Aβ resistant cells could reveal possible targets for drug therapy in the treatment of AD.

2.6 References

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Chapter 3

3 Overexpression of pyruvate dehydrogenase kinase 1 and lactate dehydrogenase A in nerve cells confers resistance to amyloid beta and other toxins by decreasing mitochondrial respiration and ROS production

3.1 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and is the most common form of age-related dementia. AD is characterized by widespread nerve cell death and the accumulation of extracellular plaques and intracellular neurofibrillary tangles within the brain (Selkoe, 2004). These plaques are primarily composed of amyloid-β-peptide (Aβ), a 39-42 amino acid peptide derived from the proteolytic cleavage of the amyloid precursor protein (APP). The Aβ peptide, particularly the 42 amino acid long variant ($A\beta_{42}$), is highly prone to undergo oligomerization and fibrillogenesis; events strongly associated with the disease state (Selkoe, 1998). The amyloid cascade hypothesis, first proposed over 20 years ago, suggests that Aβ deposition in the brain is the causative agent of AD (Hardy and Higgins, 1992; Selkoe, 1991). Although multiple clinical trials have tested agents that either prevent the cleavage of APP or promote increased clearance of Aβ, to date none of these trials have been successful in halting disease progression prompting the hunt for alternative therapies to combat AD (Ballard et al., 2011).

Aβ-deposition promotes mitochondrial dysfunction and an increase in reactive oxygen species (ROS) production resulting in oxidative damage, synaptic loss and ultimately nerve cell death (Behl et al., 1994; Butterfield et al., 2007; Markesbery, 1997; Tillement et al., 2011). However, numerous immuno-histochemical studies of brain tissue from individuals without any history of dementia showed that up to 40% of the autopsied samples had significant plaque accumulation but little or no nerve cell loss (Bouras et al., 1994; Price and Morris, 1999). It has been argued that asymptomatic individuals with high plaque accumulation likely had undiagnosed mild cognitive impairment and would have eventually developed AD had they lived long enough or had a high cognitive reserve. However, an alternative hypothesis is that these individuals may have acquired or exhibit an innate

resistance mechanism to the toxic effects of Aβ. While difficult to study in patients, models of Aβ-resistance have been generated *in vitro* following the continual exposure of cultured nerve cells to concentrations of Aβ that would otherwise be toxic and the eventual emergence of surviving clonal nerve cell populations. Analysis of these Aβ-resistant nerve cells revealed upregulation of anti-oxidant enzymes compared with the sensitive parental cells (Cumming et al., 2007; Sagara et al., 1996). Additionally these cells displayed an increased resistance to a wide array of neurotoxins, suggesting that acquisition of Aβ resistance also confers resistance to a variety of environmental stresses (Dargusch and Schubert, 2002).

Intriguingly, Aβ-resistant cells also exhibit increased glucose uptake and flux through the glycolytic pathway and heightened sensitivity to glucose deprivation (Soucek et al., 2003). These cells also appear to break down glucose in a unique manner, reminiscent of cancer cells. Cancer cells have been shown to shift metabolism from mitochondrial respiration to glycolysis and lactate production for their energy needs despite the presence of oxygen (Bonnet et al., 2007; Warburg, 1956). This phenomenon is termed the Warburg effect, or aerobic glycolysis, and is driven by hypoxia inducible factor 1 α subunit (HIF-1α) (Kim et al., 2006; Le et al., 2010; Papandreou et al., 2006; Zhou et al., 2010). HIF-1α is a heterodimeric transcription factor that regulates cellular adaptation to hypoxia and induces the transcription of pyruvate dehydrogenase kinase 1 (PDK1) (Kim et al., 2006; Papandreou et al., 2006). PDK1 phosphorylates and inhibits pyruvate dehydrogenase (PDH), an enzyme responsible for the conversion of pyruvate to acetyl-CoA (Papandreou et al., 2006). When PDH is inhibited, pyruvate is no longer an available substrate to fuel the TCA cycle and mitochondrial oxygen consumption is decreased (Papandreou et al., 2006). Additionally, HIF-1α upregulates the expression of LDHA, an enzyme responsible for the conversion of pyruvate to lactate, with the concomitant regeneration of nicotinamide adenine dinucleotide (NAD^+) (Semenza et al., 1996).

Though HIF-1α was initially believed to be only active in low oxygen environments, recent findings have suggested that HIF-1α can be upregulated under normoxic conditions in both normal and cancer cells (Lu et al., 2005; Lu et al., 2002; McFate et al., 2008; Semenza, 2010). HIF-1α regulated changes in metabolism not only allow for maintenance of energy homeostasis in prolonged low oxygen conditions but also attenuate generation of harmful

ROS at higher oxygen levels (Kim et al., 2006). By repressing mitochondrial respiration, cancer cells are less likely to produce ROS and are more resistant to mitochondrial depolarization; two events tightly linked to induction of apoptosis (Koppenol et al., 2011).

Similar to cancer cells, the observed metabolic changes in Aβ resistant cells arise through stabilization of HIF-1α (Soucek et al., 2003). In addition, Aβ-resistant nerve cells have elevated PDK1 expression as well as an increase in LDHA activity and lactate production when compared with control cells (Chapter 2, Newington et al., 2011). Moreover, mitochondrial derived ROS, which are closely associated with Aβ toxicity, are markedly diminished in resistant relative to sensitive cells (Newington et al., 2011). Chemically or genetically inhibiting LDHA or PDK1 re-sensitizes resistant cells to Aβ toxicity, suggesting that the altered glycolytic metabolism in these cells may mediate Aβ-resistance (Newington et al., 2011). Although a reduction in cerebral glucose metabolism, as measured by fluoro-2-deoxy-D-glucose (FDG) positron emission tomography (PET), is one of the most common diagnostic features of AD recent evidence suggest that glucose utilization is more complex in the AD brain (Mosconi, 2005; Vlassenko et al., 2010). Studies using modified PET imaging, which measured both glucose consumption and oxygen utilization, revealed a strong correlation between the spatial distribution of elevated aerobic glycolysis and Aβ plaques in brain tissue from patients with both AD, as well as normal individuals with high levels of Aβ-deposition but without clinical manifestation of the disease (Vlassenko et al., 2010). Additionally, the spatial distribution of Aβ deposition and increased aerobic glycolysis closely mirrors areas of high aerobic glycolysis in the normal healthy brain (Vaishnavi et al., 2010). These findings suggest that areas of the brain most susceptible to insult in AD may exhibit a Warburg effect as a protective mechanism that can be further activated in the presence of high levels of Aβ. However, this hypothesis has never formally been evaluated.

Here we show that overexpresssion of LDHA or PDK1 in the B12 central nervous system cell line confers resistance to Aβ and other neurotoxins such as H_2O_2 and staurosporine. Increased survival in cells overexpressing LDHA or PDK1 is associated with decreased mitochondrial membrane potential, oxygen consumption and ROS production, yet these cells maintain the ability to produce sufficient ATP. Expression of PDK1 and LDHA is decreased in wildtype mouse primary cortical neurons exposed to Aβ and in cortical extracts from 12

month old of AD transgenic (APP/PS1) mice. Similarly, post-mortem cortical tissue from AD patients also revealed a decrease in PDK1 expression relative to control patient brain samples. These findings suggest that loss of the adaptive advantage afforded by aerobic glycolysis may exacerbate the pathophysiological processes associated with AD.

3.2 Methods

3.2.1 Materials

Cell culture reagents including: Dulbecco's modified Eagles medium (DMEM), penicillin/streptomycin, DMEM without phenol red and Dulbecco's phosphate buffered saline (DPBS) were purchased from Biowhittaker (Walkersville, MD, USA) (Carlsbad, CA, USA). Dialyzed fetal bovine serum (FBS) and horse serum (HS) were obtained from PAA Laboratories Inc. (Etobicoke, ON, Canada). OPTIMEM I, TrypLE Express, Neurobasal Medium, N_2 Supplement, B_{27} Supplement.Glutamax-1 (100x) and Hanks Balanced Salts Solution were obtained from Invitrogen (Carlsbad, CA, USA). Amyloid beta (Aβ) peptide (25-35) was purchased from California peptide research (San Francisco, CA, USA). Poly-D-lysine, Poly-L-Ornithine, puromycin, dihydrochloride, dimethyl formamide, 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were all purchased from Sigma (St. Louis, MO, USA). DNase 1 and Trypsin Inhibitor were purchased from Roche (Laval, Quebec, Canada). G418 sulfate was purchased from Calbiochem (EMD Chemicals Inc., Darmstadt, Germany). Mitotracker Red CM-H2XRos, Tetramethyl Rhodamine Methyl Ester (TMRM), MitoSOX Red, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and the ATP determination kit were purchased from Invitrogen (Carlsbad, CA, USA). MitoXpress-Xtra-HS was purchased from Luxcel Biosciences Ltd (Cork, Ireland).

3.2.2 Cell culture

The B12, rat central nervous system cell line was obtained from Dr. David Schubert (The Salk Institute, La Jolla, CA) and cultured as previously described (Behl et al., 1994; Sagara et al., 1996). The B12 central nervous system cells are an immortalized clonal cell line derived from a nitrosoethylurea induced brain tumor in rats (Schubert et al., 1974) and have been shown to be sensitive to Aβ toxicity. These cells were grown in DMEM supplemented with 10% FBS, and 1% P/S in a humidified incubator at 37^{o}C and 5% CO_2. For toxicity studies,

the Aβ peptide (25-35) was dissolved in deionized water at 1 mg/ml, left overnight at room temperature to promote fibril formation and subsequently stored at -20^{o}C.

3.2.3 Derivation of PDK1 and LDHA overexpressing cell lines

For stable expression of PDK1 and LDHA, B12 cells were transfected with lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. Cells were plated at a density to achieve 70-80% confluency and transfected with vectors containing human *PDK1* (pCMV6-AC) or *LDHA* (pCMV6-XL4) cDNA (Origene, Rockville, MD, USA). Additionally cells were transfected with an empty vector (pcDNA) as a negative control. Following selection in G418 (1 mg/ml) for two weeks approximately 10-12 clones were picked, expanded and screened by immunoblot analysis for high level expression of either PDK1 or LDHA.

3.2.4 Derivation of PDK1 and LDHA knockdown cell lines

For stable knockdown of PDK1 and LDHA, B12 cells were transfected with HuSH 29mer shRNA (Origene, Rockville, MD, USA) constructs directed at rat *Ldha* or *Pdk1* transcripts followed by selection with puromycin as previously described (Newington et al., 2011). The shRNA construct directed against *Ldha* was: 69-GCCGAGAGCATA-ATGAAGAACCTTAGGCG and the shRNA construct directed against *Pdk1* was: 29-AATCACCAGGACAGCCAATACAAGTGGTT. A non targeting shRNA construct (scrambled) in pRS plasmid was used as a negative control.

3.2.5 Immunoblot Analysis

B12 cells from subconfluent cultures were washed twice in cold DPBS and harvested in a Tris extraction buffer (50 mM Tris pH 7.5, 2% SDS and 1 mM PMSF). Protein extracts were quantified by a Lowry assay, resolved by 12% SDS PAGE and electroblotted onto PVDF membrane (Bio-Rad Richmond, CA, USA) (Cumming et al., 2007). Membranes were probed with the following antibodies: polyclonal anti-LDHA (1:1000; Cell Signaling, Danvers, MA, USA), polyclonal anti-PDK1 (1:1000; Stressgen, San Diego, CA, USA) and a monoclonal anti-β actin (1:2000; Cell Signaling, Danvers, MA, USA) followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Richmond, CA, USA). The blots were developed using Pierce ECL western blotting

substrate (Thermo Scientific, Rockford, IL, USA) and visualized with a Bio-Rad Molecular Imager (ChemiDoc XRS, Bio-Rad, Richmond, CA, USA). Densitometric analysis was performed using Image J software. Band densities were standardized against β-actin, and the ratio of LDHA or PDK1-specific bands relative to the β-actin band was determined. Relative intensity was calculated by comparing the LDHA/β-actin or PDK1/β-actin ratios of the transfected lines to the same ratio in the control cell line.

3.2.6 Cytotoxicity assay

Aβ, hydrogen peroxide and staurosporine induced cytotoxicity was assessed by a modified MTT assay (Behl et al., 1994; Hansen et al., 1989; Sagara et al., 1996). Cells were seeded ($3x10^3$ cells/well) in a 96 well microtiter plate and $A\beta_{25-35}$ was added to the test wells at a concentration of 20 μM and incubated for 48 hours. To each well 10 μl of MTT (2.5 mg/ml dissolved in DPBS) was added and, following a 4 hour incubation, 100 μl of solubilisation solution (20% SDS in 50% dimethyl formamide pH 4.8) was added to each well. The plates were rocked at room temperature overnight then read on a microplate reader (BioRad Model 3550) using 595 nm as the test wavelength and 655 nm as the reference wavelength. The percent viability was calculated from the mean absorbance of the treated cells divided by the mean absorbance of the control cells and multiplied by 100%.

3.2.7 Oxygen Consumption

Oxygen consumption was monitored using the fluorescent oxygen probe MitoXpress-Xtra-HS. B12 cells ($1.25x10^5$) were seeded on 60 mm dishes. The following day $A\beta_{25-35}$ (20 μM) was added to the treatment dishes and cells were incubated for 48 hr. Cells were trypsinized, centrifuged, and resuspended at an appropriate density prior to the assay. Cells ($2x10^5$) were transferred to a 96 well plate and incubated with the MitoXpress oxygen probe according to the manufacturer's instructions (Luxcel Biosciences Ltd.). Oxygen consumption was monitored as a function of fluorescence (ex/em 380/650 nm) every 2 min over a 6 hr period on a time-resolved fluorescence microplate reader (Tecan, Infinite M1000). Data were analyzed using the program Mathematica as previously described (Hynes et al., 2012). Oxygen concentration in μM was calculated by the following formula, where X is the normalized fold change in fluorescence over the initial reading, t is time, S is the maximum

fluorescence in media containing no oxygen and 235 μM is the concentration of oxygen gas in fully saturated water at 30 °C:

$$[O_2](t) = \frac{235(S - X)}{X(S - 1)}$$

Linear regression over a span of 100 minutes was performed to determine the rate of oxygen consumption for each clone. Oxygen consumption rates were later standardized to cell number.

3.2.8 ATP levels

Cellular ATP was measured in B12 cells using a bioluminescence ATP determination kit. Approximately $7.5x10^4$ cells were plated in 35 mm dishes and the following day were treated with $A\beta_{25\text{-}35}$ (20 μM) for a further 48 hrs. Cells were harvested in a Tris extraction buffer (20 mM Tris pH 7.8, 2 mM EDTA, 0.5% NP40 and 25 mM NaCl). One microgram of protein sample was loaded in each well of a 96 well plate. The luciferin and luciferase buffer were prepared according to the manufacturer's instructions (Molecular Probes) and 100 μl was injected into each well. Luminescence was integrated over 10 sec using a TECAN Infinite M1000 microplate reader. ATP contents were calculated by comparing the luminescence, using an ATP standard curve.

3.2.9 Fluorescence microscopy

Mitochondrial membrane potential ($\Delta\psi_m$) was visualized by the fluorescent dye tetramethyl-rhodamine methyl-ester (TMRM) (Bonnet et al., 2007). B12 cells were seeded between $1x10^5$ and $2x10^5$ cells on 30 mm plastic tissue culture dishes pretreated with polylysine and incubated overnight. The following day, cells were treated with 20 μM $A\beta_{25\text{-}35}$ for 48 hr. Following treatment with Aβ, the media was aspirated and new media was added containing TMRM at a concentration of 200 nM. Plates were then incubated at 37°C for 20 min, washed in DPBS containing Hoechst stain (10 μg/ml), followed by an additional wash in DPBS and then placed in phenol red free DMEM. For treatment with H_2O_2 or staurosporine, cells were first stained using the above protocol. Following staining cells were treated with either H_2O_2 (200 μM) or staurosporine (200 ng/ml) for 15 min before visualization. Cells were visualized by fluorescence microscopy (Zeiss-AxioObserver, 40X objective) and

pictures were taken using a Q Imaging (Retiga 1300 monochrome 10-bit) camera with Q Capture software. Pictures were taken of three random fields of view for each experiment. TMRM fluorescence was quantified with ImageJ software.

Mitochondrial ROS was visualized by the fluorescent dye Mitotracker Red CM-H2XRos (MTR). B12 cells were plated seeded between 1 and $2x10^5$ cells on 30 mm plastic tissue culture dishes pretreated with polylysine and incubated overnight. The following day cells were treated with 20 μM $A\beta_{25-35}$ for 48 hr. Following treatment with Aβ, the media was aspirated and new media was added containing MTR at a concentration of 100 nM. Plates were then incubated at 37°C for 20 min, washed in DPBS containing Hoechst stain, followed by an additional wash in DPBS and then placed in phenol red free DMEM. For treatment with H_2O_2 or staurosporine cells were first treated with either H_2O_2 (200 μM) or staurosporine (200 ng/ml) for 30 min before visualization and then stained and visualized as described above. MTR fluorescence was quantified with ImageJ software. Mitochondrial derived superoxide was also visualized using the fluorescent dye MitoSOX Red mitochondrial superoxide indicator. Glass bottom dishes were pretreated with polylysine and cells were plated and treated as described above. Cells were stained with MitoSOX at 5 μM and incubated at 37°C for 30 min. Plates were then washed in DPBS containing Hoechst stain (10 μg/ml) and then placed in phenol red free DMEM. Cells were visualized by fluorescence microscopy (Zeiss-AxioObserver, 100X objective) and fluorescence was quantified as described above.

Overall cellular ROS was visualized by the fluorescent dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). Cells were plated and treated as described above. Cells were stained with CM-H_2DCFDA at 2.5 μM and incubated at 37°C for 15 min. Plates were then washed once in DPBS then visualized as described above. H_2DCFDA fluorescence was quantified with ImageJ software.

For time lapse microscopy B12 cells were seeded at $2x10^5$ cells in a 6 well Chamlide TC stage chamber (Live Cell Instruments, Nowan-gu, Seoul, Korea) pretreated with polylysine (50 μg/ml) and incubated overnight at 37°C in a tissue culture incubator. The following day cells were stained with TMRM as described above. Following staining the stage chamber was assembled onto the automated stage of a DMI6000 B inverted microscope (Leica

Microsystems, Wetzlar, Germany) heated to 37^{o}C and perfused with 5% CO_2 using an FC-5 CO_2/Air gas mixer (Live Cell Instrument, Nowan-gu, Seoul, Korea). Cells were treated with H_2O_2 (200 μM) and time lapse DIC and fluorescent images of cells were automatically captured from three independent fields of each well every 10 minutes over 4 hrs using a C10600 Hamamatsu Digital Camera (Meyer Instruments, Houston, TX, USA) equipped with Metamorph Software. The DIC and fluorescent images were overlaid and a time lapse video was generated using Image J software.

3.2.10 Primary nerve cell cultures

Primary cortical neurons were prepared from mouse embryonic day 14-17 as previously described (Ribeiro et al., 2005). Cells were seeded at a density of $1.6x10^6$ cells per dish and cultured in Neurobasal Media containing 2 mM glutamine, 50 units/mL P/S and N2/B27 supplements and incubated for 48 hr (Ribeiro et al., 2005). The media was changed 48 hr post plating. At this time cells were treated with 10 μM $A\beta_{25-35}$. Cells were harvested in 2% SDS buffer as described above, at 4, 8, 12, 16, 24, 36 and 48 hr post Aβ treatment. PDK1 and LDHA were visualized by Western blot analysis as described above and protein quantification was performed using Image J software.

3.2.11 Mouse and Human Tissue

APP/PS1 (APPswe,PSEN1dE9) double transgenic mice cortical brain tissues were generously provided by Dr. David Schubert (The Salk Institute, La Jolla, CA). Twelve month old tg-AD mice and age matched controls were perfused with saline and protease inhibitor cocktail, and brain regions were snap frozen and stored at -80^{o}C. Frozen tissue samples were partially thawed and ~100 mg pieces were removed and minced in a 5X weight/volume extraction buffer containing 50 mM Tris pH 7.5, 2% SDS and protease inhibitor cocktail (Cumming et al., 2007; Soucek et al., 2003). Following sonication and centrifugation supernatants were collected and protein extracts were quantified using the Lowry assay. Protein extracts (15 μg) from the frontal cortex were analyzed by immunoblot analysis as described above.

Autopsied brain samples were obtained from Drs. Carol Miller and Jenny Tang at the Alzheimer's disease Research Center (University of Southern California School of Medicine,

Los Angeles, CA). All tissue samples were extracted from the same area of the mid-frontal cortex and immediately quick frozen after removal. All AD and control cases were matched pairwise for age, sex and in most cases post-mortem interval (PMI). All AD patients had a clinical history of dementia and a plaque density (plaques per field, PPF) in the low to moderate range according to The Consortium to Establish a Registry for Alzheimer's Disease criterion (1=sparse,1-5 PPF; 3=moderate, 6-20 PPF; 5=frequent, 21-30 PPF or above). Patient details are summarized in Table 1. Frozen tissue samples were partially thawed and ~100 mg pieces were removed and minced in a 5X weight/volume extraction buffer containing 50 mM Tris pH 7.5, 2% SDS and protease inhibitor cocktail (Cumming et al., 2007; Soucek et al., 2003). Following sonication and centrifugation supernatants were collected and protein extracts were quantified using the Lowry assay. Protein extracts (15 µg) were analyzed by immunoblot analysis as described above.

3.2.12 Statistical Analysis

Data are presented as means ± SD resulting from a least three independent experiments. Data were analyzed statistically using either a two-way ANOVA followed by a Tukey test, a one-way ANOVA followed by a Dunnett test or a T-test (VassarStats). Results were considered statistically significant at $P<0.05$.

Table 1. Control and AD patient details

Patient	Diagnosis	Age	Sex	PMI[a]	PPF
C1	Normal	94	Female	4.5	0
A1	AD	95	Female	4.5	3
C2	Normal	87	Female	6	0
A2	AD	86	Female	6	1
C3	Normal	88	Male	2	0
A3	AD	85	Male	4.5	1
C4	Normal	69	Male	8	0
A4	AD	75	Male	2	5
C5	Normal	91	Female	NA[b]	0
A5	AD	90	Female	4.5	5
C6	Normal	92	Female	7	0
A6	AD	90	Female	7.5	3
C7	Normal	80	Male	12	0
A7	AD	77	Male	3	5

[a]PMI, Post mortem interval

[b]NA, Not available

PPF, plaques per field

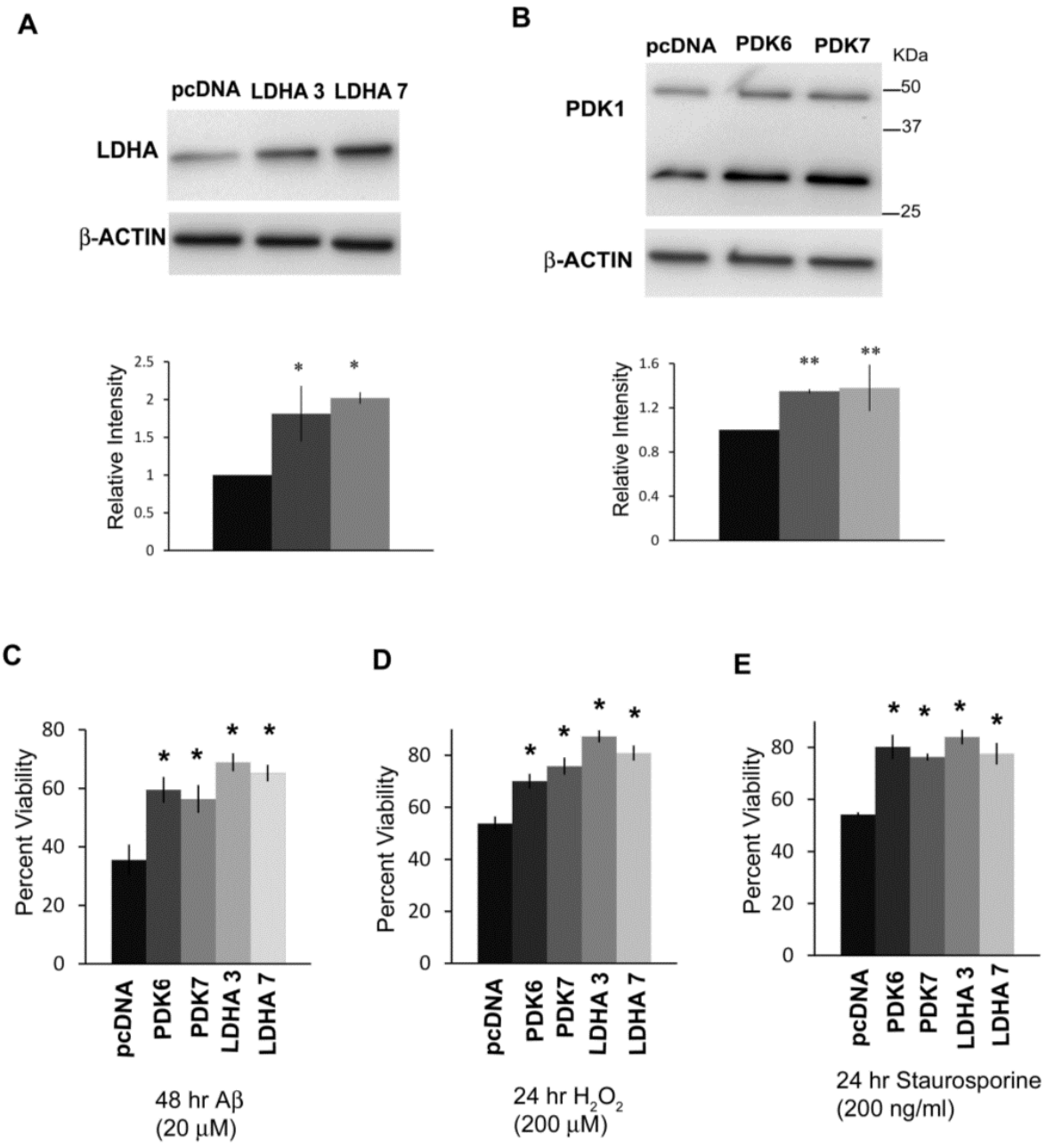
3.3 Results

3.3.1 Overexpression of LDHA or PDK1 confers resistance to Aβ, H_2O_2 and staurosporine toxicity

LDHA and PDK1 are key enzymes that mediate the Warburg effect in cancer cells (Bonnet et al., 2007; Fantin et al., 2006; Le et al., 2010; Zhou et al., 2010). Previous studies have shown that increased PDK1 expression, LDHA activity and lactate production are common features of Aβ-resistant cells (Newington et al., 2011). Therefore we sought to determine if overexpression of either LDHA or PDK1 in B12 Aβ-sensitive cells could confer resistance to Aβ toxicity. Western blot analysis of B12 cells transfected with vectors containing human *LDHA* or *PDK1* cDNAs revealed elevated constitutive expression of LDHA or PDK1 compared to cells transfected with the control plasmid (pcDNA) (Figure 3.1 A and B). B12 cells overexpressing LDHA or PDK1 exhibited a significant increase in cell viability following 48 hr exposure to Aβ (20 μM) when compared to control cells ($P<0.01$, Figure 3.1C).

Clonal nerve cell lines selected for resistance to Aβ toxicity have been shown to exhibit increased resistance to a wide array of neurotoxins (Dargusch and Schubert, 2002; Sagara et al., 1996). We were therefore interested to see if overexpression of either LDHA or PDK1 conferred resistance to other stressors. The sensitivity of LDHA and PDK1 overexpressing cells to H_2O_2, staurosporine and a variety of mitochondrial inhibitors (rotenone, antimycin and oligomycin) were examined. Interestingly, none of the overexpressing lines were more resistant to any of the mitochondrial inhibitors (data not shown). However, all LDHA and PDK1 overexpressing cell lines showed an increased resistance to H_2O_2 (200 μM) and staurosporine (200 ng/ml) ($P<0.01$, Figure 3.1 D and E). Therefore, the overexpression of either LDHA or PDK1 confers resistance to H_2O_2 and staurosporine but not to neurotoxins that specifically disrupt the mitochondrial electron transport chain (ETC).

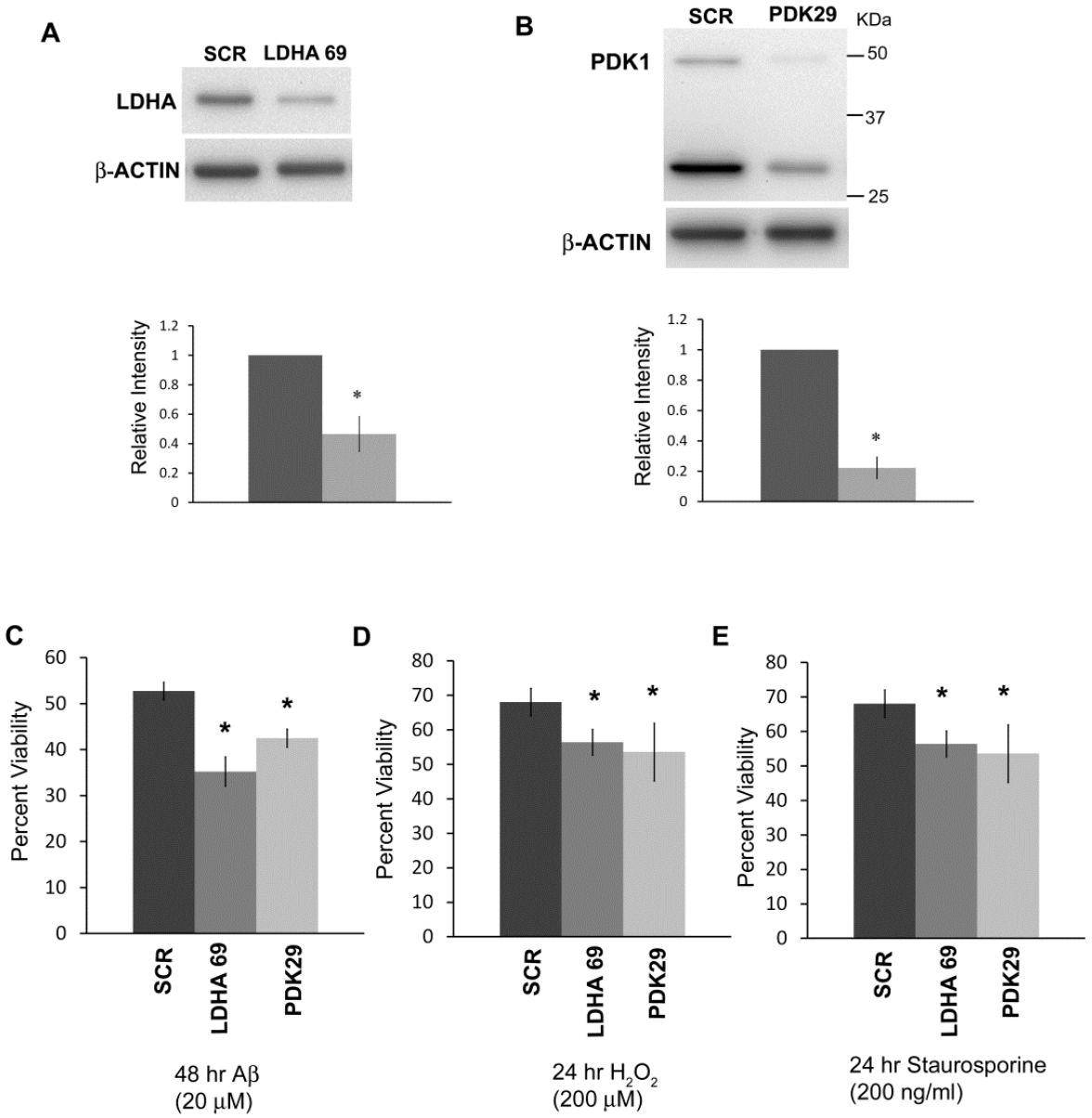
Figure 3.1 Overexpression of LDHA or PDK1 increases resistance to Aβ and other toxins in B12 sensitive cells. (A) Immunoblot analysis of B12 Aβ-sensitive cells stably transfected with either pcDNA (empty vector) or a vector containing human *LDHA* cDNA revealed two clonal cell lines (clones 3 and 7) with markedly increased LDHA protein levels (*P<0.01). **(B)** Immunoblot analysis of extracts from two clonal cell lines (clones 6 and 7) stably transfected with a *PDK1* expression vector confirmed a significant increase in PDK1 protein expression compared to the control cell line (**P<0.05). An additional 30 kDa PDK1 band was also elevated in *PDK1* transfected cells and likely represents a cleavage product. Densitometric analyses of LDHA and PDK1 band intensities relative to actin are indicated below each blot. Relative intensity was calculated by comparing the LDHA/actin or PDK1/actin ratio of the overexpressing lines to the same ratio in the control (pcNDA) cell line. Cell viability of clonal lines overexpressing either LDHA or PDK1 were significantly increased following exposure to either Aβ (20 μM) for 48 hrs **(C)**, H_2O_2 (200 μM) for 24 hrs **(D)**, or staurosporine (200 ng/ml) for 24 hrs **(E)** compared with the control cell line (* P<0.01). Data are presented as means ± SD resulting from 3 independent experiments. Data were analyzed a one-way ANOVA followed by a Dunnett's test.


A
pcDNA
LDHA 3
LDHA 7
LDHA
β-ACTIN
Relative Intensity
B
pcDNA
PDK6
PDK7
KDa
50
37
25
PDK1
β-ACTIN
Relative Intensity
C
Percent Viability
pcDNA
PDK6
PDK7
LDHA 3
LDHA 7
48 hr Aβ
(20 μM)
D
Percent Viability
24 hr H2O2
(200 μM)
E
Percent Viability
24 hr Staurosporine
(200 ng/ml)

3.3.2 Attenuated LDHA and PDK1 re-sensitizes cells to Aβ, H_2O_2 and staurosporine toxicity

Although we have previously shown that attenuation of LDHA and PDK1 in PC12 and B12 reversed Aβ toxicity in these cells, we sought to determine if specific inhibition of LDHA and PDK1 expression by shRNA–mediated knockdown could also render B12 parental cells more sensitive to Aβ (Newington et al., 2011). Immunoblot analysis confirmed B12 cell lines stably transfected with shRNA directed at rat *Ldha* or *Pdk1* transcripts exhibited decreased expression of the targeted mRNAs compared to cells transfected with a control shRNA containing a non-specific/scrambled (SCR) sequence (Figure 3.2). Knockdown of either *Ldha* or *Pdk1* in the B12 cells resulted in a significant decrease in cell viability, following 48 hr exposure to Aβ or 24 hr exposure to H_2O_2 or staurosporine when compared to the control (Figure 3.2 C, D and E, $P<0.01$). Thus attenuation of LDHA or PDK1 appears to further sensitize B12 cells to a variety of neurotoxins.

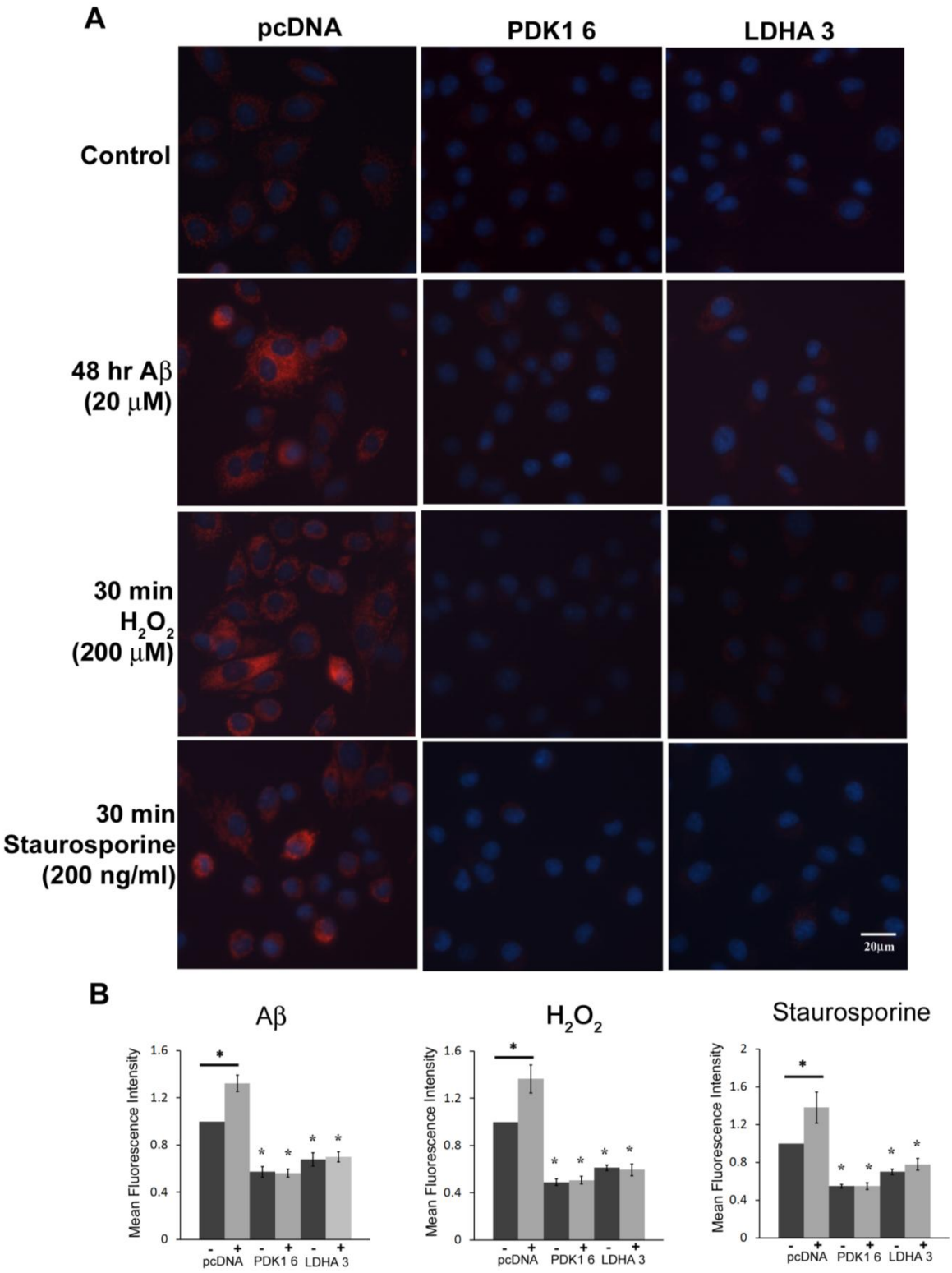
Figure 3.2 Attenuated LDHA and PDK1 expression increases sensitivity to Aβ and other neurotoxins. (A) Western blot analysis of B12 cells stably transfected with an LDHA-specific shRNA vector confirmed one clonal cell line (LDHA69) which exhibited a significant decrease in LDHA when compared to a B12 cell line transfected with a non-specific shRNA (SCR) (*P<0.01). **(B)** Immunoblot analysis also confirmed significantly decreased PDK1 expression in one clonal cell line stably expressing an shRNA vector directed at PDK1 (PDK29) when compared to control (SCR) (*P<0.01). **(C)** B12 cell lines with attenuated expression of either LDHA or PDK1 displayed a significant decrease in cell viability when treated with Aβ (20 μM) for 48 hr (*P<0.01) **(D)** Similarly cells with decreased LDHA or PDK1 had significantly decreased cell viability following 24 hr treatment with H_2O_2 or **(E)** staurosporine (200 ng/ml) (*P<0.01). Densitometric analysis of LDHA and PDK1 band densities relative to actin are found below the corresponding blot. Relative intensity was calculated by comparing the LDHA/actin or PDK1/actin ratio of the knockdown lines to the same ratio in the scrambled shRNA cell line. Data represent the mean ± SD of three independent experiments. Data were analyzed by a one-tailed T test (A and B) or by a one-way ANOVA followed by a Dunnett test (C-E).


A
SCR
LDHA 69
LDHA
β-ACTIN
Relative Intensity
B
SCR
PDK29
KDa
PDK1
50
37
25
β-ACTIN
Relative Intensity
C
Percent Viability
SCR
LDHA 69
PDK29
48 hr Aβ
(20 μM)
D
Percent Viability
SCR
LDHA 69
PDK29
24 hr H2O2
(200 μM)
E
Percent Viability
SCR
LDHA 69
PDK29
24 hr Staurosporine
(200 ng/ml)

3.3.3 Decreased mitochondrial membrane potential in LDHA and PDK1 overexpressing cells

The transfer of electrons through the ETC in the inner mitochondrial membrane provides the energy to drive H^+ against their concentration gradient into the intermembrane space. The resulting increase in H^+ outside the membrane creates a negative mitochondrial membrane potential ($\Delta\psi_m$). The stored energy of the $\Delta\psi_m$ and the flow of H^+ back into the mitochondria are used to synthesize ATP through the F_1/F_0 ATP-synthase, thus a decrease in mitochondrial membrane potential is indicative of decreased electron transport and OXOPHOS activity (Bonnet et al., 2007). Both elevated PDK1 and LDHA expression have been tied to reduced mitochondrial OXOPHOS activity, therefore we sought to determine if overexpression of LDHA or PDK1 could result in decreased $\Delta\psi_m$ following treatment with Aβ, H_2O_2, and staurosporine. Indeed, all overexpressing cell lines had significantly lower $\Delta\psi_m$ as measured by reduced TMRM fluorescence, compared to control cells expressing plasmid alone (Figure 3.3). When control cells were treated for 48 hr with Aβ (20 μM) there was a significant increase in $\Delta\psi_m$ ($P<0.001$) whereas $\Delta\psi_m$ remained unaltered in both LDHA and PDK1 overexpressing cell lines compared to untreated cells. Similar results were obtained when cells were treated with either H_2O_2 (200 μM) or staurosporine (200 ng/ml) for 30 min; namely the control cells showed a significant increase in membrane potential ($P<0.001$) whereas all the LDHA and PDK1 overexpressing cell lines maintained significantly $\Delta\psi_m$. Timelapse microscopy revealed that control cells displayed increased $\Delta\psi_m$ or hyper-polarization, followed by rapid depolarization and cell death when exposed to H_2O_2 (200 μM) (data not shown). In contrast, LDHA and PDK1 overexpressing cells showed decreased $\Delta\psi$m under control conditions which was maintained when cells were exposed to H_2O_2 (200 μM). Furthermore, the majority of cells overexpressing either LDHA or PDK1 did not undergo mitochondrial membrane depolarization and subsequent cell death following treatment with H_2O_2. Taken together, overexpression of either LDHA or PDK1 results in decreased $\Delta\psi$m which is maintained following exposure to a variety of stressors.

Figure 3.3 Decreased mitochondrial membrane potential in LDHA and PDK1 overexpressing cells. (A) Mitochondrial membrane potential ($\Delta\psi_m$) was measured in B12 cells following staining with the red fluorescing dye Tetramethyl Rhodamine Methyl Ester (TMRM). Both PDK1 and LDHA overexpressing B12 cell lines exhibited a significant reduction in $\Delta\psi_m$ under normal culture conditions or following exposure to Aβ (20 μM), H_2O_2 (200 μM) or staurosporine (200 ng/ml) when compared to the control cell line expressing the empty vector (pcDNA) under similar culture conditions (*P<0.001). Cells expressing the empty vector (pcDNA) showed a significant increase in $\Delta\psi_m$ following exposure to Aβ, H_2O_2 or staurosporine (*P<0.001). As a counterstain, nuclei were stained with Hoescht (Blue) and visualized by fluorescence microscopy at 400X magnification. **(B)** Quantification of TMRM fluorescence intensity revealed that $\Delta\psi_m$ was consistently lower in PDK1 and LDHA overexpressing cells in the absence (-) or presence (+) of the indicated stressor when compared to the pcDNA control cell line. Pictures were taken from 3 random fields of view for each treatment. Data are presented as means ± SD resulting from a least three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.

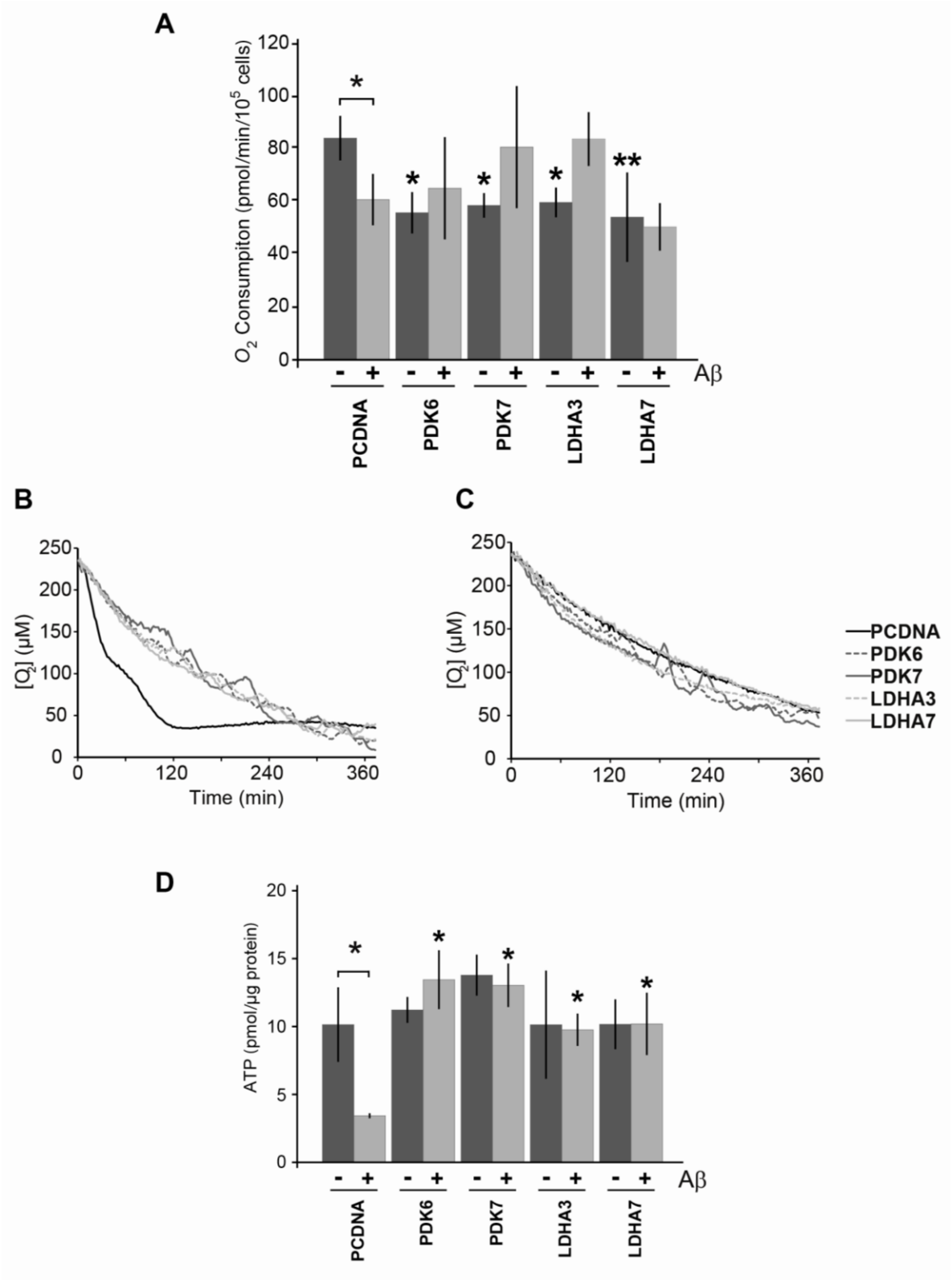

A
pcDNA
PDK1 6
LDHA 3
Control
48 hr Aβ (20 μM)
30 min H2O2 (200 μM)
30 min Staurosporine (200 ng/ml)
20μm
B
Aβ
H2O2
Staurosporine
Mean Fluorescence Intensity
pcDNA
PDK1 6
LDHA 3

3.3.4 Overexpression of LDHA or PDK1 decreases oxygen consumption but maintains ATP levels in the presence of Aβ

The overexpression PDK1 or LDHA is believed to be sufficient to shift metabolism away from mitochondrial respiration towards increased lactate production (Bonnet et al., 2007; Fantin et al., 2006). Thus we sought to determine whether the overexpression of these enzymes in B12 cells would result in a reduction in oxygen (O_2) consumption, a measure of mitochondrial respiration. O_2 consumption was monitored in B12 cells stably overexpressing LDHA or PDK1 using MitoXpress-Xtra-HS, a fluorescent oxygen probe. Under control conditions all PDK1 and LDHA expressing clonal lines exhibited significantly decreased O_2 consumption compared to the pcDNA control (Figure 3.4 A, $P<0.05$). Interestingly, 48 hr $A\beta_{25-35}$ (20 μM) treatment resulted in decreased O_2 consumption in the pcDNA control line but had little effect on the overexpressing clones ($P<0.01$).

Considering that mitochondrial respiration is a far more efficient way of producing energy compared to lactate production, we sought to determine if the observed decreased O_2 consumption affected the levels ATP in cells overexpressing LDHA or PDK1. Intracellular ATP levels were measured in the B12 overexpressing cell lines cultured in the absence or presence of 20 μM Aβ after 48 hours. Under control conditions all B12 cell lines had similar levels of ATP (Figure 3.4 D). However, Aβ treatment resulted in a 50% reduction in ATP levels in B12 control cells (pcDNA) ($P<0.01$). Interestingly all PDK1 and LDHA overexpressing clones maintained significantly higher levels of ATP production in the presence of Aβ compared to control cells under the same conditions ($P<0.05$). These results suggest that cells which are less dependent on mitochondrial respiration are also less sensitive to Aβ-induced alterations in glucose metabolism and are able to maintain ATP levels in the presence of Aβ.

Figure 3.4 Respiration is decreased but ATP levels are maintained in cells overexpressing LDHA and PDK1. (A) Oxygen consumption was monitored in B12 cell lines using the MitoXpress-Xtra HS fluorescent probe. All clonal cell lines overexpressing LDHA and PDK1 displayed significantly lower levels of oxygen consumption under control conditions compared with cells expressing an empty vector (pcDNA) (*P<0.01,**P<0.05). Oxygen consumption significantly decreased in pcDNA control cells following 48 hr treatment with $A\beta_{25-35}$ (20 μM) compared to untreated conditions (*P<0.01). In contrast, cells overexpressing LDHA or PDK1 maintain or increase their oxygen consumption following 48 hr Aβ exposure. **(B)** A representative example of oxygen consumption over time for the indicated B12 cell lines. **(C)** A representative example of oxygen consumption over time following 48 hr $A\beta_{25-35}$ (20 μM) treatment. **(D)** Cells overexpressing LDHA or PDK1 had similar levels of ATP when compared to control cells under normal culture conditions. Cells expressing empty vector had significantly lower levels of ATP following exposure to Aβ (*P<0.05) whereas LDHA and PDK1 overexpressing cells maintained significantly higher ATP levels than the control following treatment with Aβ (*P<0.01). Data represent the mean ± SD of three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.


A
O_2 Consumpiton (pmol/min/10^5 cells)
120
100
80
60
40
20
0
*
*
*
*
**
- + - + - + - + - +
Aβ
PCDNA
PDK6
PDK7
LDHA3
LDHA7
B
[O_2] (μM)
250
200
150
100
50
0
0
120
240
360
Time (min)
C
[O_2] (μM)
250
200
150
100
50
0
0
120
240
360
Time (min)
PCDNA
PDK6
PDK7
LDHA3
LDHA7
D
ATP (pmol/μg protein)
20
15
10
5
0
*
*
*
*
*
- + - + - + - + - +
Aβ
PCDNA
PDK6
PDK7
LDHA3
LDHA7

3.3.5 Attenuated mitochondrial and cellular ROS in LDHA and PDK1 overexpressing B12 cells

Reduced mitochondrial ROS is associated with the Warburg effect and was previously observed in Aβ resistant cells with innately high PDK1 and LDHA activity (Bonnet et al., 2007; Michelakis et al., 2010; Newington et al., 2011). Therefore, we sought to determine if overexpression of either PDK1 or LDHA in Aβ-sensitive cells could reduce mitochondrial ROS when exposed to Aβ or other stressors. We examined mitochondrial ROS in control and LDHA or PDK1 overexpressing cells using Mitotracker Red CM-H2XRos (MTR); a mitochondrial specific dye that fluoresces when oxidized by ROS (Bonnet et al., 2007). Under control conditions, all LDHA and PDK1 overexpressing cells showed significantly less mitochondrial ROS, as measured by mean MTR fluorescence, when compared to the control cells expressing an empty vector (Figure 3.5 A and B, $P<0.001$). Moreover, LDHA and PDK1 overexpressing cell lines exhibited significantly lower levels of mitochondrial ROS when treated with Aβ (20 μM) for 48 hours compared to the vector control cells ($P<0.001$). In contrast, the parental cells expressing vector alone showed a significant increase in mitochondrial ROS following exposure to Aβ ($P<0.001$). Similarly, LDHA and PDK1 overexpressing cells generated significantly less mitochondrial ROS when exposed to either H_2O_2 or staurosporine for 30 minutes compared to vector control cells treated in the same manner ($P<0.001$). Similar results were obtained using MitoSOX; a mitochondrial specific superoxide indicator (Figure 3.5 C and D). Thus overexpression of either LDHA or PDK1 results in a significant reduction in mitochondrial ROS which is maintained following toxin exposure.

Intracellular ROS levels have been previously shown to increase when cells are treated with stressors such as Aβ (Behl et al., 1994; Butterfield et al., 2007). We therefore sought to examine if the cellular levels of ROS were altered in B12 cells overexpressing LDHA or PDK1. We used the live cell fluorescent ROS indicator CM-H_2DCFDA to determine the overall levels of ROS. Under control conditions cells overexpressing either LDHA or PDK1 showed significantly decreased basal levels of ROS when compared to the parental cell expressing the empty vector (Figure 3.6 $P<0.001$). Interestingly, significantly lower levels of ROS were maintained in these cells when exposed to Aβ, H_2O_2 or staurosporine (Figure 3.6, $P<0.001$). In contrast B12 cell expressing empty vector alone exhibited a significant increase

cellular ROS following 48 hr exposure to Aβ ($P<0.01$) or when treated with either H_2O_2 or staurosporine for 24 hr (Figure 3.6, $P<0.001$). Thus cells overexpressing either LDHA or PDK1 exhibit lower levels of both mitochondrial and cellular ROS when compared to control cells which likely contributes to their broad resistance to oxidant promoting neurotoxins.

3.3.6 Decreased LDHA and PDK1 expression in primary nerve cells following exposure to Aβ

Expression of both PDK1 and LDHA in wild type mouse primary cortical nerve cell cultures was examined following Aβ exposure over a 48 hr period (Figure 3.7). A significant decrease in the expression of both these proteins was observed by 48 hrs following exposure to Aβ when compared to untreated cells harvested at the same time points ($P<0.01$). Decreased PDK1 and LDHA expression was unlikely due to loss of cells because minimal cell death was observed up to 48 hrs (data not shown). Therefore it appears Aβ exposure inhibits expression of Warburg effect enzymes in primary neurons.

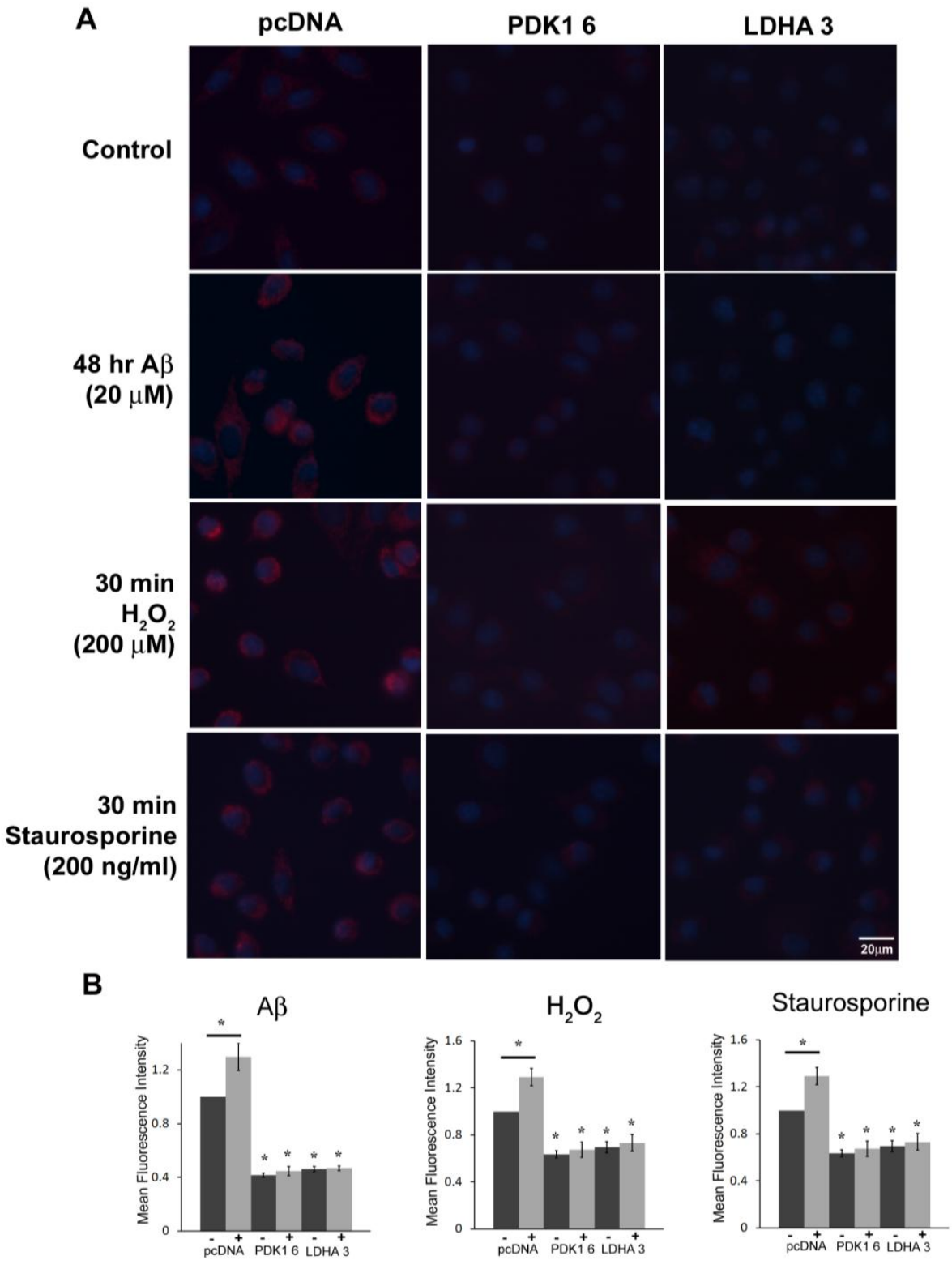
3.3.7 Decreased LDHA and PDK1 expression in APP/PS1 transgenic mice at 12 months

The APPswe/PS1dE9 double transgenic mouse strain (tg-AD) exhibits pronounced amyloid plaque accumulation and memory deficits at 12 months of age compared to age matched controls (Cardoso et al., 2001; Dineley et al., 2002). Based on the neuroprotective properties of PDK1 and LDHA in culture, we sought to determine if the levels of both enzymes were altered in cortical extracts from tg-AD mice relative to non-tg littermate controls. Immunoblot analysis (Figure 3.8) revealed a significant reduction in overall levels of LDHA and PDK1 in tg-AD mice compared to controls ($P<0.001$).

3.3.8 Decreased PDK1 expression in human AD cortical samples

Glycolytic activity and enzymes involved in glycolysis are upregulated in the AD brain (Soucek et al., 2003). We therefore examined the levels of LDHA and PDK1 in extracts from post-mortem control and AD frontal cortex tissue. We observed no significant difference in the overall protein levels of LDHA (data not shown). However, a significant decrease in PDK1 protein expression was detected in AD brain samples when compared to age matched controls (Figure 3.9, $P<0.01$).

Figure 3.5 Decreased mitochondrial ROS in LDHA and PDK 1 overexpressing cells. (A) Mitochondrial ROS production was measured in B12 cell lines following labeling with the red fluorescent dye MitoTracker-ROS Red (MTR). **(B)** Quantification of MTR fluorescent images revealed that B12 clonal cell lines overexpressing PDK1 or LDHA exhibited a significant reduction in mitochondrial ROS (red) compared to the parental cell line expressing the empty vector (pcDNA) under both normal culture conditions and following exposure to $A\beta_{25-35}$ (20 µM), H_2O_2 (200 µM) or staurosporine (200 ng/ml) for the indicated time periods (*P<0.001). Mitochondrial ROS significantly increased in parental cells expressing empty vector (pcDNA) when treated with $A\beta_{25-35}$, H_2O_2 or staurosporine (*P<0.001). **(C)** Mitochondrial derived superoxide was measured by staining cells with the fluorescent dye MitoSOX. All cells overexpressing LDHA or PDK1 displayed significantly decreased mitochondrial derived superoxide (red) when compared to the parental line (pcDNA) under control conditions or following exposure to $A\beta_{25-35}$ (20 µM), H_2O_2 (200 µM) or staurosporine (200 ng/ml) for the indicated time periods (*P<0.001). **(D)** Quantification of MitoSOX fluorescent images revealed that mitochondrial ROS levels significantly increased in the pcDNA control line when treated with Aβ, H_2O_2 and staurosporine (*P<0.001). Nuclei were stained with Hoescht (Blue) and cells were visualized by fluorescence microscopy at 400X magnification for MTR and at 1000X for MitoSOX. MTR and MitoSOX fluorescence were quantified with ImageJ software. Pictures were taken from 3 random fields of view for each treatment. Data represent the mean ± SD of three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.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pcDNA
PDK1 6
LDHA 3
Control
48 hr Aβ (20 μM)
30 min H2O2 (200 μM)
30 min Staurosporine (200 ng/ml)
20μm
B
Aβ
H2O2
Staurosporine
Mean Fluorescence Intensity
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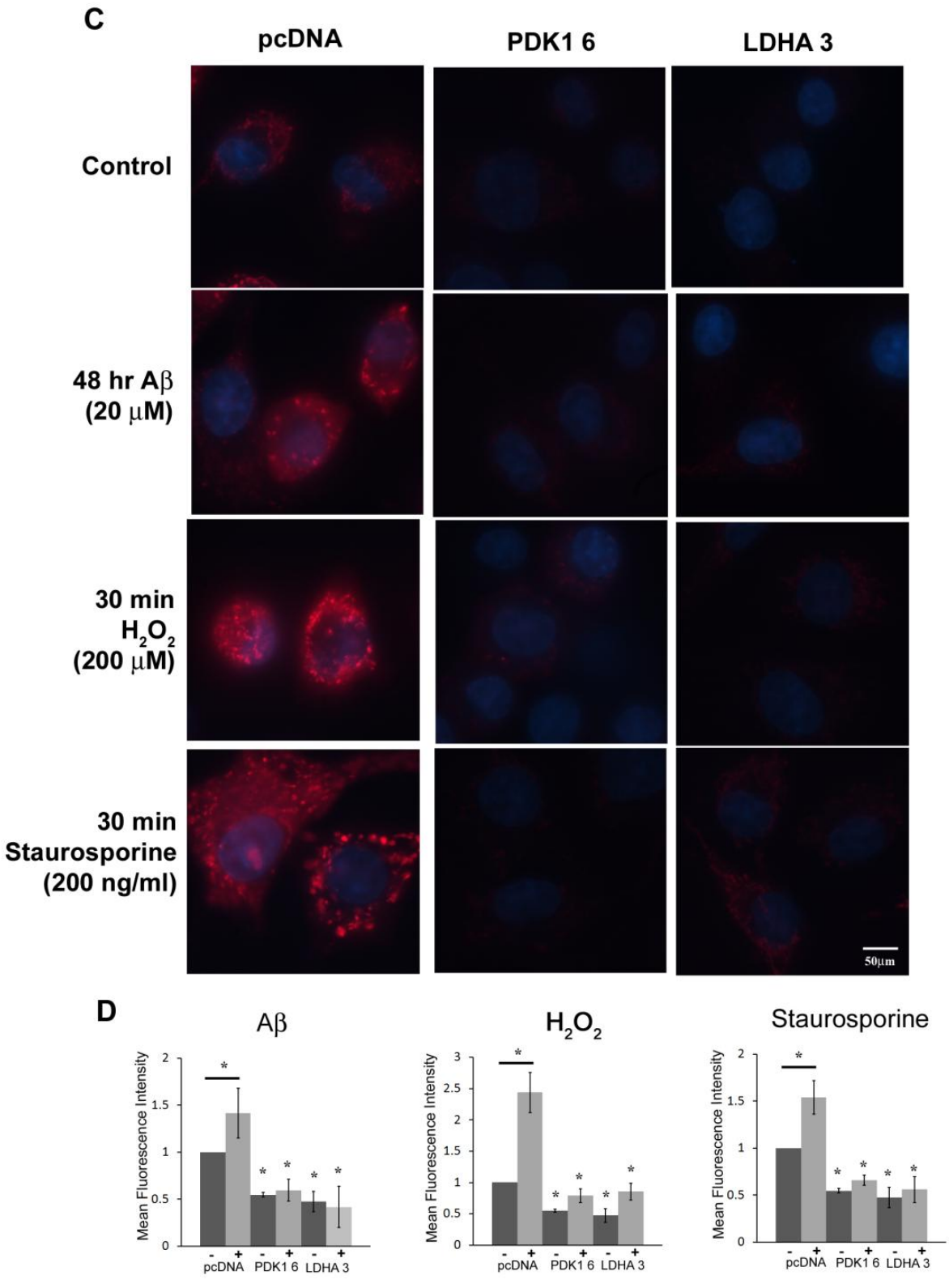
0.4
0.8
1.2
1.6
- + - + - +
pcDNA PDK1 6 LDHA 3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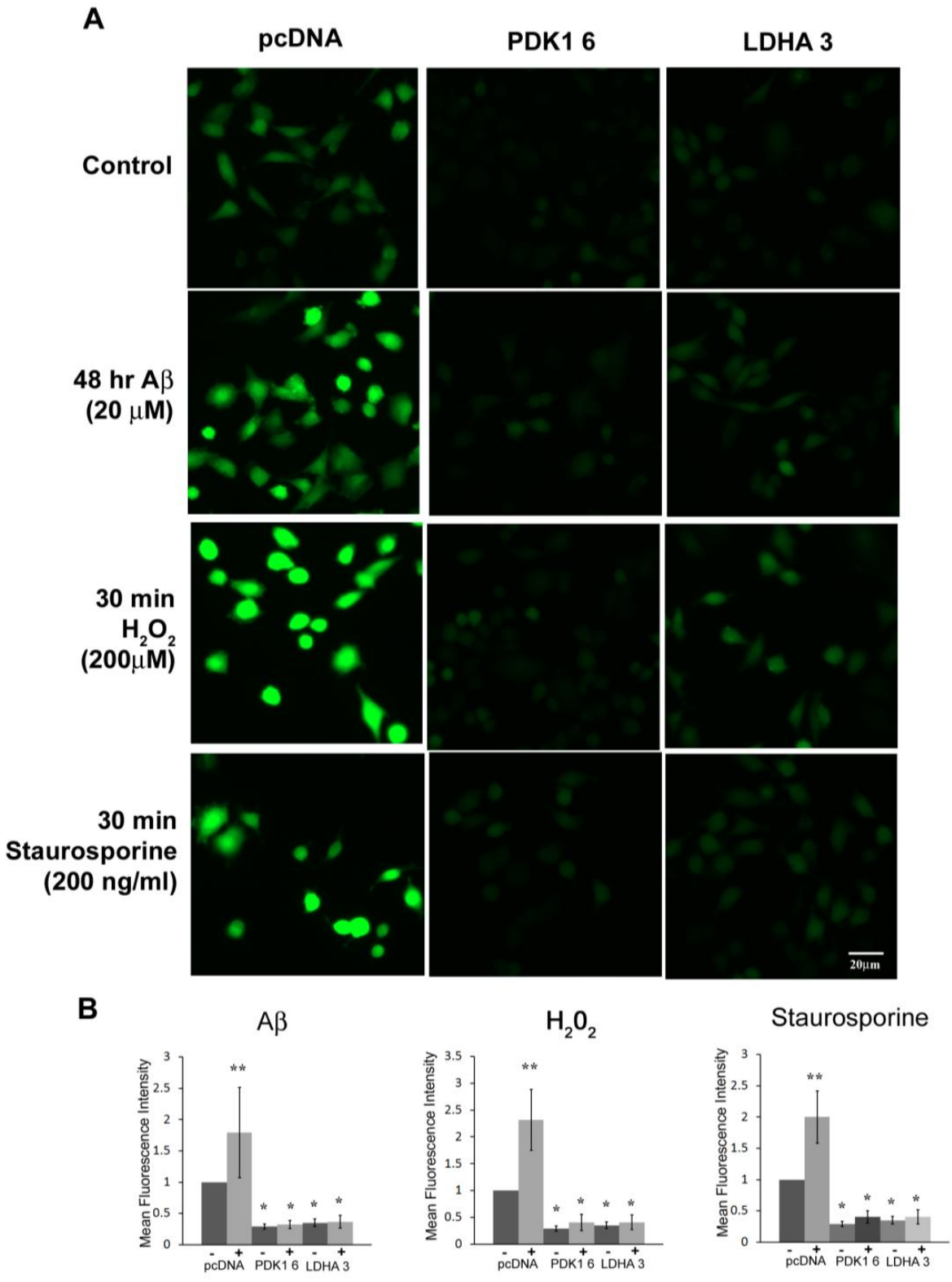
PDK1 6
LDHA 3
Control
48 hr Aβ (20 μM)
30 min H2O2 (200 μM)
30 min Staurosporine (200 ng/ml)
50μm
D
Aβ
H2O2
Staurosporine
Mean Fluorescence Intensity
pcDNA
PDK1 6
LDHA 3

Figure 3.6 Decreased cellular ROS in LDHA and PDK1 overexpressing cells. (A) Cellular ROS was measured in B12 cells by staining with the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). **(B)** Quantification of H_2DCFDA fluorescence revealed B12 clonal cell lines overexpressing PDK1 or LDHA exhibited a significant reduction in cellular ROS (green) compared to the parental cell line expressing the empty vector (pcDNA) under both normal culture conditions and following exposure to $A\beta_{25-35}$ (20 μM), H_20_2 (200 μM) or staurosporine (200 ng/ml) for the indicated time periods (*P<0.001). B12 parental cells (pcDNA) exhibited a significant increase in H_2DCFDA fluorescence when treated with Aβ (P<0.01), H_20_2 or staurosporine (*P<0.001). Cells were stained with H_2DCFDA (2.5 μM) and fluorescent images were taken at 400X magnification and quantified with ImageJ software. Pictures were taken from 3 random fields of view for each treatment. Data represent the mean ± SD of three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.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LDHA 3
Control
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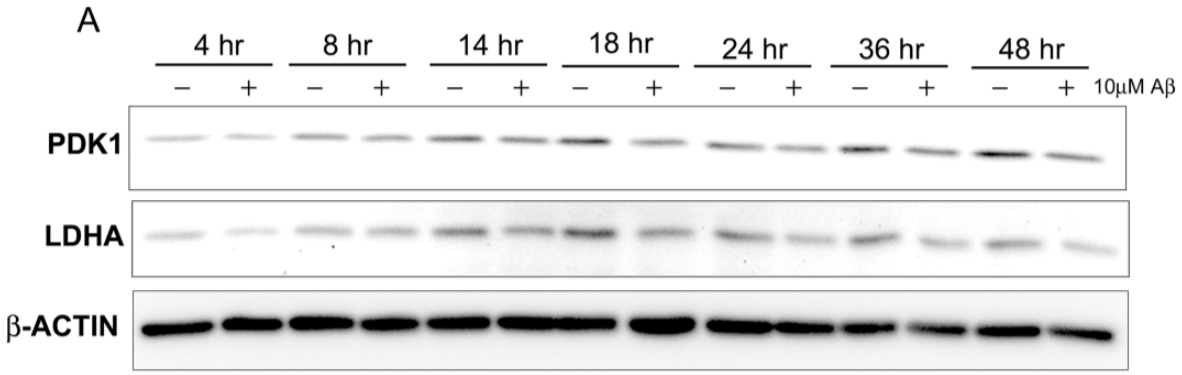
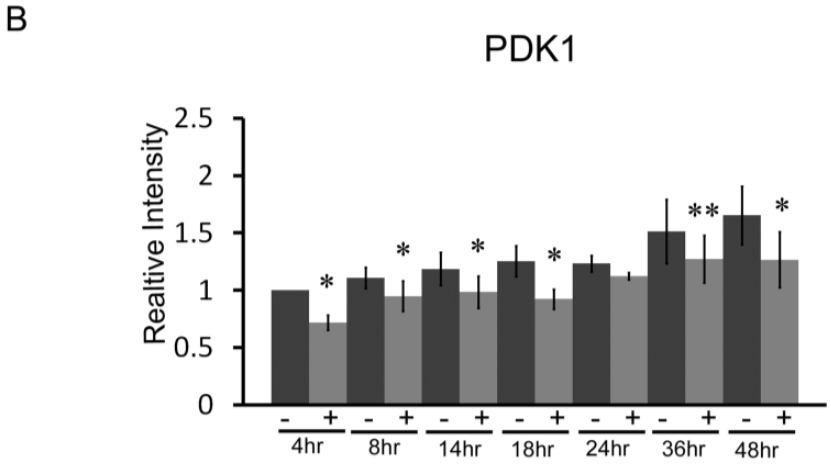
(20 μM)
30 min
H2O2
(200μM)
30 min
Staurosporine
(200 ng/ml)
20μm
B
Aβ
H202
Staurosporine
Mean Fluorescence Intensity
pcDNA
PDK1 6
LDHA 3

Figure 3.7 Aβ exposure inhibits expression of Warburg effect enzymes in mouse primary cortical neurons. (A) Primary cortical nerve cell cultures were exposed to $A\beta_{25-35}$ (10 μM) and harvested at the indicated time points over a 48 hour period. Western blot analysis revealed that both PDK1 and LDHA expression decreased in cortical neurons exposed to Aβ compared to untreated cells. Densitometric analyses of PDK1 **(B)** and LDHA **(C)** band intensities relative to actin are indicated. A significant decrease in both PDK1 and LDHA expression in Aβ treated cells, relative to untreated cells, was observed at a number of time points (*P<0.01, **P<0.05, #P<0.001). Data represent the mean ± SD of three independent experiments. Data were analyzed by a two-way ANOVA followed by a Tukey test.


A
4 hr
8 hr
14 hr
18 hr
24 hr
36 hr
48 hr
– + – + – + – + – + – + – + 10μM Aβ
PDK1
LDHA
β-ACTIN


B
PDK1
Realtive Intensity
2.5
2
1.5
1
0.5
0
- + - + - + - + - + - + - +
4hr
8hr
14hr
18hr
24hr
36hr
48hr

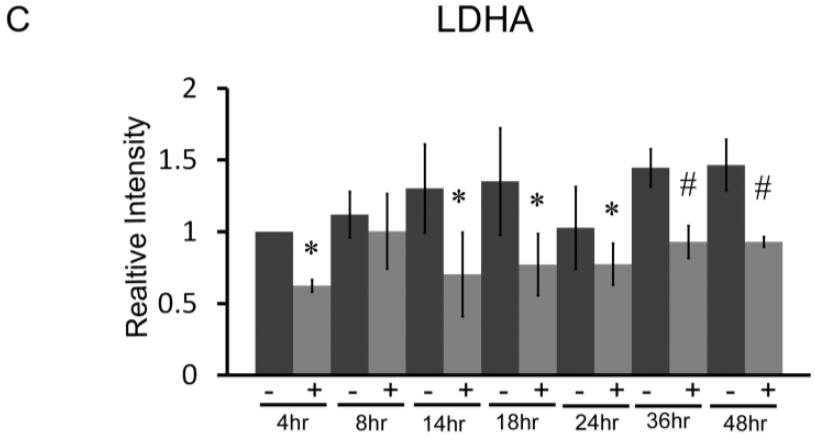

C
LDHA
Realtive Intensity
2
1.5
1
0.5
0
- + - + - + - + - + - + - +
4hr
8hr
14hr
18hr
24hr
36hr
48hr

Figure 3.8 PDK1 and LDHA protein levels are decreased in APP/PS1 transgenic mice. **(A)** Immunoblot analysis of cortical extracts from 12 month-old mice revealed that PDK1 and LDHA protein levels were markedly decreased in APP/PS1 transgenic mice (tg-AD) mice when compared to non-transgenic littermate controls. **(B)** Densitometric analysis revealed significantly decreased PDK1 and LDHA expression in APP/PS1 transgenic mice compared to littermate controls (*$P<0.001$). Data were analyzed by a one-tailed T-Test.

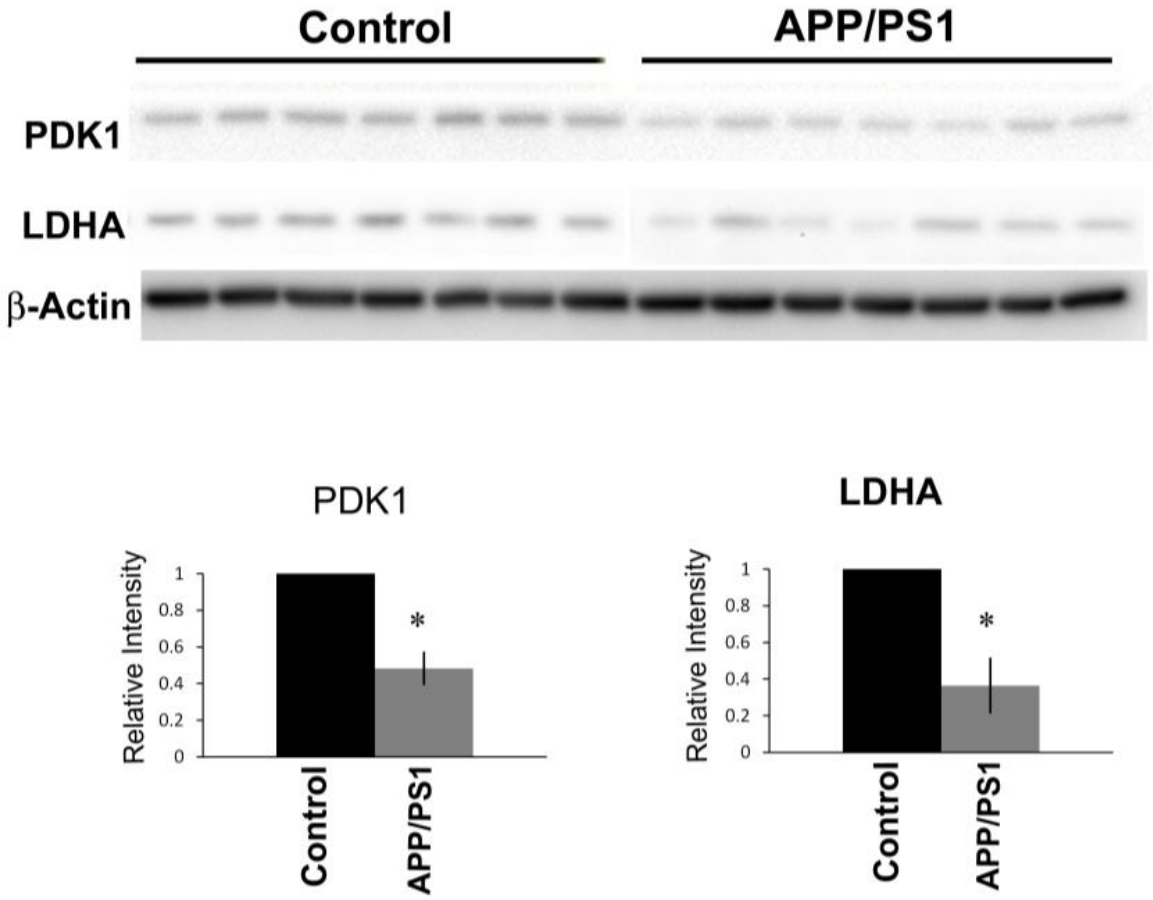
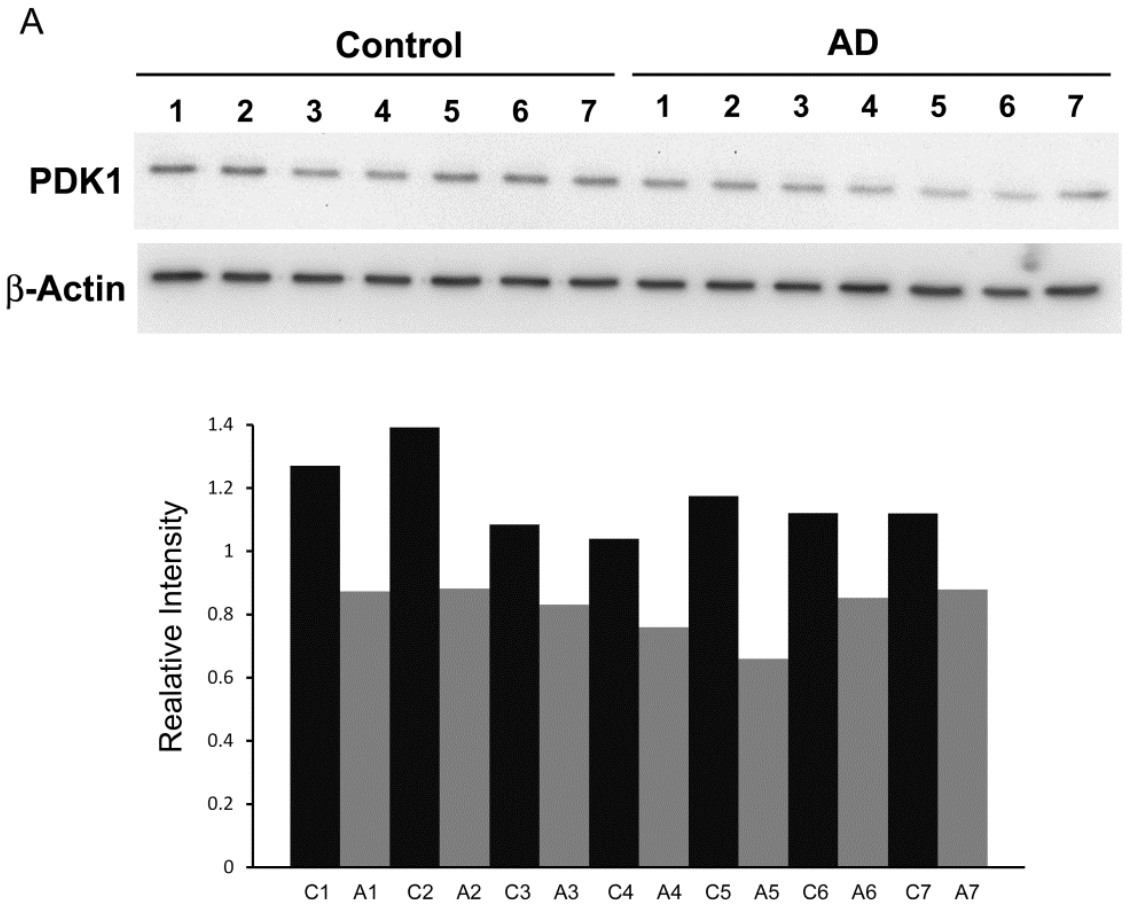

Control
APP/PS1
PDK1
LDHA
β-Actin
PDK1
Relative Intensity
1
0.8
0.6
0.4
0.2
0
*
Control
APP/PS1
LDHA
Relative Intensity
1
0.8
0.6
0.4
0.2
0
*
Control
APP/PS1

Figure 3.9 PDK1 is decreased in cortical extracts from AD patients. (A) Immunoblot analysis of post-mortem human cortical extracts revealed decreased PDK1 levels in human AD brain samples (A1-A7) when compared to the age and sex matched controls (C1-C7), individual densitometric values are shown below. **(B)** Average densitometric values of the above blot revealed a significant decrease in PDK1 expression in AD patients compared to controls (*P<0.01). **(C)** Densitometric analysis revealed there was a significant difference in PDK1 expression in female AD patients and male AD patients when compared to controls (*P<0.01; **P<0.05). Data were analyzed by a one-tailed T-Test or a one-way ANOVA followed by a Tukey test.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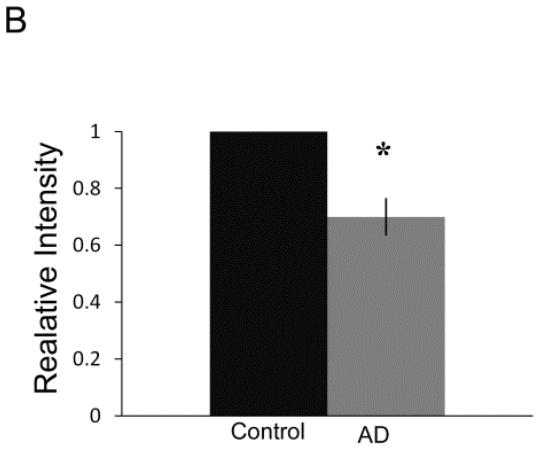
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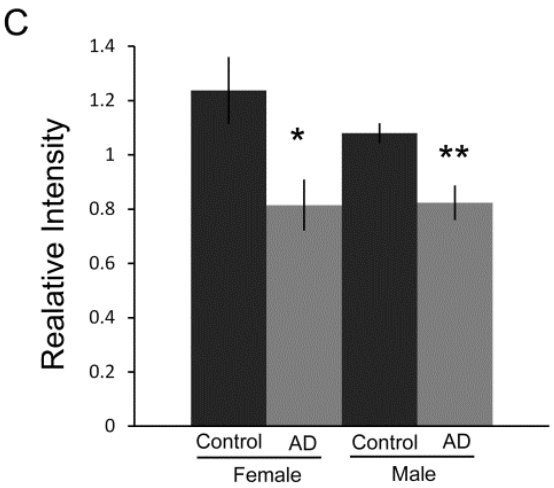
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3.4 Discussion

3.4.1 Mitochondrial membrane potential, ROS production and Aβ sensitivity

Mitochondrial dysfunction is a hallmark of AD, and is thought to be central to Aβ toxicity. ROS can be produced by the leakage of electrons from the mitochondrial ETC which results in the partial reduction of molecular oxygen and the subsequent generation of superoxide radicals ($O_2^{\cdot}$). Chronic increases in ROS production, as seen in AD, can lead to the oxidation and damage of macromolecules such as proteins, lipids and nucleic acids and is strongly associated with the induction of apoptosis. Interestingly, cells depleted of mitochondrial DNA lacking critical subunits of the respiratory chain are not sensitive to the toxic effects of Aβ suggesting that Aβ relies on a functional mitochondrial respiratory chain in order to elicit toxicity (Cardoso et al., 2001). Aβ has been shown to accumulate within the mitochondria of AD patients and transgenic mice (Caspersen et al., 2005; Fernandez-Vizarra et al., 2004; Lustbader et al., 2004; Manczak et al., 2006). The direct binding Aβ to the mitochondrial protein alcohol dehydrogenase (ABAD) promotes leakage of electrons, mitochondrial dysfunction, increased ROS production and ultimately cell death (Lustbader et al., 2004). However, overexpression of the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD) in tg-AD mice improved resistance to Aβ and attenuated the AD phenotype, suggesting that mitochondrial toxicity is central to Aβ induced cell death (Dumont et al., 2009). Here we show that overexpression of LDHA or PDK1 in nerve cells results in both decreased $\Delta\psi_m$ and O_2 consumption which is associated with reduced mitochondrial ROS production and attenuated cell death following exposure to various toxins including Aβ. Interestingly, the reduction in mitochondrial respiration does not appear to negatively affect ATP levels in cells overexpressing LDHA or PDK1. These cells are likely able to maintain high levels of cellular ATP through increased flux through the glycolytic pathway, similar to Aβ resistant cells (Soucek et al., 2003). We propose that decreased ETC activity and ROS production associated with LDHA and PDK1 expression is central to conferring protection against Aβ and other toxins. This is further supported by the observation that knockdown of either LDHA or PDK1 in B12 cells further potentiated sensitivity to Aβ and other neurotoxins. Moreover, genetic silencing of either LDHA or

PDK1 in Aβ-resistant cells resulted in re-sensitization to Aβ toxicity, suggesting that these enzymes play an important role in protecting cells against Aβ toxicity (Newington et al., 2011).

3.4.2 Protective role of LDHA and PDK1

Elevated LDHA and PDK1 expression may confer resistance to Aβ, H_2O_2 and staurosporine by a variety of mechanisms. Although Aβ is known to trigger an increase in H_2O_2 production resulting in free radical damage and cell death, H_2O_2 accumulation is not observed in Aβ resistant cells (Sagara et al., 1996). Moreover, cells selected for resistance against Aβ toxicity are also resistant to exogenously applied H_2O_2 and neurotoxins known to induce oxidative stress, suggesting that Aβ and H_2O_2 promote cell death by a similar mechanism (Dargusch and Schubert, 2002; Sagara et al., 1996). Additionally, the observation that pre-treatment of CNS primary cultures or PC12 and B12 cells with catalase, an antioxidant that detoxifies H_2O_2, results in protection against Aβ-induced cell death further suggests that H_2O_2 mediates Aβ toxicity (Behl et al., 1994). H_2O_2 treatment has been shown to induce transient $\Delta\psi_m$ hyperpolarization and a subsequent delayed burst of endogenous ROS in mouse primary neurons and human neuroblastoma cells. Furthermore, chemical inhibition of mitochondrial hyperpolarization was shown to protect neuronal cells from oxidative stress-induced cell death (Qin et al., 2011). In this study, PDK1 or LDHA overexpression also prevented the transient increase in $\Delta\psi_m$ following H_2O_2 exposure.

Interestingly, overexpression of LDHA or PDK1 also resulted in increased resistance to staurosporine, an apoptosis inducing agent that was initially believed to promote toxicity in a ROS-independent manner (Ruegg and Burgess, 1989). Staurosporine has been well characterized as a potent inducer of apoptosis through inhibition of protein kinases (Herbert et al., 1990; Ruegg and Burgess, 1989). However, several studies have shown that staurosporine-induced apoptosis in neurons is partly dependent on mitochondrial derived ROS (Kruman et al., 1998; Morais Cardoso et al., 2002; Pong et al., 2001). Given that oxidative stress is tightly associated with Aβ and H_2O_2 induced cell death, overexpression of LDHA or PDK1 is likely to protect cells by reducing mitochondrial ETC activity and associated ROS production.

In this study we observed decreased $\Delta\psi_m$ and O_2 consumption in cells overexpressing LDHA or PDK1 compared to control cells which was maintained following toxin exposure. In contrast, control cells underwent a sharp increase in $\Delta\psi_m$ followed by rapid depolarization and cell death in the presence of all toxins. A positive $\Delta\psi_m$ is created by the ETC which transfers protons (H^+) into the intermembrane space. The resulting electrochemical gradient is subsequently used to synthesize ATP. Thus if ETC activity is low, in the case of reduced mitochondrial respiration, then $\Delta\psi_m$ would also be low. Loss of $\Delta\psi_m$ altogether mediates the release of proapoptotic factors through the mitochondrial transition pore. In cells overexpressing PDK1, the observed decrease in $\Delta\psi_m$ and O_2 consumption is likely reflective of a decrease in ETC activity as a result of PDH inhibition (McFate et al., 2008). PDK1-mediated inhibition of PDH results in decreased entry of pyruvate into the TCA cycle and a subsequent decrease in the production of the electron donors NADH and $FADH_2$ necessary for ETC activity. In LDHA overexpressing cells, LDHA competes with the mitochondrial NADH/NAD^+ shuttle systems to regenerate NAD^+ (Golshani-Hebroni and Bessman, 1997). Therefore, the overexpression of LDHA or PDK1 likely limits the availability of both pyruvate and NADH in the mitochondria thereby decreasing respiration and $\Delta\psi_m$ (Fantin et al., 2006).

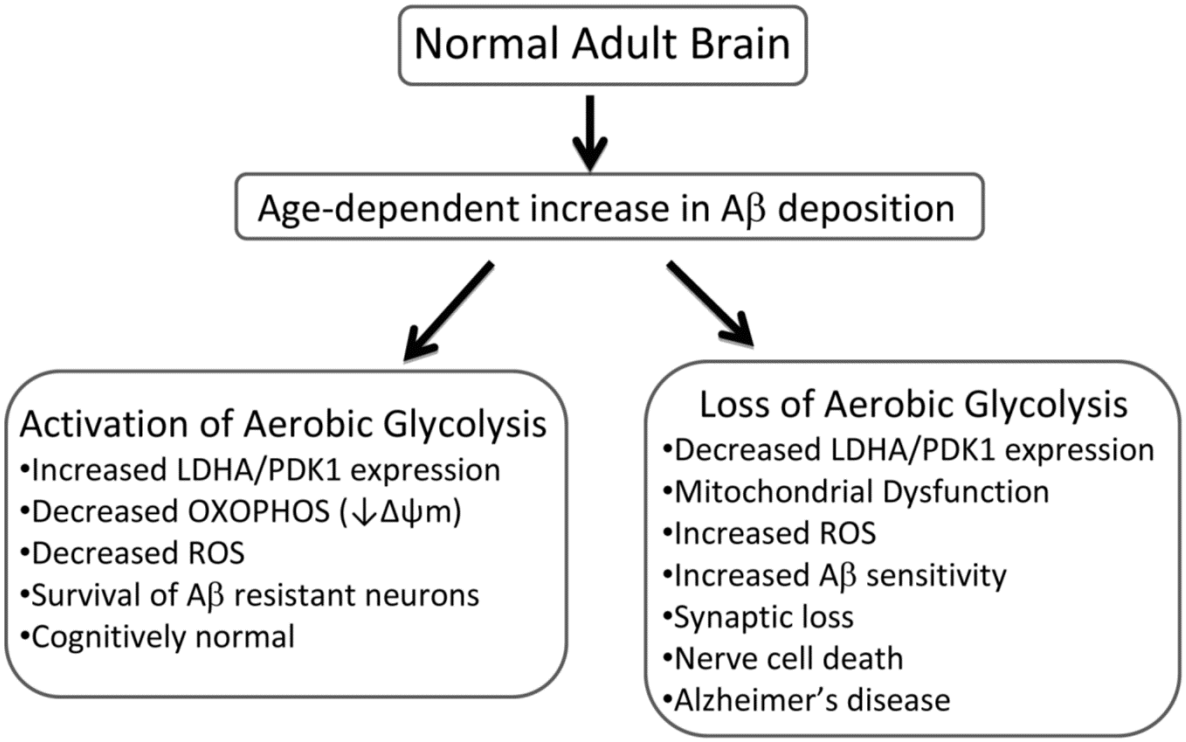
Previous studies revealed that more ROS is generated at higher mitochondrial membrane potentials, with dramatic increases in ROS being produced when mitochondrial membranes reach potentials of 140 mV or more (Korshunov et al., 1997). Conversely a small decrease (10mV) in $\Delta\psi_m$ significantly attenuates ROS production (up to 70%) via complex I of the ETC, suggesting that moderate attenuation of membrane potential can result in significant changes in the oxidative potential of the cell (Miwa and Brand, 2003). Interestingly we observed that cells overexpressing LDHA or PDK1 exhibited decreased mitochondrial membrane potentials with or without exposure to toxins, which likely contributed to their resistance through the associated decrease in mitochondrial ROS. Moreover, the observed hyperpolarization prior to depolarization and cell death in H_2O_2 exposed control cells was likely associated with a burst in ROS production. Collectively, our findings suggest that cells exhibiting a lower $\Delta\psi_m$ and decreased mitochondrial ROS production under basal conditions may have a unique advantage over cells exhibiting higher $\Delta\psi_m$ when faced with a toxic stressor such as Aβ.

3.4.3 Glucose metabolism in the AD brain

Recent [^{18}F]-FDG PET imaging studies in tg-AD ($APP_{SweLon}/PS1_{M146L}$) mice revealed a dynamic picture of glucose utilization within the brain (Poisnel et al., 2011). In 3, 6 and 12 month old tg-AD mice, it was found that there was an age-dependent increase in glucose uptake in the cortex, hippocampus and striatum; areas associated with high plaque accumulation (Poisnel et al., 2011). However, this study did not discern what proportion of glucose was processed by aerobic glycolysis versus mitochondrial respiration. Here we looked at 12 month old APP/PS1 mice and observed a decrease in both LDHA and PDK1 expression in the frontal cortex when compared to age matched controls. Thus, it is possible that at early stages of pathogenesis in tg-AD mice, nerve cells exploit the Warburg effect and increase glucose uptake to protect against Aβ toxicity. However, if LDHA and PDK1 expression decreases in older mice then more glycoltyic flux would be processed through the mitochondria leading to increased ROS production, elevated apoptosis and ultimately cognitive impairment. A more intensive longitudinal study of the Warburg effect in AD mice is necessary to offer more insight into the metabolic state of affected neurons.

Interestingly, a study measuring the regional distribution of aerobic glycolysis in the human brain revealed that areas most susceptible to amyloid toxicity exhibit high aerobic glycolysis (Vlassenko et al., 2010). In the developing nervous system, aerobic glycolysis is believed to account for 90% of glucose consumed (Powers et al., 1998). During childhood this fraction accounts for 35% of glucose utilization and finally drops to 10-12% in the adult brain (Vaishnavi et al., 2010). PET studies of cognitively normal individuals have shown an age-associated decrease in FDG uptake in regions of the brain frequently affected in AD, although these studies did not determine what proportion of glucose was processed by aerobic glycolysis versus oxidative phosphorylation (Cunnane et al., 2011). However, a recent neuroimaging study revealed a strong correlation between the spatial distribution of Aβ deposition and aerobic glycolysis in both cognitively normal individuals and AD patients (Vlassenko et al., 2010). Thus, aerobic glycolysis may be elevated in areas of the brain most susceptible to insult as a pre-emptive protective mechanism or in response to Aβ accumulation during aging (Figure 3.10). Loss of this protective mechanism may render certain areas of the brain susceptible to Aβ-induced neurotoxicity.

Figure 3.10 Proposed model describing the relationship between aerobic glycolysis and AD. In the normal young adult brain aerobic glycolysis (the Warburg effect) is elevated in regions known to be susceptible to Aβ deposition. Aerobic glycolysis is maintained, in part, by increased lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) expression. LDHA converts pyruvate to lactate with the concomitant regeneration of nicotinamide adenine dinucleotide (NAD^+) which is necessary to sustain glycolysis. PDK1 phosphorylates and inhibits pyruvate dehydrogenase resulting in decreased oxidative phosphorylation (OXOPHOS), mitochondrial membrane potential ($\Delta\psi_m$) and reactive oxygen species (ROS) production. The age-associated increase in Aβ deposition and concomitant decrease in aerobic glycolysis may render certain populations of neurons vulnerable to Aβ toxicity in the elderly. In cognitively normal individuals, gradual Aβ deposition triggers increased expression of LDHA and PDK1 resulting in elevated aerobic glycolysis, lowered $\Delta\psi_m$ and diminished ROS. As a result of increased aerobic glycolysis nerve cells become resistant to Aβ toxicity. In individuals who develop Alzheimer's disease the inability to either activate or maintain aerobic glycolysis renders nerve cells more susceptible to Aβ-mediated mitochondrial dysfunction and increased ROS production leading to synaptic loss and ultimately widespread nerve cell death.


Normal Adult Brain
Age-dependent increase in Aβ deposition
Activation of Aerobic Glycolysis
•Increased LDHA/PDK1 expression
•Decreased OXOPHOS (↓Δψm)
•Decreased ROS
•Survival of Aβ resistant neurons
•Cognitively normal
Loss of Aerobic Glycolysis
•Decreased LDHA/PDK1 expression
•Mitochondrial Dysfunction
•Increased ROS
•Increased Aβ sensitivity
•Synaptic loss
•Nerve cell death
•Alzheimer's disease

This study examined AD post mortem tissue and observed a decrease in PDK1 expression in the frontal cortex. Decreased PDK1 expression, may contribute to loss of aerobic glycolysis in brain areas with Aβ deposition which, in turn, may trigger an increase in mitochondrial respiration, ROS production and nerve cell death. A recent study showed that inhibition of respiratory complexes in a cell culture model leads to an increase in both ROS and Aβ production, further potentiating toxicity (Leuner et al., 2012). Similarly, AD mice treated with a complex I inhibitor also exhibit an increase in Aβ levels (Leuner et al., 2012). In both models, ROS-dependent accumulation of Aβ was reduced by treatment with antioxidants. Therefore mitochondrial ROS production appears to be tightly associated with Aβ production *in vitro* and *in vivo* (Leuner et al., 2012). The decreased ROS production afforded by the Warburg effect may not only be intrinsically neuroprotective but may actually attenuate Aβ production in AD. In future studies it will be important to perform immunohistochemical analysis on brain tissues to determine the spatial relationship between PDK1 expression and Aβ deposition.

Although we propose that glucose is the main fuel source for Aβ-resistant neurons, alternative substrates, such as lactate, may be also used to meet the energy demands of brain cells. The astrocyte-neuron lactate shuttle theory postulates that glucose is predominately taken up by glia and metabolized glycolytically to lactate which is subsequently secreted and taken up by neurons (Aubert et al., 2005). In neurons, lactate is converted to pyruvate which enters the TCA cycle and drives oxidative phosphorylation. Preliminary studies have shown that addition of lactate to the culture media of either PC12 or B12 cells failed to alleviate Aβ sensitivity (unpublished observations). Furthermore, exogenous lactate failed to rescue the elevated sensitivity of Aβ-resistant PC12 and B12 clonal nerve cell lines to glucose deprivation (unpublished data). These findings suggest that exogenous lactate itself is unlikely to fuel the neuroprotective response associated with aerobic glycolysis.

3.5 Conclusions

PDK1 and LDHA appear to be central mediators of Aβ-resistance by altering mitochondrial activity which results in a decrease in both $\Delta\psi_m$ and mitochondrial ROS production. In addition, overexpression of either of these enzymes confers resistance to other stressors

including H_2O_2 and staurosporine. Overexpression of these key Warburg effect enzymes decreases mitochondrial respiration while maintaining ATP production which appears to contribute to the protective role of these proteins. Decreased expression of LDHA and PDK1 in mouse primary cortical neurons may also contribute to their sensitivity to Aβ. Likewise, decreased expression of both LDHA and PDK1 in 12 month old tg-AD (APP/PS1) mice suggests that loss of this neuroprotective mechanism may potentiate cognitive impairment. Loss of PDK1-mediated aerobic glycolysis in AD patients may hasten both memory loss and nerve cell death and could be used as a biomarker of disease progression. Moreover, identification of compounds that mimic or augment PDK1 activity may have clinical relevance for the treatment of AD.

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Chapter 4

4 Novel drugs protect against Aβ and glutamate toxicity in nerve cells and sustain the expression of Warburg effect enzymes

4.1 Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disease that results in progressive cognitive decline. AD is the most common form of dementia, with an estimated 500,000 Canadians currently suffering from the disease or related dementias (Honjo et al., 2012). With our aging population, the number of people living with AD is expected to more than double over the next 25 years making the treatment of AD a concern of global proportions (Honjo et al., 2012). At the cellular level AD is characterized by the accumulation of extracellular deposits of amyloid beta (Aβ) peptide, intracellular tangles composed of hyperphosphorylated tau protein and wide spread nerve cell loss (Selkoe, 2004). Despite over a century of research there is still no cure for the disease and treatment strategies remain limited. Moreover, except in rare familial cases of AD, the exact cause of sporadic forms of the disease remains unknown posing additional difficulty when searching for viable treatment options. To date, most drug development strategies in AD research have been based on a pathology-centered approach which focuses on the identification of a molecular target related to disease pathology and the development of a novel drug targeted at a single candidate molecule (Pangalos et al., 2007). However, AD is not likely to be caused by a single molecular target and drug development strategies that focus on multiple targets in a favored pathway or single targets in multiple pathways are likely to show more efficacy (Pangalos et al., 2007). Unfortunately, this drug development strategy has, to date, yielded few novel drugs for the treatment of AD (Pangalos et al., 2007). Recently, a research group at the Salk Institute in California took an innovative approach to drug discovery which led to the development of new neurotrophic drugs that hold promise for the treatment of AD (Chen et al., 2011; Liu et al., 2008).

Curcumin is a curcuminoid; a polyphenolic compound found in the ancient curry spice turmeric and has long been used in Indian traditional medicine to treat various ailments

(Chainani-Wu, 2003; Grynkiewicz and Slifirski, 2012). Interestingly, curcumin can protect PC12 cells, a rat pheochromocytoma derived cell line, against Aβ induced toxicity and reduce oxidative stress and damage in these cells (Kim et al., 2001). Curcumin has also been shown to inhibit Aβ aggregation into fibrils and destabilize existing fibrils *in vitro*, likely contributing to its protective effects (Jiang et al., 2012). Additionally, curcumin inhibits key players involved in the activation of inflammation which is known to play a role in the pathogenesis of AD (Ammon et al., 1993; Pan et al., 2000; Xu et al., 1997). Collectively, *in vitro* evidence suggests that curcumin may act as a neuroprotective compound as a result of its anti-oxidant, anti-inflammatory and anti-aggregate activity (Cole et al., 2007; Ringman et al., 2005). More recent animal studies indicate that curcumin may have therapeutic potential for treating diverse neurodegenerative diseases including AD (Cole et al., 2007). More specifically, curcumin has been shown to reduce oxidative damage, inflammation and plaque burden by up to 50% in an AD transgenic mouse model carrying the familial linked APP Swedish mutation (APPswe) (Lim et al., 2001; Yang et al., 2005). Similar results were obtained when 22 month old Sprague-Dawley rats were fed curcumin while receiving central nervous system (CNS) infusions of Aβ (Frautschy et al., 2001). Moreover, dietary curcumin prevented Aβ induced memory deficits in the same rodent model (Frautschy et al., 2001).

Although curcumin has good therapeutic potential due to its safety, extensive history of use and inexpensive cost, it failed to protect nerve cells against neurotrophic factor withdraw which is closely associated with AD pathology (Elliott and Ginzburg, 2006; Liu et al., 2008; Wainer, 1989). Additionally, the EC_{50}, half maximal effective concentration, at which curcumin is effective in cell culture assays is high (10-100 μM) (Liu et al., 2008). These shortcomings of curcumin prompted researchers to synthesize a hybrid molecule of curcumin, CNB-001, in an attempt to improve its effectiveness as a neuroprotective compound. CNB-001 was selected from a variety of curcumin derivatives for its ability to protect against loss of neurotrophic factor support, oxidative stress, glucose starvation and Aβ toxicity (Liu et al., 2008). Moreover, CNB-001 enhanced long term potentiation and memory in rats (Maher et al., 2010). However, the researchers sought to further improve the potency and stability of CNB-001 by developing a derivative called J147 (Chen et al., 2011). J147 is a potent (EC_{50} almost 100X lower than CNB-001), orally active compound that not only to prevented memory loss in AD transgenic mice, but also enhanced memory in

cognitively normal mice (Chen et al., 2011). Furthermore, J147 was shown to reduce soluble Aβ levels in the hippocampus (Chen et al., 2011). At the cellular level, J147 appears to reduce the levels of pro-oxidant enzymes and heat shock proteins associated with stress and increase the expression of proteins involved in synaptic function (Chen et al, 2011). However, the exact molecular mechanisms by which J147 elicits neuroprotection is poorly defined (Chen et al., 2011). Interestingly, mice fed J147 showed increased expression of brain derived neurotrophic factor (BDNF)(Chen et al., 2011), a member of the nerve growth factor family which plays a central role in the support and survival of neurons and maintenance of synaptic plasticity in the CNS, particularly in affected brain regions in AD patients (Huang and Reichardt, 2001). BDNF plays an essential role in long term potentiation and memory (Bekinschtein et al., 2008; Figurov et al., 1996) and the progression of AD is associated with the progressive loss in BDNF (Laske et al., 2006). Therefore, the ability of J147 to enhance memory in normal mice and prevent memory loss in AD mice may, in part, be due to the increased expression of BDNF and the subsequent activation of downstream signalling pathways (Chen et al., 2011). In light of the fact that CNB-001 and J147 are derivatives of curcumin, they are likely to have pleiotrophic effects by binding to various molecular targets. Further investigation into these molecular targets is essential for understanding the mechanisms by which both CNB-001 and J147 are neuroprotective.

Interestingly, we have recently shown that activation of aerobic glycolysis or the Warburg effect in nerve cells facilitates resistance to Aβ toxicity (Newington et al., 2011). Aerobic glycolysis results in increased lactate production in the presence of oxygen and is a common feature of cancer cells (Bonnet et al., 2007; Warburg, 1956). Though aerobic glycolysis is a much less efficient means of producing ATP, it appears to offer a survival advantage for cancer cells, in addition to nerve cells exposed to a variety of neurotoxins including Aβ (Fantin et al., 2006; McFate et al., 2008; Michelakis et al., 2010; Michelakis et al., 2008; Newington et al., 2011; Newington et al., 2012). Aerobic glycolysis in Aβ resistant nerve cells is mediated by lactate dehydrogenase A (LDHA), the enzyme responsible for the conversion of pyruvate to lactate, and pyruvate dehydrogenase kinase 1 (PDK1), an enzyme which phosphorylates and inhibits pyruvate dehydrogenase (PDH) thereby actively repressing mitochondrial metabolism (Newington et al., 2011). PDH converts pyruvate to acetyl-CoA, a metabolite which drives the citric acid cycle thereby increasing the production

of the electron donors NADH and $FADH_2$. NADH and $FADH_2$ subsequently donate their electrons to the electron transport chain (ETC). Thus inhibiting the production of acetyl CoA at the level of PDH reduces the overall rate of respiration and production of reactive oxygen species (ROS), a toxic species which is closely associated with Aβ induced cell death. Interestingly, the overexpression of either LDHA or PDK1 in sensitive cells confers resistance to Aβ and other neurotoxins and is accompanied by decreased mitochondrial ROS (Newington et al., 2012). Moreover loss of PDK1 is observed in the brains of AD transgenic (APPswe/PS1dE9) mice and post mortem brain tissue from human patients with AD (Newington et al., 2012). Drugs which augment aerobic glycolysis through upregulation of PDK1 and LDHA may protect against nerve cell loss and associated cognitive decline in patients with AD. However, there currently are no known drugs which effectively regulate the expression of these enzymes. Thus we sought to investigate whether CNB-001 or J147 could increase the expression of these enzymes which could contribute to their neuroprotective effects. In this study we show that CNB-001 or J147 protect HT22 cells and primary nerve cells against glutamate toxicity and Aβ induced cell death respectively. Treatment with either of these drugs was associated with maintenance of LDHA and PDK1 expression following exposure to neurotoxins. Moreover, PDK1 expression, which decreases in 12 month old AD transgenic mice, is rescued when mice were fed CNB-001. These results indicate a possible role for LDHA and PDK1 in the protective effects elicited by CNB-001 and J147.

4.2 Methods

4.2.1 Materials

Cell culture reagents including: Dulbecco's modified Eagles medium (DMEM), penicillin/streptomycin (P/S), DMEM without phenol red and Dulbecco's phosphate buffered saline (DPBS) were purchased from Biowhittaker (Walkersville, MD, USA) (Carlsbad, CA, USA). Dialyzed fetal bovine serum (FBS) was obtained from PAA Laboratories Inc. (Etobicoke, ON, Canada). TrypLE Express, Neurobasal Medium, N_2 Supplement, B_{27} Supplement, Glutamax-1 (100X) and Hanks Balanced Salts Solution were obtained from Invitrogen (Carlsbad, CA, USA). Protease inhibitor cocktail (100X) was purchased from Cell Signaling (Danvers, MA, USA). Amyloid beta (Aβ) peptide (25-35) was purchased

from California peptide research (San Francisco, CA, USA). Poly-L-Ornithine, L-glutamic acid, Propidium Iodide (PI), dihydrochloride, dimethyl formamide, dimethyl sulfoxide (DMSO), 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were all purchased from Sigma (St. Louis, MO, USA). DNase 1 and Trypsin Inhibitor were purchased from Roche (Laval, Quebec, Canada). Cell Trace^{TM} Calcein Green, AM (Molecular probes) was purchased from Invitrogen (Carlsbad, CA, USA).

4-((1E)-2-(5-(4-hydroxy-3-methoxystyryl-)-1-phenyl-1H-pyrazoyl-3-yl)vinyl)-2-methoxy-phenol) (CNB-001) and 1-(2,4-dimethylphenyl)-2-(3-methoxybenzylidene) hydrazine chloride (J147) were provided by Dr. David Schubert (The Salk Institute, La Jolla, CA) and synthesized as described (Chen et al., 2011). For cell culture experiments CNB-001 and J147 were dissolved in DMSO at appropriate stock concentrations and stored at -80^{o}C. Stock solutions were diluted with water to reach working concentrations. Aβ was prepared at a concentration of 1 mM in sterile deionized water and left to rock overnight at room temperature to promote fibril formation. The following day, Aβ was aliquoted and stored at -20^{o}C.

4.2.2 Cell Culture

HT22 cells are an immortalized mouse hippocampal cholinergic line subcloned from the HT4 line which has been selected for its sensitivity to glutamate toxicity (Morimoto and Koshland, 1990). HT22 cells were obtained from Dr. David Schubert (The Salk Institute, La Jolla, CA) and cultured in DMEM supplemented with 10% FBS and 1% P/S as previously described (Chen et al., 2011; Dargusch and Schubert, 2002). Primary cortical neurons were prepared from day 17 mouse embryos as previously described (Ribeiro et al., 2005). Primary cultures were maintained in Neurobasal Media supplemented with 2 mM glutamine, 50 units/mL P/S and N2/B27 supplements and incubated for 48 hr (Newington et al., 2012; Ribeiro et al., 2005). All cells were grown in a humidified incubator at 37^{o}C and 5% CO_2.

For cell viability experiments HT22 cells were seeded at 2x10^{3} cells per well in 96 well dishes and incubated for 24 hr. The following day CNB-001 was added at a final concentration of 0.5 μM or 1 μM to the appropriate test wells and cells were incubated at 37^{o}C. J147 was added a concentration of 50 nM or 100 nM to the appropriate test wells and cells were incubated at 37^{o}C. Two hours following the administration of either drug, 2 mM

glutamate was added to the appropriate test wells and cells were incubated overnight. The following day, a MTT assay was performed as previously described (Newington et al., 2012). The absorption values at 595 nm were measured on a microplate reader (BioRad Model 3550). The results are presented as the percentage of the control untreated cells.

4.2.3 Immunoblot analysis

HT22 and mouse primary cortical cells from treated and untreated subconfluent cultures were washed twice in cold DPBS and harvested in an SDS extraction buffer (50 mM Tris pH 7.5, 2% SDS, 1 mM PMSF and 1X protease inhibitor cocktail). Protein extracts were quantified by a Lowry assay and equal [protein] were loaded and resolved by 12% SDS PAGE and electroblotted onto PVDF membrane (Bio-Rad Richmond, CA, USA). Membranes were probed with the following antibodies: polyclonal anti-LDHA (1:1000; Cell Signaling, Danvers, MA, USA), polyclonal anti-PDK1 (1:1000; Stressgen, San Diego, CA, USA) and a monoclonal anti-β actin (1:2000; Cell Signaling, Danvers, MA, USA) followed by incubation with an appropriate horseradish peroxidase (HRP) -conjugated secondary antibody (Bio-Rad, Richmond, CA, USA). The blots were developed using Pierce ECL western blotting substrate (Thermo Scientific, Rockford, IL, USA) and visualized with a Bio-Rad Molecular Imager (ChemiDoc XRS, Bio-Rad, Richmond, CA, USA). Densitometric analysis was performed using Image J software. Band densities were standardized against β-actin, and the ratio of LDHA or PDK1-specific bands relative to the β-actin band was determined.

4.2.4 Fluorescence Microscopy

Cell viability in primary nerve cell cultures was determined by staining with propidium iodide (PI) and calcein green. Primary cells were seeded at $1.6x10^6$ cells per 35 mm dish and incubated for 48 hr at 37^oC. Following an initial incubation period the media was changed and either CNB-001 (0.5 μM or 1 μM) or J147 (10 nM, 50 nM or 100 nM) was added to the appropriate test dishes. Following a 2 hr incubation cells were treated with $A\beta_{25–35}$ (10 μM) and were put back in the incubator for 48 hr. At this time the media was aspirated and replaced with DPBS containing PI (0.1 μg/ml) and calcein green (1 μM) and incubated for 30 min at 37^oC. Following incubation, cells were washed with 1X DPBS and placed back in fresh DPBS. Cells were visualized by fluorescence microscopy (Zeiss-AxioObserver, 40X

objective) and pictures were taken using a Q Imaging (Retiga 1300 monochrome 10-bit) camera and Q Capture software. Pictures were taken of three random fields of view.

4.2.5 AD mouse tissue

The APP/PS1 transgenic mouse line both expresses chimeric mouse/human APP (Mo/HuAPP695swe) and mutant human presenilin 1 (PS1-dE9) in the CNS. Both or these mutations are associated with the familial forms of the disease. At three months of age male transgenic mice and their non-transgenic littermates were fed a high fat diet with or without CNB-001 (500 ppm). Food consumption and body weights were monitored weekly, to ensure there were no differences between groups. The mice were sacrificed at 12 months of age. Cortical tissue from these mice fed CNB-001 was generously provided by Dr. David Schubert (The Salk Institute, La Jolla, CA). Brain tissue was extracted and frozen as previously described (Cumming et al., 2007; Soucek et al., 2003). Cortical tissue was processed for western blot analysis as previously described (Newington et al., 2012). Protein extracts were analyzed by immunoblot analysis as described above.

4.2.6 Statistical Analysis

Data are presented as means ± SD resulting from a least three independent experiments. Data were analyzed statistically using a one-way ANOVA followed by a Tukey test or by a T-test (VassarStats). Results were considered statistically significant at $P<0.05$.

4.3 Results

4.3.1 CNB-001 and J147 protect cells exposed to glutamate and preserves PDK1 and LDHA expression

CNB-001 protects against glutamate induced excitotoxicity in mouse primary cortical neurons (Liu et al., 2008). Similarly, J147 protects rat cortical neurons (E18 cells) from glutamate toxicity (Chen et al., 2011). We sought to determine if CNB-001 or J147 could also protect a hippocampal nerve cell line, HT22, from glutamate induced toxicity. Treatment of HT22 cells with glutamate (2 mM) for 24 hr resulted in a 55% reduction in cell viability (Figure 4.1, $P<0.01$). Interestingly, incubation of HT22 cells with J147 or CNB-001 prior to glutamate exposure resulted in a significant increase in cell viability (Figure 4.1, $P<0.01$). Immunoblot analysis of HT22 cells treated with 2 mM glutamate for 24 hr revealed

a significant loss of both PDK1 and LDHA expression (Figure 4.2, $P<0.01$). Densitometric analysis revealed a 36% reduction in PDK1 expression and a 58% reduction in LDHA expression. Expression of these enzymes was maintained by pre-treatment with either J147 or CNB-001 at varying concentrations. More specifically, treatment of HT22 cells with 100 nM J147 or 1 μM CNB-001 prior to glutamate exposure significantly increased the expression of both PDK1 and LDHA to levels similar or higher than the control ($P<0.01$). Therefore J147 and CNB-001 appear to be protective against glutamate toxicity in HT22 cells. Moreover, treatment with either of these drugs preserves PDK1 and LDHA expression following glutamate exposure, which may contribute, in part, to their protective effects.

Figure 4.1 CNB-001 and J147 protect HT22 cells from glutamate toxicity. Cell viability of HT22 cells was significantly decreased following 24 hr exposure to 2 mM glutamate (*P<0.01). Cell viability significantly increased compared to glutamate treatment alone when cells were pretreated for 2 hr with CNB-001 (001) at 0.5 μM or 1 μM prior to glutamate treatment (*P<0.01). Similarly pre-treatment with J147 (50 nM or 100 nM) prior to glutamate exposure significantly increased cell viability when compared to glutamate treatment alone (*P<0.01). There was a significant increase in cell viability with increased concentrations of CNB-001 or J147 (*P<0.01). Cell viability was measured by MTT assay. Data represent the mean ± SD of three independent experiments. Data were analyzed by a one-way ANOVA followed by a Tukey test.

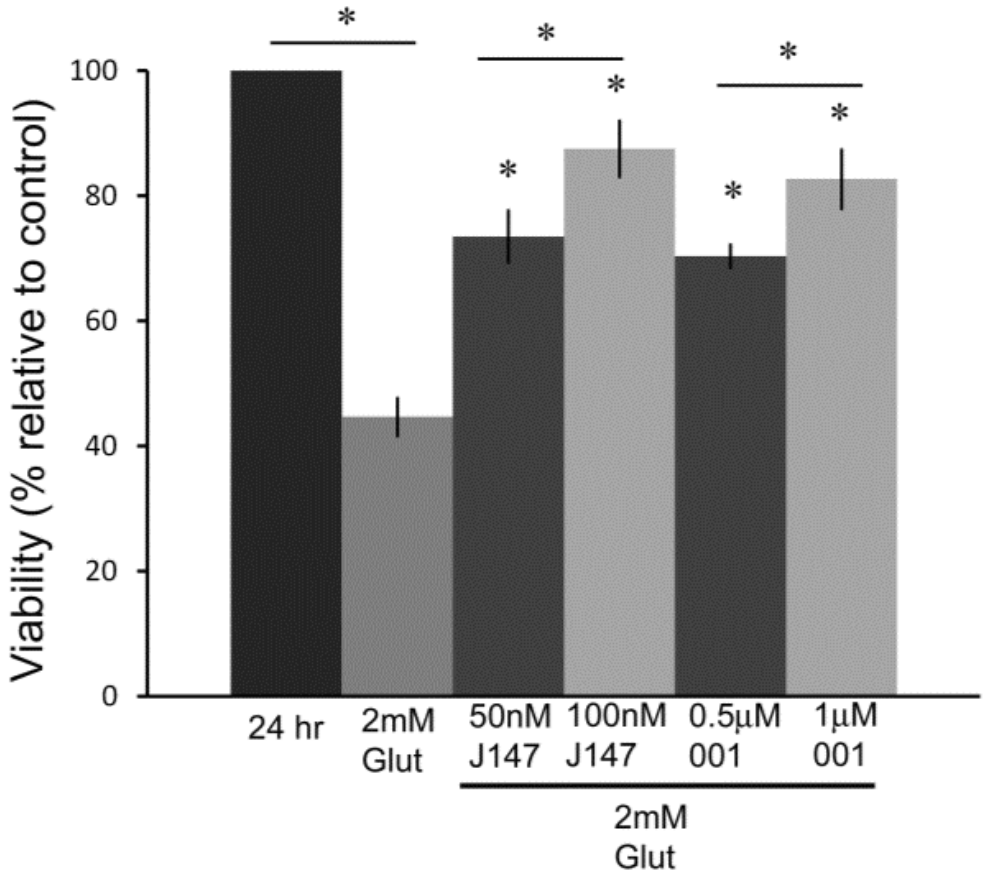
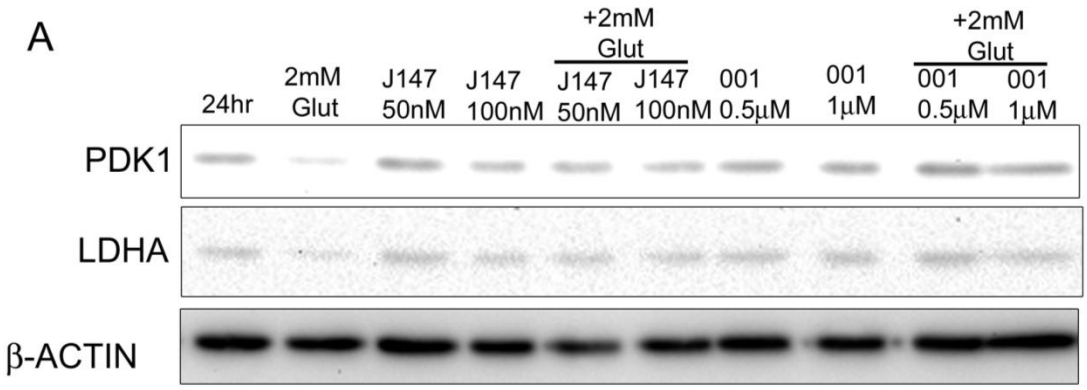
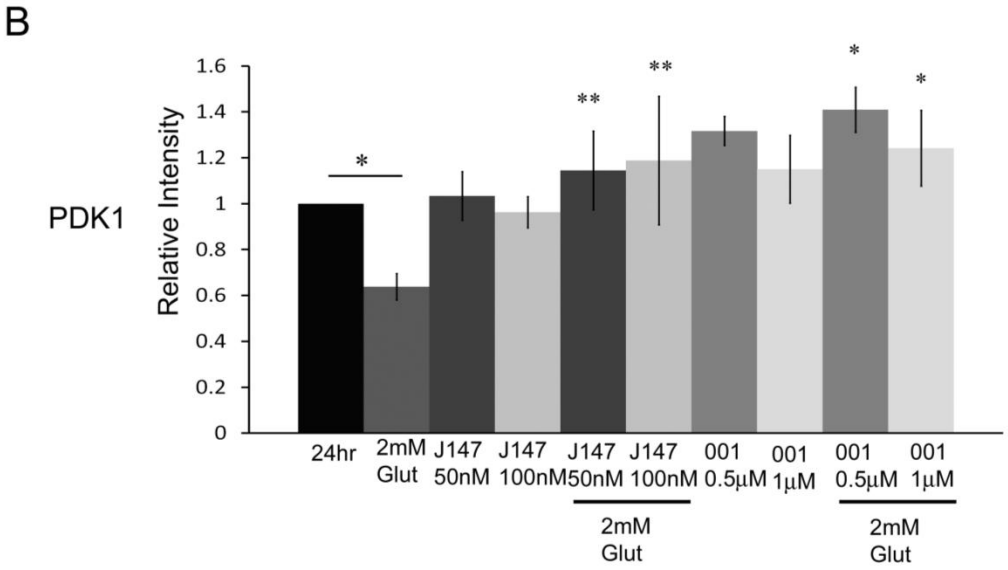
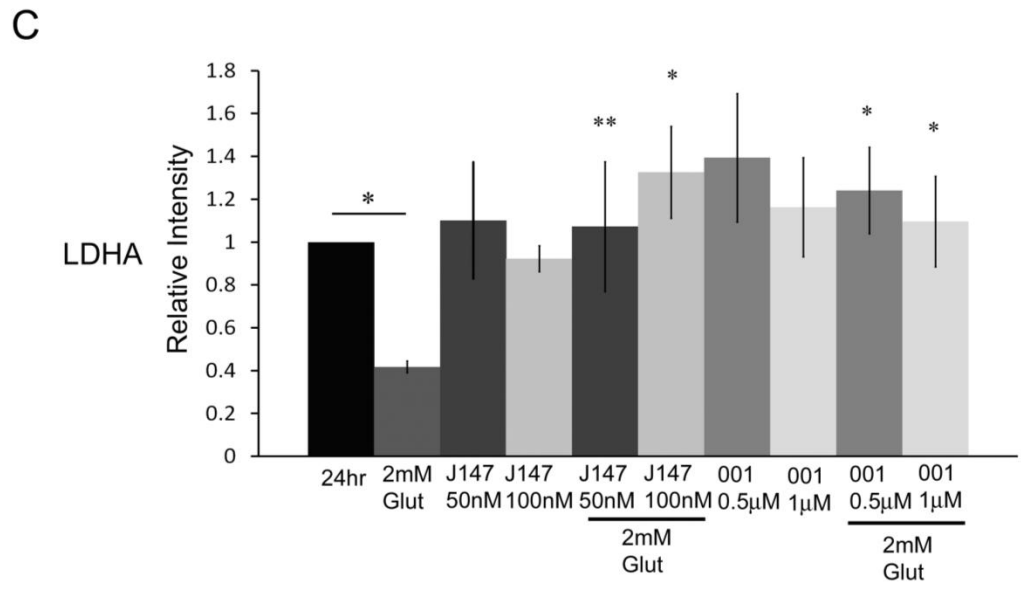

Viability (% relative to control)
100
80
60
40
20
0
*
*
*
*
*
*
*
24 hr
2mM Glut
50nM J147
100nM J147
0.5µM 001
1µM 001
2mM Glut

Figure 4.2 CNB-001 and J147 maintain PDK1 and LDHA expression in HT22 cells exposed to glutamate. (A) Western blot analysis of HT22 cells treated with glutamate (2 mM) for 24 hr revealed a significant decrease in the expression of both PDK1 and LDHA (*P<0.01). In contrast, pre-treatment for 2 hr with J147 (50 nM or 100 nM) prior to glutamate exposure (2 mM) significantly increased the expression of PDK1 (**P<0.05) and LDHA (50 nM, **P<0.05; 100 nM, *P<0.01) when compared to cells treated with glutamate alone. Similarly, cells exposed to CNB-001 (001; 0.5 μM or 1 μM) prior to treatment with glutamate exhibited significantly higher levels of PDK1 and LDHA (*P<0.01) compared to glutamate treatment alone. Densitometric analysis of PDK1 and LDHA band intensities relative to actin are found below (**B** and **C** respectively). Relative intensity was calculated by comparing the LDHA/actin or PDK1/actin ratio of the treated cells to the same ratio of the control cell line (24 hr). Data represent the mean ± SD of three independent experiments. Data were analyzed by a one-way ANOVA followed by a Tukey test.


A
+2mM Glut
+2mM Glut
24hr
2mM Glut
J147 50nM
J147 100nM
J147 50nM
J147 100nM
001 0.5μM
001 1μM
001 0.5μM
001 1μM
PDK1
LDHA
β-ACTIN

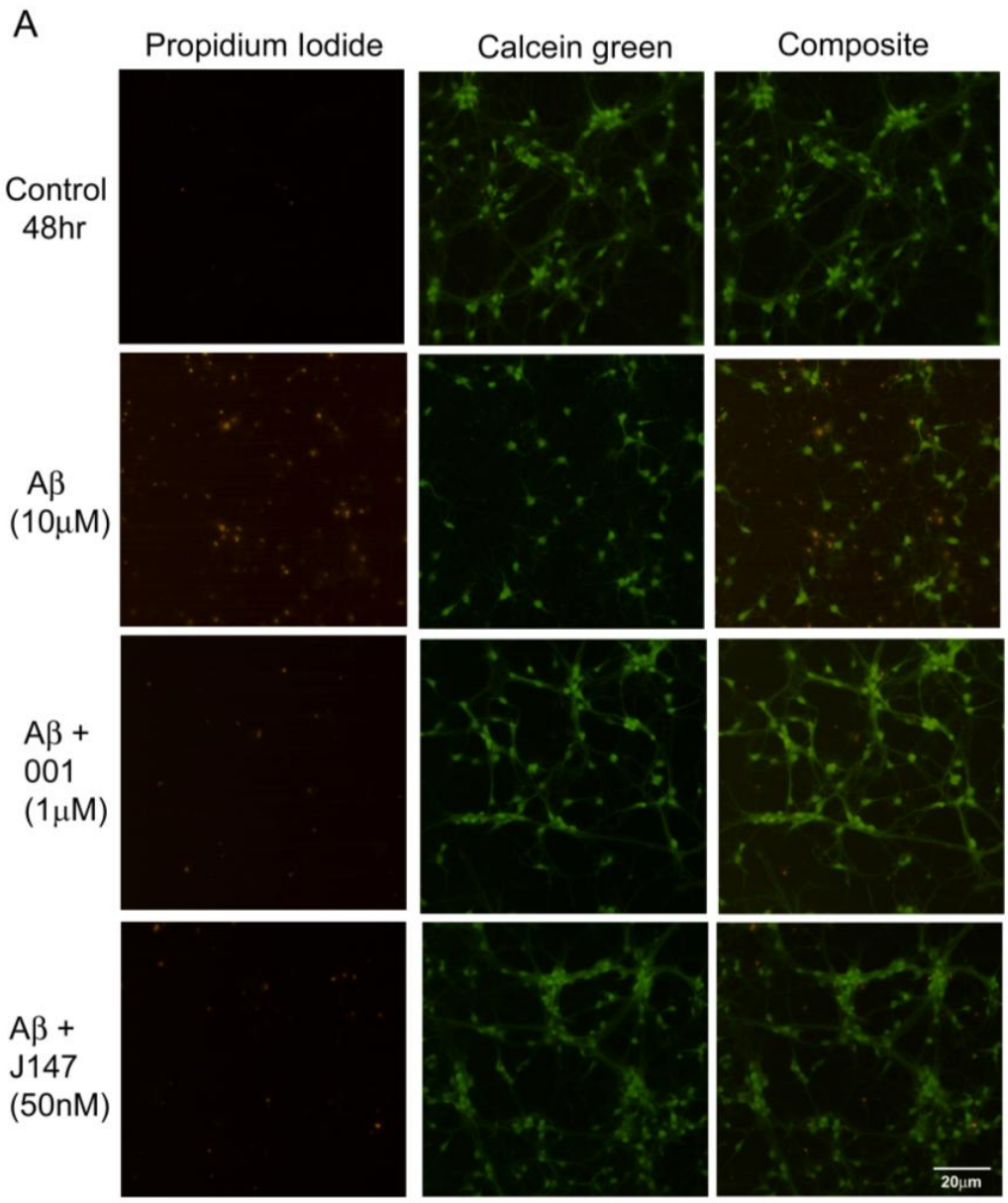

B
PDK1
Relative Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
*
*
24hr
2mM Glut
J147 50nM
J147 100nM
J147 50nM
J147 100nM
001 0.5μM
001 1μM
001 0.5μM
001 1μM
2mM Glut
2mM Glut


C
LDHA
Relative Intensity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
*
*
*
24hr
2mM Glut
J147 50nM
J147 100nM
J147 50nM
J147 100nM
001 0.5μM
001 1μM
001 0.5μM
001 1μM
2mM Glut
2mM Glut

4.3.2 CNB-001 or J147 protects against Aβ toxicity and maintains PDK1 and LDHA expression in primary nerve cells

Treatment of primary rat hippocampal neurons with either CNB-001 or J147 prevents Aβ toxicity (Chen et al., 2011; Liu et al., 2008). We examined whether CNB-001 or J147 could elicit similar protective effects in mouse primary cortical neurons. Cell viability was examined using the fluorescent dyes Calcein Green and PI, and visualized by fluorescent microscopy. Cells treated with $A\beta_{25-35}$ (10 μM) for 48 hr showed a significant increase in PI positive cells, indicative of non viable or dead cells (Figure 4.3 A and B, $P<0.01$). When cells were incubated with either CNB-001 (1 μM) or J147 (50 nM) prior to treatment with Aβ, there were significantly less dead cells (PI positive cells) and more live cells (Calcein Green, positive) suggesting that both compounds are protective in mouse primary cortical nerve cells (Figure 4.4 A and B, $P<0.01$). Immunoblot analysis of primary cortical neurons treated with $A\beta_{25-35}$ (10 μM) for 48 hr revealed a significant decrease in PDK1 and LDHA when compared to untreated cells (Figure 4.4, $P<0.05$). Interestingly, exposure to CNB-001 prior to treatment with Aβ prevented decreases in PDK1 and LDHA expression. Treatment with J147 prior to exposure to Aβ also maintained expression of PDK1 and LDHA at levels similar to control cells (Figure 4.5). Densitometric analysis revealed that PDK1 expression was maintained at a significantly higher level following pre-treatment with 100nM J147 and that LDHA expression was maintained to a significantly higher degree following pre-treatment with 50 nM J147 prior to Aβ exposure ($P<0.01$ and $P<0.05$ respectively). Thus, exposure to CNB-001 or J147 maintains expression of PDK1 and LDHA following treatment with Aβ; events which may contribute to the neuroprotective properties of these drugs.

Figure 4.3 CNB-001 and J147 protect primary cortical neurons from Aβ toxicity. (A) Cell viability was measured in mouse primary cortical neurons by staining cells with Calcein green which labels viable cells and propidium iodide (PI) which labels dead cells. Cell viability was reduced when cells are incubated with $A\beta_{25-35}$ (10 μM) for 48 hr. An increase in cell survival was observed when cells were treated with CNB-001 (001; 1μM) or J147 (50 nM) for 2 hr prior to treatment with Aβ. **(B)** There was a significant increase in PI positive cells treated with $A\beta_{25-35}$ (10 μM) for 48 hr when compared to control cells (*P<0.01). In contrast there was a significant decrease in PI positive cells when cells were pre-treated with 001 or J147 prior to exposure to Aβ, when compared to cells treated with Aβ alone (∗P<0.01). Pictures were taken from 3 random fields of view for each treatment. Data represent the mean ± SD of three independent experiments. Data were analyzed by a one-way ANOVA followed by a Tukey test.


A
Propidium Iodide
Calcein green
Composite
Control
48hr
Aβ
(10μM)
Aβ +
001
(1μM)
Aβ +
J147
(50nM)
20μm

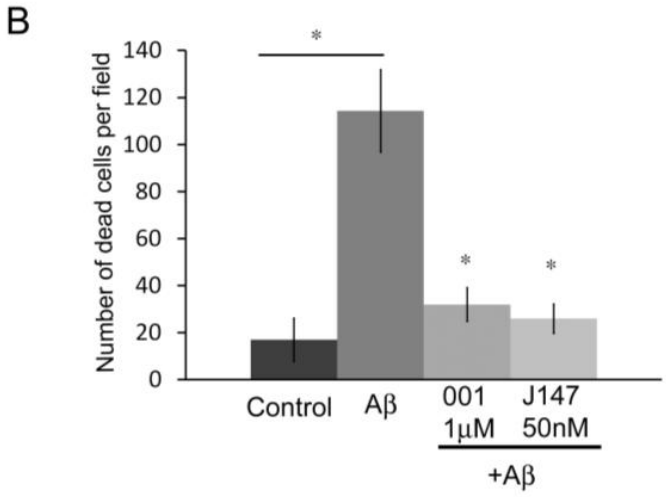
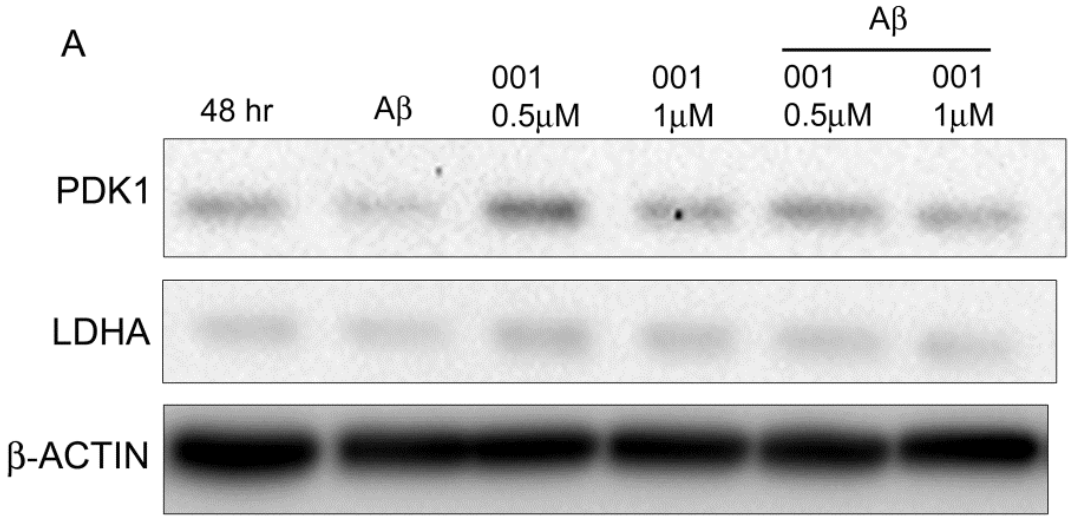
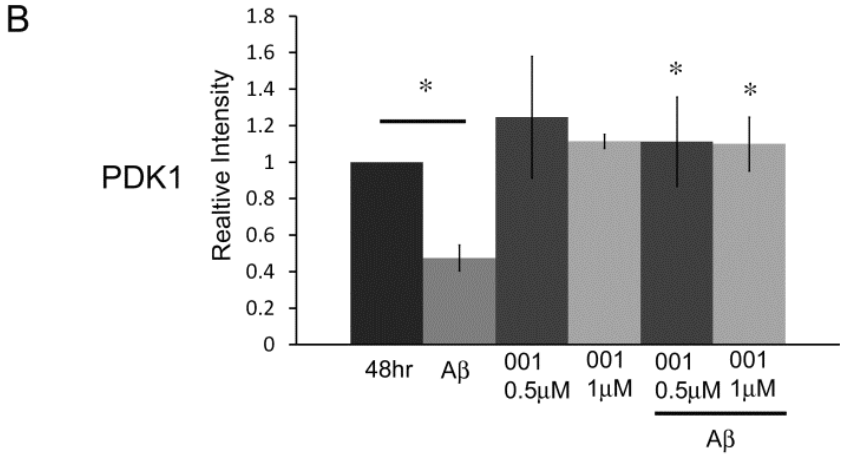

B
Number of dead cells per field
140
120
100
80
60
40
20
0
*
*
*
Control
Aβ
001
1μM
J147
50nM
+Aβ

Figure 4.4 CNB-001 increases PDK1 and LDHA in primary cortical neurons treated with Aβ. **(A)** Immunoblot analysis of primary cortical neurons revealed a significant decrease in both PDK1 and LDHA when cells were treated with $A\beta_{25-35}$ (10 μM) for 48 hr when compared to control cells (*P<0.01). **(B)** Densitometric analysis revealed incubating cells with CNB-001 (001; 0.5 μM or 1 μM) for 2 hr prior to treatment with Aβ significantly increased the expression of PDK1 at 48 hr compared to cells treated with Aβ alone (*P<0.01). **(C)** A significant increase in the expression of LDHA is also observed when cells are pre-treated with 001 compared to cells treated only with Aβ (**P<0.05). Relative intensity was calculated by comparing the LDHA/actin or PDK1/actin ratio of treated cells to the same ratio of the control cell line (48 hr). Data represent the mean ± SD of three independent experiments. Data were analyzed by a one-way ANOVA followed by a Tukey test.


A
Aβ
48 hr
Aβ
001 0.5μM
001 1μM
001 0.5μM
001 1μM
PDK1
LDHA
β-ACTIN


B
PDK1
Realtive Intensity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
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*
*
48hr
Aβ
001 0.5μM
001 1μM
001 0.5μM
001 1μM
Aβ

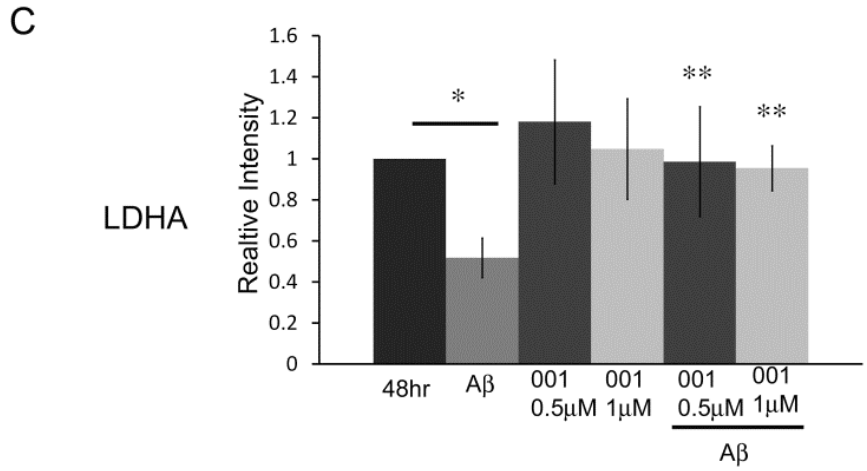
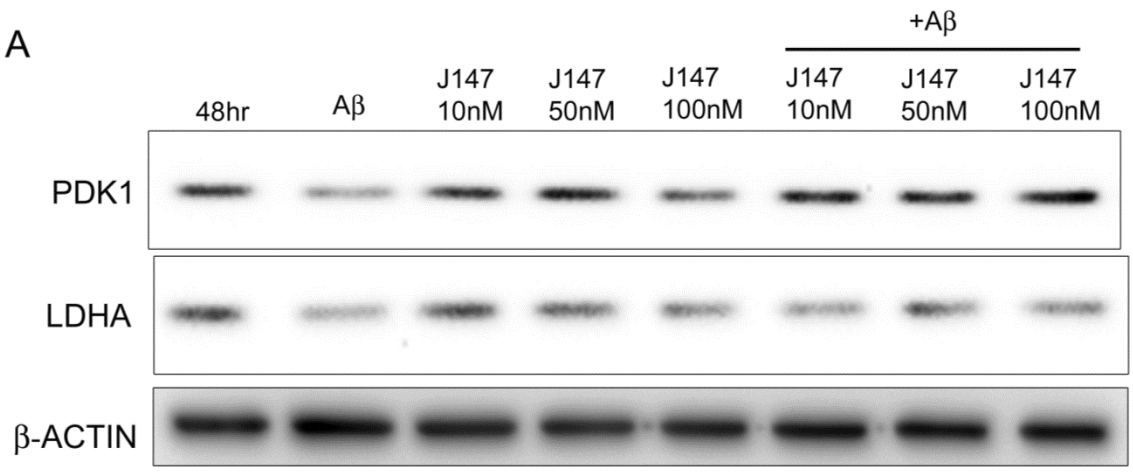
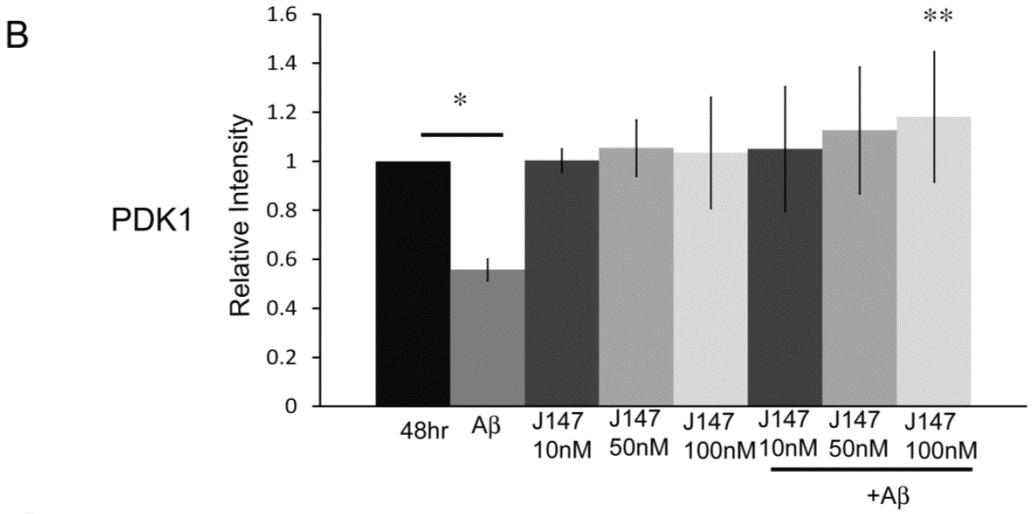

C
LDHA
Realtive Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
**
48hr
Aβ
001 0.5μM
001 1μM
001 0.5μM
001 1μM
Aβ

Figure 4.5 J147 maintains the expression of PDK1 and LDHA in primary cortical neurons exposed to Aβ. **(A)** Western blot analysis of primary cortical neurons treated with $A\beta_{25-35}$ (10 μM) for 48 hrs exhibited a significant decrease in the expression of PDK1 and LDHA compared to untreated cells (*P<0.01). PDK1 and LDHA expression increased when cells were treated with different concentrations of J147 for 2 hr prior to exposure to Aβ. **(B)** Densitometric analysis revealed a significant increase in PDK1 was observed when cells were pre-treated with 100 nM J147 prior to exposure to Aβ when compared to cells treated with Aβ (**P<0.05). **(C)** A significant increase in LDHA was observed when cells were treated with 50 nM J147 prior to Aβ treatment compared to cells treated with Aβ alone (**P<0.05). Relative intensity was calculated by comparing the LDHA/actin or PDK1/actin ratio of the treated cells to the same ratio of the control cell line (48 hr). Data represent the mean ± SD of three independent experiments. Data were analyzed by a one-way ANOVA followed by a Tukey test.


A
+Aβ
48hr
Aβ
J147 10nM
J147 50nM
J147 100nM
J147 10nM
J147 50nM
J147 100nM
PDK1
LDHA
β-ACTIN


B
PDK1
Relative Intensity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
48hr
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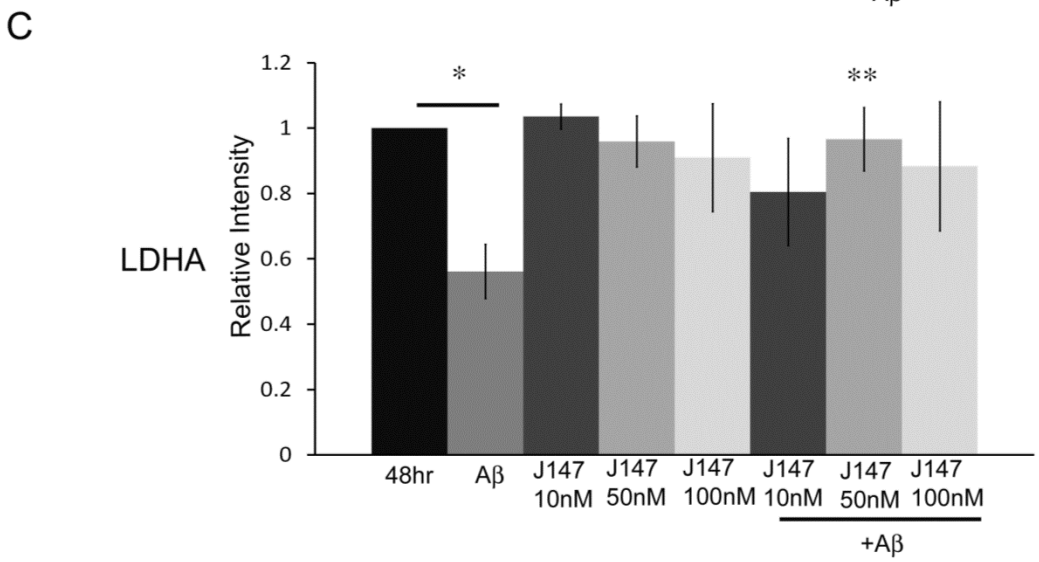
J147 10nM
J147 50nM
J147 100nM
J147 10nM
J147 50nM
J147 100nM
+Aβ


C
LDHA
Relative Intensity
1.2
1
0.8
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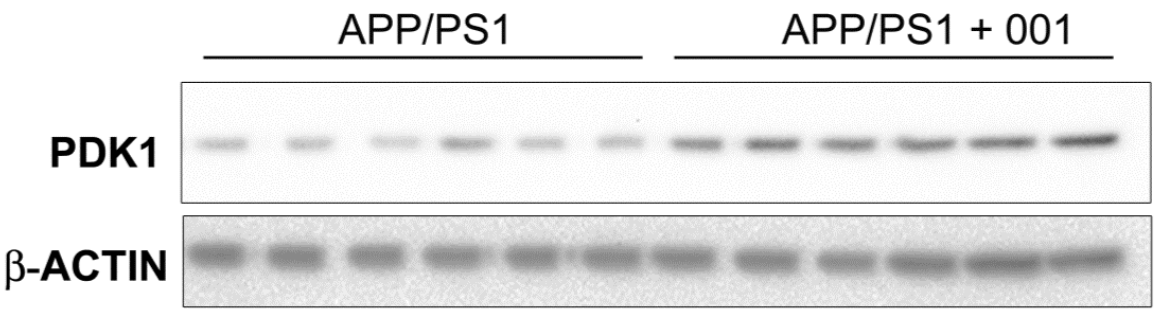
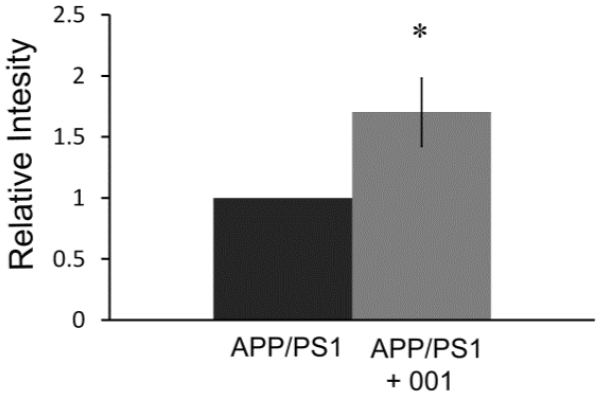
48hr
Aβ
J147 10nM
J147 50nM
J147 100nM
J147 10nM
J147 50nM
J147 100nM
+Aβ

4.3.3 CNB-001 maintains PDK1 expression in APP/PS1 transgenic mice

CNB-001 enhances both long term potentiation and memory in rats (Maher et al., 2010). In addition, this compound protects against Aβ toxicity in primary hippocampal nerve cells (Liu et al., 2008). Interestingly we have previously shown that 12 month old AD transgenic (APPswe/PS1dE9) mice exhibit a loss of PDK1 and LDHA expression when compared to control mice (Newington et al., 2012). Therefore, we wondered whether AD transgenic mice fed CNB-001 would show altered expression of PDK1 or LDHA. Interestingly, 12 month old mice fed CNB-001, starting at 3 months of age, displayed a significant increase in PDK1 when compared to AD untreated mice (Figure 4.6, $P<0.001$). More specifically we observed a 1.7 fold increase in PDK1 expression. We did not however observe any significant change in LDHA expression when mice were fed CNB-001 (data not shown). These results suggest that the ability of CNB-001 to sustain PDK1 expression in AD transgenic mice may play a role in the memory enhancing properties of this compound.

Figure 4.6 CNB-001 preserves PDK1 expression in APP/PS1 transgenic mice. Immunoblot analysis of cortical tissue from 12 month old AD transgenic (APP/PS1) mice fed a high fat diet with or without CNB-001 (001; 500 ppm) revealed a significant increase in PDK1 expression in mice fed 001 compared to control mice (*P<0.001). Relative intensity was calculated by comparing the PDK1/actin ratio of the AD mice fed CNB-001 to the same ratio of the control (APP/PS1). Data were analyzed by a one-tailed T-Test.


APP/PS1
APP/PS1 + 001
PDK1
β-ACTIN


Relative Intesity
2.5
2
1.5
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0.5
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*
APP/PS1
APP/PS1 + 001

4.4 Discussion

Uncovering new drugs for the effective treatment or prevention of AD is crucial to reducing the financial, physical and emotional burden of this devastating disease. In previous studies it was shown that curcumin elicited neuroprotective effects and showed therapeutic potential for the treatment of AD in transgenic mice models of the disease (Lim et al., 2001; Yang et al., 2005). The engineered curcumin derivatives CNB-001 and J147 displayed enhanced neuroprotective properties against common stressors associated with AD and promoted improved memory in wild type mice (Chen et al., 2011; Liu et al., 2008; Maher et al., 2010). Moreover, J147 prevented synaptic loss and cognitive decline in AD transgenic mice (Chen et al., 2011). Despite evidence indicating reductions in pro-oxidant enzymes and a reduction in soluble Aβ, the mechanism by which CNB-001 or J147 protects nerve cells against AD pathology including Aβ toxicity remains unclear (Chen et al., 2011). Importantly, these compounds, similar to curcumin, exhibit a range of biological activities suggesting that they have affinity for multiple molecular targets (Chen et al., 2011; Liu et al., 2008). Interestingly, the data presented here suggests that PDK1 and possibly LDHA could potentially be targets, either direct or indirect, for these drugs. The maintained expression or activity of these proteins when faced with stressors associated with AD may contribute to the protective effects afforded by these compounds.

4.4.1 Glutamate toxicity and aerobic glycolysis

Glutamate is the principle excitatory neurotransmitter in the CNS and plays an important role in learning and memory (Collingridge and Lester, 1989). Overstimulation of glutamate receptors or exicitoxicity as a result of excessive glutamate release can cause nerve cell death and plays an important role in neurodegeneration and AD (Hynd et al., 2004; Sattler and Tymianski, 2001). Additionally, extracellular glutamate can induce a unique oxidative stress-induced programmed cell death termed oxytosis (Tan et al., 2001). Excess glutamate can affect the oxidative potential of the cell through a cystine/glutamate antiporter system (Bannai and Kitamura, 1980; Murphy et al., 1989; Sato et al., 1999). Cystine uptake, which plays an essential role in the production of the antioxidant glutathione (GSH), is inhibited by excess extracellular glutamate resulting in GSH depletion, oxidative stress and cell death (Murphy et al., 1989). Oxidative glutamate toxicity or oxytosis appears to also play an

important role in nerve cell death and neurodegenerative disorders including AD (Tan et al., 2001). Oxytosis has been modeled and studied in hippocampal HT22 nerve cells which lack glutamate receptors but die within 48 hours following exposure to glutamate (Dargusch and Schubert, 2002; Tan et al., 2001). Similar to HT22 cells, many neurons in the brain lack glutamate receptors and are susceptible to oxytosis induced by excess glutamate associated with ischemia, trauma and neurodegeneration (Maher, 2006). Thus glutamate toxicity in HT22 cells is a valuable model for testing protective molecules for the treatment of AD. Here it is shown that glutamate exposure results in decreased levels of the Warburg effect enzymes PDK1 and LDHA in HT22 cells.

Both PDK1 and LDHA expression have shown to be protective against a variety of neurotoxins that induce oxidative stress and cell death (Newington et al., 2012). Moreover, overexpression of either PDK1 or LDHA reduces mitochondrial ROS production, thus loss of these proteins in HT22 cells following glutamate exposure could further exacerbate oxidative stress induced by this molecule (Newington et al., 2012). Interestingly, both CNB-001 and J147 protect against glutamate toxicity which is associated with maintenance of PDK1 and LDHA levels. Although it could be argued that CNB-001 and J147 mediated effects on PDK1 and LDHA expression in glutamate treated cells are merely a reflection of increased survival, this is unlikely because even cells treated with glutamate alone were harvested under conditions in which at least 90% of the remaining adherent cells, following washes, were alive (data not shown). Both PDK1 and LDHA have been previously shown to play an important role in protection against Aβ toxicity (Newington et al., 2011). Moreover, Aβ resistant cells and glutamate resistant HT22 cells display similar cross resistance to a variety of other neurotoxins suggesting similar resistance mechanisms are elicited by these cells (Dargusch and Schubert, 2002). Therefore it is plausible that, similar to Aβ resistant nerve cells, PDK1 and LDHA may contribute to protection against glutamate toxicity in HT22 cells. Furthermore, maintenance of these proteins through treatment with CNB-001 or J147 could potentially contribute to the survivability of nerve cells lacking ionotropic glutamate receptors in the AD brain.

4.4.2 Protective role for maintenance of a Warburg effect in nerve cells exposed to Aβ

We have previously shown a time dependent loss of both PDK1 and LDHA expression when primary cortical neurons are exposed to Aβ (Newington et al., 2012). Furthermore, expression of these enzymes is decreased in the cortex of 12 month old AD transgenic (APPswe/PS1dE9) mice a time at which cognitive impairments are present (Newington et al., 2012). Moreover, there is a significant decrease in the levels of PDK1 in post mortem tissue taken from the frontal cortex of individuals diagnosed with AD (Newington et al., 2012). Both PDK1 and LDHA expression divert energy metabolism away from mitochondria which are known to be dysfunctional in AD (Lezi and Swerdlow, 2012). Importantly, cells which have been depleted of endogenous mtDNA, and which lack ETC activity, are insensitive to the toxic effects of Aβ stressing the importance of functional mitochondria in Aβ induced cell death (Cardoso et al., 2001). Aβ has been shown to physically associate with mitochondria in AD transgenic mice, suggesting that the peptide causes impaired mitochondrial metabolism by promoting the increased offloading of electrons from the ETC thereby increasing H_2O_2 levels in the cell (Manczak et al., 2006). Blocking the ability of Aβ to associate with mitochondria inhibits Aβ-induced nerve cell death and associated ROS production (Lustbader et al., 2004). Interesting, preliminary studies in our lab have found that Aβ-resistant PC12 cells exhibit dramatically decreased intracellular accumulation of Aβ (T. Rappon, personal communication). The role of elevated Warburg effect enzymes in promoting increased clearance of intracellular Aβ, including mitochondrial localized Aβ, awaits further investigation.

Deficiencies in essential enzymes involved in mitochondrial respiration have been reported in the brains of patients with AD (Brooks et al., 2007; Gibson et al., 1998; Kish, 1997; Liang et al., 2008). Thus redirecting energy metabolism away from the mitochondria through increased PDK1 and/or LDHA activity not only reduces toxic ROS production but would allow for adenosine triphosphate (ATP) production in cells with dysfunctional mitochondria. The expression of these enzymes when cells are exposed to Aβ would likely direct the conversion of pyruvate to lactate and reduce metabolic flux in the mitochondria. A subsequent reduction in mitochondrial ETC activity would reduce the production of superoxide, a natural byproduct of ETC activity due to the early offloading of electrons onto

molecular oxygen. Moreover, when mitochondria become dysfunctional they cannot produce enough ATP required by a cell for survival, thus diverting energy production to aerobic glycolysis through the activities of PDK1 and LDHA would allow for maintenance of energy supply despite the loss of mitochondrial metabolism. Decreased levels of PDK1 are observed in 12 month old AD transgenic mice. Interestingly, PDK1 expression is preserved in the cortex of AD transgenic mice fed CNB-001. Unfortunately, we did not have control mice (non transgenic) or control mice fed 001 to make a full analysis of the effects of CNB-001 on PDK1 expression. There was no observable difference in LDHA expression, however LDHA expression is often not reflective of LDHA activity thus future studies may look to examine LDHA activity in these brain samples (Newington et al., 2012). However, loss of PDK1 and not LDHA expression is observed in the human AD post-mortem brain thus preservation of PDK1 may be more important to confer protection against nerve cell death associated with AD.

4.4.3 Activation of PDK1 or LDHA by CNB-001 and J147

Though it appears that both CNB-001 and J147 have the ability to sustain the expression of the protective enzymes LDHA and PDK1, it remains unclear whether these drugs actually play a direct role in maintaining their expression. Given that both CNB-001 and J147 are derivatives of curcumin, we hypothesize that these compounds are likely to act in a similar manner and share common molecular targets. Curcumin has been shown to modulate a number of molecular targets including growth factors and their receptors, transcription factors, cytokines, enzymes and genes (Pari et al., 2008). In examining the current identified targets of curcumin there does not appear to be a clear link to transcriptional or post transcriptional regulation of these enzymes. In fact curcumin appears to negatively regulate transcription factors known to play a role in the transcription of these enzymes (Pari et al., 2008). For example hypoxia inducible factor 1 (HIF-1) is a well known transcriptional regulator of both PDK1 and LDHA that is activated in low oxygen conditions to mediate the switch from oxidative metabolism to lactate production (Kim et al., 2006; Semenza et al., 1996). Treatment of breast cancer cells with curcumin inhibited HIF-1α gene expression resulting in decreased HIF-1 activity (Bae et al., 2006; Choi et al., 2006; Thomas et al., 2008). Moreover, treatment of HT22 cells with CNB-001 or J147 failed to induce the expression of transfected luciferase reporter driven by the HIF-1α promoter with or without

glutamate treatment (data not shown). In addition to regulating enzymes involved in glucose metabolism, HIF-1 activates enzymes that play a critical role in cancer biology including enzymes involved in invasion, cell survival, angiogenesis and drug resistance (Semenza, 2003). Thus with more than 60 identified transcriptional targets of HIF-1, it does not appear to be an appealing candidate to target increased expression of PDK1 and LDHA for the treatment of AD (Semenza, 2003). Moreover, HIF-1 has been shown to activate enzymes responsible for increased production of Aβ (Wang et al., 2006; Zhang et al., 2007). Therefore, a mechanism in which PDK1 and LDHA are activated independent of HIF-1 could offer significant therapeutic potential.

Importantly, these results suggest that maintenance of PDK1 and LDHA expression in HT22 cells and primary cortical neurons treated with CNB-001 and J147 likely occurs independent of HIF-1. This however this does not exclude the possibility that these drugs could play a role in their transcriptional regulation. Interestingly, some preliminary evidence in our lab suggests that CNB-001 and J147 promote increased transcription of *PDK1* (R. Harris, personal communication) but further investigation is necessary to confirm these findings. Alternatively, it is possible that CNB-001 and J147 maintain LDHA and PDK1 levels through post translational mechanisms which may be important to examine in future studies. Though we could not find a clear mechanism for maintenance of these proteins by examining the known molecular targets of curcumin it is important to note that these newly derived drugs are derivatives of curcumin which may possess novel molecular targets that have not yet been identified such as LDHA and PDK1. Determining the molecular targets of both CNB-001 and J147 could offer further insight into how these drugs sustain PDK1 and LDHA expression when cells are exposed to stressors associated with AD. Moreover testing these drugs in cells with decreased PDK1 and LDHA expression could help in determining if these proteins are necessary for the protection afforded by these compounds. Unfortunately, attempts to use shRNA mediated knockdown of either LDHA or PDK1 transcripts in primary neurons where not successful due to the limited transfection/nucleofection properties of this cell type. Future experiments using adenoviral or lentiviral technologies to silence *LDHA* or *PDK1* expression will help resolve the importance of these enzymes in mediating CNB-001 and J147 neuroprotective effects.

4.5 Conclusions

We are in need of developing new compounds for the treatment of AD, which effectively reduce nerve cell loss and cognitive decline associated with the disease. CNB-001 and J147 are promising candidates with the potential of slowing disease progression and providing immediate cognitive benefits to patients (Chen et al., 2011). Their ability to maintain the expression of the protective enzymes PDK1 and LDHA in both HT22 cells and primary cortical neurons suggests that these compounds might promote increased neuron survival *in vivo* when exposed to diverse neurotoxins associated with AD. Moreover, maintenance of PDK1 in AD transgenic mice fed CNB-001 may contribute to decreases in the production ROS, memory deficits and eventual nerve cell death. It remains unclear how these protective compounds sustain the expression of these enzymes, but it likely occurs independent of HIF-1. Activation of these enzymes independent of HIF-1 may have clinical relevance in the treatment of AD. Future research into determining the molecular targets of these drugs may reveal novel candidates for the treatment of AD and lead to a further understanding of how they elicit their protective effects. Moreover, further investigation of compounds, in addition to CNB-001 and J147, which activate PDK1 and LDHA expression may offer a neuroprotective strategy for the treatment of AD and other neurodegenerative disorders.

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Chapter 5

5 General Discussion and Conclusions

5.1 Summary

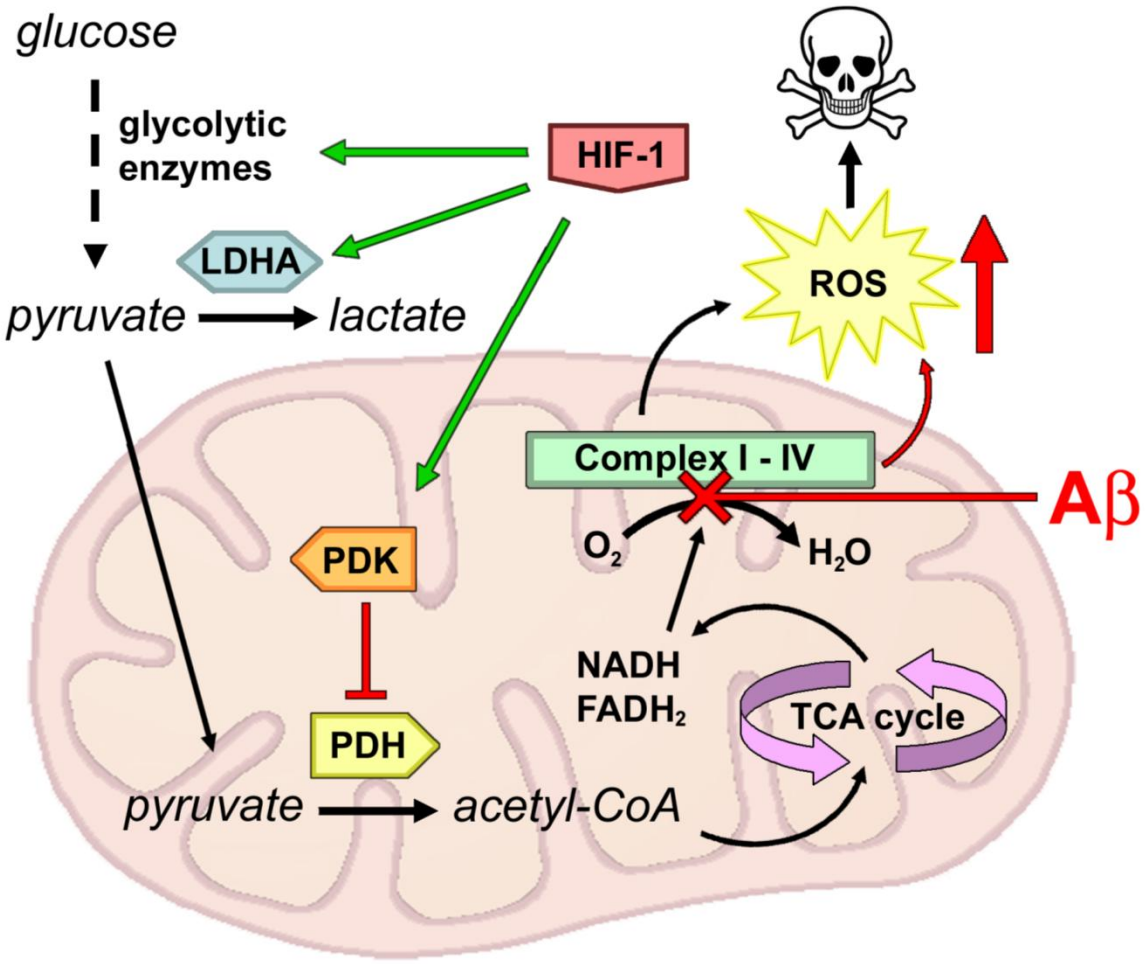
5.1.1 Summary of Findings

Understanding the mechanisms by which nerve cells become resistant to the toxic effects of Aβ could reveal novel targets for the treatment and/or prevention of AD. By examining nerve cells selected for resistance against Aβ toxicity I have shown that these cells exhibit a shift in metabolism to favor increased lactate production reminiscent of the Warburg effect in cancer cells. Interestingly, Aβ-resistant nerve cells express increased levels of PDK1 accompanied by increased LDHA activity and lactate production when compared to control cells. As a result of these metabolic changes, the production of mitochondrial derived ROS, which is closely associated with Aβ toxicity, is markedly diminished in resistant relative to sensitive cells. Inhibiting LDHA or PDK1 expression or activity re-sensitizes resistant cells to Aβ induced cell death, suggesting that these enzymes play a central role in conferring resistance to Aβ. Importantly, PDK1 and LDHA overexpressing cells exhibit decreased ROS production and oxygen consumption but maintain ATP pools under both normal culture conditions and following Aβ treatment. These findings indicate that activation of aerobic glycolysis in nerve cells confers resistance to Aβ, while maintaining sufficient energy reserves for cell survival. Similar to cancer cells, the stabilization of HIF-1α likely accounts for the observed metabolic changes in Aβ resistant cells (Soucek et al., 2003). Intriguingly, decreased expression of both LDHA and PDK1 was observed in cortical extracts of 12 month old AD transgenic (APPswe/PSEN1dE9) mice. A loss of PDK1 expression was also observed in post-mortem cortical tissue from AD patients. Collectively, these findings indicate that PDK1- or LDHA-mediated aerobic glycolysis protects against Aβ-toxicity while maintaining cellular ATP levels (Figure 5.1). Loss of these proteins may contribute to the cognitive decline and nerve cell death observed in AD. Moreover, overexpression of either PDK1 or LDHA in a rat CNS cell line confers resistance to not only Aβ but a variety of other neurotoxins. These findings suggest that expression of these enzymes may be linked to a

broad acting neurotoxin resistance mechanism and may have relevance to other neurodegenerative diseases.

Currently there is no cure for AD and treatment remains limited to reducing the symptoms associated with the disease, rather than slowing disease progression. Here I have identified a novel resistance mechanism in nerve cells which confers resistance to Aβ toxicity *in vitro.* Identifying molecules or drugs that activate this pathway in nerve cells may have important therapeutic potential. I tested the effects of two newly derived neurotrophic drugs (CNB-001 and J147) on expression levels of rate limiting Warburg effect enzymes in nerve cells exposed to glutamate and Aβ; two stressors closely associated with AD. Interestingly, treatment with either CNB-001 or J147 maintained the expression of the protective enzymes LDHA and PDK1 when nerve cells were exposed to either glutamate or Aβ. Moreover, transgenic AD mice fed CNB-001 from 3 to 12 months of age exhibited increased PDK1 expression when compared to control fed mice. These results suggest that one mechanism by which these drugs exhibit a neuroprotective effect may occur via the upregulation or maintenance of Warburg effect enzymes. Activation of aerobic glycolysis in mice fed J147 may not only elicit neuroprotective effects, but may also contribute to the immediate cognitive benefits including improvements in learning and memory (Chen et al., 2011).

Figure 5.1 Aerobic glycolysis in Aβ resistant cells. The stabilization of hypoxia inducible factor 1 α (HIF1α) in amyloid beta (Aβ) resistant cells stimulates increased expression of glucose transporters and glycolytic enzymes thereby increasing the conversion of glucose to pyruvate. Additionally, HIF-1 induces the transcription of lactate dehydrogenase A (LDHA), resulting in an increase in the conversion of pyruvate to lactate. Futhermore HIF-1 suppresses mitochondrial respiration by upregulating pyruvate dehydrogenase kinase 1 (PDK1). PDK1 phosphorylates and inhibits pyruvate dehydrogenase (PDH) resulting in decreased flux through the tricarboxcylic acid (TCA) cycle and repressed oxidative phosphorylation (OXOHOS). Decreased OXOPHOS attenuates mitochondrial ROS production rendering cells more resistant to apoptosis in the presence of Aβ. In cells failing to undergo aerobic glycolysis, increased mitochondrial respiration potentiates Aβ mediated ROS production to toxic levels resulting in cell death.


glucose
glycolytic enzymes
HIF-1
LDHA
pyruvate
lactate
ROS
Complex I - IV
Aβ
PDK
O2
H2O
NADH
FADH2
TCA cycle
PDH
pyruvate
acetyl-CoA

5.1.2 Limitations of study

A major limitation to this study was the heavy reliance on modeling in immortalized cell lines. Though these cell lines behave similar to nerve cells, they have undergone transformation which allows them to proliferate indefinitely through the acquisition of spontaneous or induced mutations which can drastically alter the biology of the cell. Importantly, mature adult nerve cells *in vivo* do not proliferate following differentiation. Future studies should focus on recapitulating the findings presented here in primary nerve cell cultures as this cell type represents a better, more physiologically relevant *in vitro* model. Though I made attempts to both over express and knockdown the expression of PDK1 and LDHA in primary nerve cells I was unable to achieve successful results with the plasmids and transfection technologies that I had available. Future studies which make use of adenoviral vectors to overexpress or knockdown LDHA or PDK1 and test for Aβ sensitivity and resistance would lend further support to aerobic glycolysis as a protective mechanism elicited by nerve cells against Aβ toxicity.

The use of $A\beta_{25-35}$ instead of $A\beta_{1-42}$ was another major limitation to this study. Though $A\beta_{25-35}$ does elicit toxicity and exhibits similar properties of the full length peptide, it is not present in the brains AD patients. Thus the use of $A\beta_{1-42}$ would offer more physiological relevance to the findings presented here. It is important to note that $A\beta_{1-42}$ was used to verify the effects elicited by $A\beta_{25-35}$ exposure, but not in replicates of 3. In addition, this study did not examine the effects of a scrambled peptide (non-functional Aβ peptide) on toxicity as a negative control. The use of this peptide would lend support to the specific toxicity of $A\beta_{25-35}$. However, previous studies examining Aβ toxicity in PC12 cells, showed the use of a $A\beta_{25-35}$ scrambled peptide sequence had no effect on cell viability (Behl et al. 1994). Additionally in the present study cell viability assays were carried out with MTT. MTT is a tetrazole that is reduced to a purple formazan by mitochondrial reductase in viable cells. Given that this study examines mitochondrial function and mitochondrial reductase enzymes are found in the electron transport chain it is possible that a reduction in the amount of formazan (purple) formed could be a result of reduced mitochondrial activity independent of viability. The consistent use of trypan blue or propidium iodide/calcein green live dead assays throughout this study may have provided a more reliable indicator of cell viability or

death. Importantly, cells were examined by light microscopy prior to adding MTT to dishes to visually confirm decreased cell numbers relative to untreated cells. Moreover, when trypan blue exclusion analysis was performed, the results were in concordance with the MTT assay, suggesting that the use of MTT as a measure of cell viability was not a significant issue.

Another major limitation to this study was the lack of *in vivo* evidence supporting a role for the Warburg effect in neuronal resistance to Aβ. Moreover, I examined whole tissue extracts from cortical brain regions taken from transgenic AD mice and AD patients, which did not allow the ability to distinguish between glial cells and nerve cells. In light of the fact that some areas of the brain contain glial cells in excess of nerve cells by a 10 to 1 ratio, it would be valuable to examine the longitudinal expression patterns of PDK1 and LDHA in surviving neurons and astrocytes in AD brain to determine if a cell type specific expression pattern correlates with Aβ sensitivity. Furthermore, it would also be of interest to examine astrocytic resistance to Aβ *in vitro* to determine if these cells exploit a similar resistance mechanism. To determine if aerobic glycolysis contributes to resistance *in vivo* it would be useful to monitor the effects of attenuated LDHA and/or PDK1 levels on memory and neurodegeneration in AD mice. By crossing Cre/lox mediated conditional knockout models, in which LDHA or PDK1 genes are deleted in neuronal or glial cell compartments, to AD transgenic mice this could be achieved. One could then examine the effect of attenuated neural or glial expression of Warburg effect enzymes on susceptibility to Aβ toxicity. Though aerobic glycolysis appears to contribute to resistance against Aβ *in vitro*, it is imperative to demonstrate a similar effect *in vivo.*

Though preliminary studies with CNB-001 and J147, suggest that these compounds may activate and/or sustain a Warburg effect in nerve cells, these studies failed to determine if the neuroprotective effects elicited by these drugs rely on LDHA and/or PDK1. Thus in the future it would be important to determine if the neuroprotective effects of CNB-001 and J147 still exist in either nerve cell lines or primary nerve cultures in which LDHA and PDK1 is knocked down using siRNAs specific to each transcript. In addition, examining the longitudinal expression of these enzymes in mice fed either drug would be valuable in determining their role with respect to neuroprotection. Given that there does not appear to be

a direct link between CNB-001 or J147 and LDHA or PDK1 expression, further investigation in the mechanisms by which these drugs activate and/or maintain the expression of these enzymes is crucial to fully understanding their neuroprotective properties.

5.2 Global perspective of findings

5.2.1 Aerobic glycolysis and the AD brain

Aging and AD are associated with a decreased rate of cerebral glucose consumption, possibly contributing to both nerve cell loss and memory decline (Heiss et al., 1991; Mielke et al., 1998). Although a reduction in cerebral glucose metabolism, as measured by FDG-PET, is commonly used in the diagnosis of AD, recent evidence suggest that glucose utilization is more complex in the AD brain (Mosconi, 2005; Vlassenko et al., 2010). A recent PET imaging study which measured both glucose consumption and oxygen utilization, revealed a strong correlation between the spatial distribution of elevated aerobic glycolysis and Aβ plaques in brain tissue from patients with AD, as well as normal individuals with high levels of Aβ-deposition but without clinical manifestation of the disease (Vlassenko et al., 2010). In the developing nervous system, aerobic glycolysis is believed to account for 90% of glucose consumed (Powers et al., 1998). During childhood this fraction accounts for 35% of glucose utilization and finally drops to 10-12% in the adult brain (Vaishnavi et al., 2010). PET studies of cognitively normal individuals have shown an age-associated decrease in FDG uptake in regions of the brain frequently affected in AD, although these studies did not determine what proportion of glucose was processed by aerobic glycolysis versus oxidative phosphorylation (Cunnane et al., 2011). Moreover, recent imaging analysis of patients with AD revealed regional variations in atrophy, hypometabolism and Aβ deposition (La Joie et al., 2012). These observed variations are likely reflective of region specific pathological or compensatory mechanisms (La Joie et al., 2012). Interestingly, a recent neuroimaging study that attempted to correlate multimodal neuronal parameters (cortical thickness, glucose metabolism and hippocampal volume) with Aβ deposition (PIB-PET) in cognitively normal older individuals, found no association between PIB uptake and the multimodal neurodegenerative biomarkers (Wirth et al., 2013). Surprisingly, improved neuronal integrity and cognitive function correlated with the presence of high Aβ burden in brain regions that are most affected in AD (Wirth et al., 2013). In another recent study of cognitively normal elderly subjects, using both PIB-PET and FDG-PET, it was

revealed that increased glucose uptake was associated with better verbal episodic memory in individuals with elevated amyloid levels (Ossenkoppele et al., 2013). These observations prompted to authors to speculate that Aβ can trigger a neural compensation mechanism that enables elderly, presumably on the path to AD, to at least temporarily suppress the neurotoxic effects of Aβ and preserve cognitive function by increasing brain activity (Ossenkoppele et al., 2013). These findings lend support to our own observations and suggest that aerobic glycolysis may be elevated in areas of the brain most susceptible to insult as a pre-emptive protective mechanism or in response to Aβ accumulation during aging. Loss of this protective mechanism may render certain areas of the brain susceptible to Aβ-induced neurotoxicity.

5.2.2 AD and cancer

Interestingly, cancer survivors have a lower risk of developing AD than those without cancer (Driver et al., 2012). In contrast, patients who suffered from AD had a lower incident of cancer (Driver et al., 2012). It is possible that individuals with cancer also have a higher propensity to activate aerobic glycolysis, as this form of metabolism confers a growth and survival advantage (i.e. anti-apoptotic function) to cancer cells (Koppenol et al., 2011). However, individuals who survive cancer may still have higher innate levels of aerobic glycolysis, presumably in areas of the brain, which may protect against the development of AD. In contrast, patients with AD may have lower levels of aerobic glycolysis, which not only renders them susceptible to the toxic effects of Aβ but also leads to decreased susceptibility to developing cancer. A similar trend has been observed in Parkinson's disease (Bajaj et al., 2010; Driver et al., 2007a; Driver et al., 2007b). Thus it may be valuable in the future to examine aerobic glycolysis in a Parkinson's disease context. Taken together these data suggest an inverse association between cancer and the development of neurodegenerative diseases. Future studies that examine the direct relationship between aerobic glycolysis, AD and cancer may provide more insight into this fascinating inverse relationship.

5.2.3 Lactate is a neuroprotective metabolite

Although aerobic glycolysis and associated lactate production has been shown to enhance memory, the effect of this metabolism on age-dependent or AD-related memory decline and neuronal loss has never formally been examined. Interestingly, L-lactate treatment following an ischemic insult is neuroprotective and attenuates neurological deficits in mice (Berthet et al.,

2012; Berthet et al., 2009). Intracerebroventricular or intravenous injection of lactate has also been shown to exert a neuroprotective effect during experimentally induced hypoglycemia or cerebral ischemia (Berthet et al., 2012; Berthet et al., 2009; Wyss et al., 2011). Lactate also exerts neuroprotective effects via transcriptional activation of brain-derived neurotrophic factor (BDNF) expression in human astrocytes and the SH-SY5Y cell line (Coco et al., 2012). BDNF is a necessary factor for the survival of nerve cells within the CNS, and is also essential for long term memory storage (Bekinschtein et al., 2008). In addition, under normoxic conditions lactate can promote HIF-1 stabilization by inhibiting prolylhydroxylase 2 activity, the enzyme responsible for HIF-1α degradation (De Saedeleer et al., 2012). Stabilization of HIF-1 increases glycolysis and lactate production; events associated with resistance to Aβ toxicity. Moreover, exogenous lactate has been shown to increase both MCT1 and COX mRNA and protein expression in L6 cells (Hashimoto et al., 2007). Thus lactate can elicit a number of events leading to activation of transcription factors known to elicit protective effects. Furthermore, several studies have reported that lactate increases vasodilation (Gordon et al., 2008; Yamanishi et al., 2006); and continuous lactate production in the activated brain may serve as a signaling mechanism to increase blood flow and fuel delivery to the brain. Therefore lactate may function as a versatile signaling molecule by both activating neuroprotective metabolism and promoting increased blood flow to certain regions of the brain. These changes mediated by increased lactate production may contribute to resistance against the toxic effects of Aβ in AD brain.

5.2.4 Astrocytic lactate production and memory

Lactate has long been considered a metabolic dead end; a harmful metabolite for the CNS (Chih et al., 2001; Chih and Roberts Jr, 2003). However, this viewpoint has changed in light of growing evidence indicating that lactate transport from astrocytes to neurons is essential for long term memory (Alberini, 2009; Newman et al., 2011). Memory is a process in which information is encoded, stored and retrieved. Short term memories involve the retention of information for a brief period of time and are dependent on post translational modifications of proteins (Silva et al., 1998; Yin and Tully, 1996). Long term memories are formed after learning, retention, and consolidation which require the activation of signalling cascades that lead to gene activation, protein synthesis and the growth of new synaptic connections (Silva et al., 1998; Yin and Tully, 1996). The cAMP response element binding protein (CREB) is a

nuclear protein that modulates transcription and plays a central role in long term memory following phosphorylation dependent activation (Gibbs et al., 2006; Silva et al., 1998; Yin and Tully, 1996). Not surprisingly, memory and learning are metabolically demanding processes, which appear in part to be dependent on glycogen metabolism (Belanger et al., 2011; Newman et al., 2011; Rafiki et al., 2003). Glycogen represents the major energy reserve in the brain and is stored exclusively in astrocytes, not neurons (Brown et al., 2004; Newman et al., 2011; Vilchez et al., 2007). During periods of low glucose or increased activity glycogen is broken down to lactate to fuel neuronal metabolism (Brown et al., 2004). A role for glycogenolysis in long term memory formation was first observed by Gibbs and colleagues who found that intracerebral injection of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) (a glycogen phosphorylase inhibitor), in day-old chickens resulted in a dose-dependent inhibition of long term memory (Rafiki et al., 2003). More recently a more intensive study investigating the importance of astrocytic glycogenolysis and long term memory was performed by Suzuki et al. who examined learning and memory in rats using an inhibitory avoidance (IA) test. To test for the importance of glycogenolysis in hippocampal astrocytes rats were injected with DAB either 15 min before or immediately after IA training (Belanger et al., 2011). Training led to a significant increase in extracellular lactate in the hippocampus which was abolished by DAB administration (Belanger et al., 2011; Newman et al., 2011). DAB had no effect on short term memory (tested an hour after) but significantly blocked long term memory (tested at 24hr)(Belanger et al., 2011). Importantly, L-lactate co-administered with DAB significantly rescues memory loss (Belanger et al., 2011). Similar results were obtained testing spatial working memory in rats using spontaneous alteration tasks (Newman et al., 2011). Furthermore, astrocytic glycogenolysis also appears to be required for phosphorylation of CREB (pCREB) a key molecular event linked to memory formation (Belanger et al., 2011). DAB-induced reduction of pCREB activation was also rescued by exogenous L-lactate suggesting a possible signalling role for lactate (Belanger et al., 2011; Belanger and Magistretti, 2009).

The ability to shuttle (uptake and release) lactate to various regions of the brain is dependent on MCT activity. Examination of both MCT mRNA and protein levels in mouse cortical tissues, revealed that MCT1 and MCT4 were expressed almost exclusively in astrocytes, whereas MCT2 was strongly expressed in neurons (Debernardi et al., 2003; Pellerin et al., 2005). Based on these finding Suzuki and colleagues demonstrated the importance of CNS lactate transport

on memory by using intrahippocampal injections of antisense oligodeoxynucleotides to individually decrease expression of MCT1, MCT2 and MCT4 (Belanger et al., 2011). Decreased expression of MCT1 or MCT4 in astrocytes resulted in disrupted long-term memory formation that was rescued by exogenous administration of lactate but not glucose (Belanger et al., 2011). Disrupting the expression of neuronal MCT2 also resulted in loss of long-term memory which was not rescued by exogenous lactate or glucose, indicating that transport of lactate into neurons is required for long-term memory formation (Belanger et al., 2011). Taken together these results suggest that the astrocytic lactate export by MCT-1 and/or MCT-4, and subsequent import into neurons through MCT2, is essential for long term memory (Belanger et al., 2011). However, the role of lactate transport in memory loss associated with neurodegenerative diseases has remained largely unexplored. In examining cortical tissue from the AD transgenic mice or AD postmortem tissue, we did not distinguish between nerve and glial cell when examining the expression of the Warburg effect enzymes. Loss of LDHA and/or PDK1 in astrocytes may contribute to memory loss associated with AD.

5.2.5 Exercise induced lactate production enhances memory

During periods of physical exertion such as exercise, systemic lactate levels increase. Under resting conditions the brain releases small amounts of lactate which increases during exercise or hypoxia (Overgaard et al., 2012). During exercise the cerebral uptake of lactate also increases. As such, the brain plays an active role in the clearance of excessive lactate during exercise (Ide et al., 2000; Overgaard et al., 2012; Quistorff et al., 2008; Rasmussen et al., 2011; van Hall et al., 2009). The oxidation of lactate in the brain may account for as much as 33% the total energy substrate used by the brain (Overgaard et al., 2012). In contrast, cerebral glucose uptake is reduced by ~25% when cerebral lactate uptake is increased, suggesting that the brain preferentially consumes lactate during exercise (Rasmussen et al., 2011). Therefore, it appears that lactate is an important fuel source for brain metabolism both under normal conditions and during exercise. Given the importance of glycogen derived lactate for long term memory, it is feasible that exercise may benefit memory and cognitive function. Indeed, a study that examined the effects of a single bout of exercise on motor memory found that subjects that exercised before or after practice of a motor skill displayed significantly better retention of that skill 24 hrs and 7 days after practicing compared to subjects that did not exercise (Roig et al., 2012). These findings suggest a single bout of exercise before or after learning a motor skill

can improve long-term retention of that skill. Moreover, regular exercise has not only been shown to have a positive effect on memory retention but also appears to reduce the risk of developing neurodegenerative diseases including AD. Notably, exercise ameliorated memory deficits and Aβ deposition in APP transgenic mice (Maesako et al., 2012). In addition, a longitudinal study that followed 716 older individuals without dementia over 4 years assessed the link between exercise and AD (Buchman et al., 2012). The outcome of the study revealed that a higher level of total daily physical activity was associated with a reduced risk of AD (Buchman et al., 2012). Similar results were obtained when individuals were followed over a 14 year period (Scarmeas et al., 2009).

Interestingly BDNF is significantly elevated in response to exercise, possibly through increased lactate production, which may also account for some of the neuroprotective effects of exercise (Ferris et al., 2007). It should be noted that intravenous sodium-lactate administration in AD patients failed to improve cognitive functioning, although it did slightly improve semantic memory (Kalman et al., 2005). However, this study only examined the effects of a single 20 min intravenous administration of sodium-lactate on cognitive function. Because lactate was only administered for a short period it is unknown if longer periods or multiple administrations would improve cognitive function in AD patients. The systematic administration of glucose to AD patients does however improve memory (Manning et al., 1993). In contrast, insulin deficiency in AD transgenic mice exacerbates the AD phenotype (Wang et al., 2010). Notably, AD patients are at an increased risk for Type II diabetes, indicating an important association between glucose uptake and disease progression (Janson et al., 2004). Furthermore, patients with Type II diabetes are at increased risk for developing cognitive defects, AD or related dementias (Biessels and Kappelle, 2005; Whitmer, 2007). Collectively, these observations suggest that glucose uptake, aerobic glycolysis and associated lactate production may play a key role in promoting neuronal survival and preventing memory loss during aging and in AD. However, the role of aerobic glycolysis in maintaining CNS neuronal function during aging and preventing AD progression has never experimentally been examined. Clearly alterations in brain metabolism are tightly linked to AD and future research should focus on mechanisms that either enhance glucose uptake, aerobic glycolysis or lactate production.

5.3 Conclusions and Future Studies

5.3.1 Conclusions and suggestions for future research

In light of recent research, lactate has emerged as an important metabolite in the brain. These new findings accompanied by the findings presented here have altered the context in which we should look at the brain and its functioning. Importantly, given that metabolic dysfunction is tightly linked to neurodegenerative diseases, including AD, further studies measuring aerobic glycolysis and lactate production *in vitro* and *in vivo* are warranted. Examining the effects of exogenous lactate on Aβ sensitivity in primary nerve cells may lend support that this metabolite plays an important role in neuroprotection. Moreover, determining the effects of knocking down the expression of cellular lactate transporters (MCT's) in both Aβ resistant cells and primary nerve cells (MCT's) may also reveal the importance of this metabolite to modulating Aβ sensitivity. Considering the relationship between aerobic glycolysis in the AD brain, as well as the observed protective effect of aerobic glycolysis against Aβ *in vitro*, it would be valuable to perform a longitudinal study of aerobic glycolysis in normal and AD patients using FDG-PET to determine if elevated or sustained aerobic glycolysis correlates with better clinical outcome. Recent results from the dominantly inherited Alzheimer's network (DIAN) study showed that Aβ accumulation preceded detectable atrophy and hypometabolism by decades (Bateman et al., 2012). Interestingly, the caudate nucleus had very high levels of Aβ deposition but did not exhibit hypometabolism or neuronal loss throughout the course of disease progression (Bateman et al., 2012). Assuming that the caudate nucleus is resistant to the toxic effects of AD it would be valuable to determine if aerobic glycolysis is also elevated in this brain region.

A significant proportion of elderly individual's exhibit sufficient plaque accumulation warranting a neuropathology-based classification as probable AD, yet are normal by cognitive assessments (Bouras et al., 1994; Price and Morris, 1999). Assuming Aβ accumulation produces neurotoxicity and dementia, then increased CNS aerobic glycolysis may arise as a protective mechanism to enable these individuals to evade cognitive decline. Examining the brains of non-demented individuals with AD neuropathology (NDAN), may shed light on these neuroprotective mechanisms. Given the protective nature of LDHA and PDK1 *in vitro*, it would be of interest to examine the expression patterns of these enzymes in NDAN individuals.

Moreover, AD transgenic mice also may provide an interesting model for uncovering neuroprotective mechanisms that arise from the constitutive and progressive production of Aβ in the CNS. Although AD transgenic mice have high plaque loads by 6 months of age, most fail to exhibit nerve cell loss and cognitive defects until 12 months, a timepoint in which we see a decline in PDK1 expression (Savonenko et al., 2005). Thus young AD mice may offer a model of Aβ-resistance. Moreover, the creation of transgenic mice expressing enzymes that regulate aerobic glycolysis in the CNS, crossed to AD-transgenic mice, may help further define the role of this metabolism in preventing cognitive decline in older transgenic mice. These studies would provide a strong rationale for identifying compounds which activate aerobic glycolysis and enhance CNS function. The wealth of new research demonstrating the importance of aerobic glycolysis in the brain holds promise that activation of this form of metabolism may offer a new therapeutic strategy for the treatment of AD and other neurodegenerative diseases.

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Curriculum Vitae

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Honours and Awards:	Ontario Graduate Scholarship 2012-2013
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	Graduate Research Award in Alzheimer's disease and Dementia Alzheimer's Society London and Middlesex 2011-2012
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Related Work Experience	**Undergraduate Course Instructor** The University of Western Ontario 2012

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Publications:

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Arseneault, R., Chien, A., **Newington, J.,** Rappon, T., Harris, R., and Cumming, R. Attenuation of LDHA expression in cancer cells leads to redox-dependent alterations in cytoskeletal structure and cell migration. *Cancer Letters* (In Press)

Newington, J., Rappon, T., Albers,, S., Wong, D.Y., Rylett, R. J., and Cumming R. (2012) Overexpression of pyruvate dehydrogenase kinase and lactate dehydrogenase A in nerve cells confers resistance to amyloid beta and other toxins by decreasing mitochondrial respiration and ROS production**.** *J. Biol. Chem.* 287:37245-37258.

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Pitts, A., Dailey, K., **Newington, J.,** Chien, A., Arseneault, R., Cann, T., Thompson, L., and Cumming R. (2012) Dithiol-based compounds maintain expression of antioxidant protein peroxiredoxin1 that counteracts toxicity of mutant huntingtin. *J. Biol. Chem.* Vol.287 No. 27 p22717-22729.

Presentations:

Newington, J. Genetic engineering in animals. (2012) Guest lecture for Genetic Engineering -3593. Western University

Newington, J. Nerve cells and Alzheimer's disease. (2012) Guest lecture for Virtual Researcher on call. Western University

***Newington, J.,** and Cumming, R. PDK1 and LDHA confer resistance to neurotoxins including Aβ through mitochondrial remodeling. (2011) Society for Neuroscience Annual Conference. Washington DC, Poster presentation.

***Newington, J.,** and Cumming, R. Amyloid beta resistance and the Warburg effect: Re-examining Alzheimer's disease from a cancer perspective. (2011) Biology Graduate Research Forum.Western University, Oral Presentation.

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Academic Services:

September 2012-2013: Graduate Education Committee Representative

September 2012-2013: Advisory Committee member for two 4th year honours projects

September 2011-2012: Advisory Committee member for two 4th year honours projects

January 2011: Society of Graduate Students Election Appeal Board

September 2010-2012: Society of Graduate Students Representative

September 2010-2011: Advisory Committee member for one 4th year honours project

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Volunteer Experience:

2011-present: SARI Therapeutic Riding Center Program Committee. Sari Therapeutic Riding Centre, London, Ontario

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