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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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THE ROLE OF LACTATE DEHYDROGENASE B IN AEROBIC GLYCOLYSIS-MEDIATED RESISTANCE TO AMYLOID β TOXICITY IN PC12 CELLS

(Thesis format: Monograph)

by

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Graduate Program in Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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Abstract

Alzheimer's disease is a progressive, neurodegenerative disorder characterized by the accumulation of amyloid β (Aβ) plaques in affected brain regions. Strong evidence indicates that Aβ exerts neurotoxic effects by promoting mitochondrial dysfunction and ROS production, leading to widespread oxidative damage and activation of pro-apoptotic mechanisms. Past investigations suggest that neuronal resistance to Aβ toxicity is partly mediated by a Warburg Effect-like metabolism, in which cells exhibit elevated glycolytic activity and lactate production, while limiting mitochondrial respiration. Elevated lactate dehydrogenase A (LDHA) activity, which catalyzes lactate production from pyruvate, has been demonstrated to counter Aβ-induced oxidative stress and neurotoxicity, however the role of LDHB, which catalyzes the reverse reaction, has not been determined in this regard. This study utilized Aβ-sensitive and resistant PC12 cell lines, derived from a tumour of the rat adrenal medulla, and sought to determine the effect of altered LDHB protein levels on mitochondrial membrane potential, mitochondrial ROS levels, and cell viability following treatment with Aβ. Elevated levels of LDHB were detected in Aβ-resistant PC12 cells, compared to sensitive cells. Attenuation of LDHB expression using shRNA-mediated silencing resulted in increased mitochondrial membrane potential, ROS levels, and neurotoxicity in Aβ-resistant PC12 cells. Additionally, the effect of monocarboxylate (pyruvate and lactate) transport inhibition on PC12 cell viability, was investigated. In contrast to Aβ-sensitive PC12 cells, resistant cells were tolerant of monocarboxylate transporter inhibition while all cell lines were intolerant of mitochondrial pyruvate transporter inhibition. These findings indicate that LDHB may regulate mitochondrial respiration and sensitivity to Aβ in PC12 cells.

Key words: Lactate dehydrogenase isoform B, Reactive oxygen species, mitochondrial membrane potential, amyloid beta, hydrogen peroxide, monocarboxylate transport

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List of Abbreviations

AD	Alzheimer’s disease
ATP	Adenosine Triphosphate
DMSO	Dimethyl Sulfoxide
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
HS	Horse Serum
IMM	Inner Mitochondrial Membrane
IMS	Intermembrane Space
kDa	Kilo Dalton
LDHA	Lactate Dehydrogenase Isoform A
LDHB	Lactate Dehydrogenase Isoform B
MCT	Monocarboxylate Transporter
mM	Millimolar
MPC	Mitochondrial Pyruvate Carrier
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
NFT	Neurofibrillary Tangle
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
OMM	Outer Mitochondrial Membrane
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene Fluoride
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide Dismutase
VDAC	Voltage Dependent Anion Channel
µM	Micromolar
$\Delta\psi_m$	Mitochondrial Membrane Potential

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Chapter 1: Introduction

1.1 – Alzheimer's disease Overview

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders impacting the aging population and is characterized by progressive memory loss, cognitive decline and, eventually death. Initially described by Alois Alzheimer in 1906, AD accounts for up to 80% of all cases of dementia and has a tremendous financial impact on society (Alzheimer's Association 2014). In 2010, over 36 million individuals were estimated to be suffering from dementia worldwide, with treatment costs of patients suffering from AD and related dementias estimated at $604 billion (Alzheimer's Disease International 2012). By 2050, it is estimated that 115 million individuals will suffer from AD, and associated healthcare costs are expected to increase dramatically as a result (Alzheimer's Disease International 2012).

Currently, there is no cure for AD and treatment options remain limited, necessitating further research into causes and potential new therapies for the disease. Increasing age remains the greatest risk factor for developing AD, with the majority of affected individuals developing the disease sporadically after 65 years of age (late onset AD). However, mutations in *amyloid precursor protein (APP)*, *Presenilin 1 (PSEN1)* or *Presenilin 2 (PSEN2)* lead to an early onset familial form of AD (Chartier-Harlin *et al.* 1991; Levy-Lahad *et al.* 1995). AD pathology is characterized by the accumulation of extracellular amyloid beta (Aβ) plaques and intraneuronal neurofibrillary tangles (NFTs) in the brain; two events associated with neuronal cell death.

The cortex and hippocampus, which govern higher cognitive functions and memory, respectively, are brain regions that degenerate during AD progression and exhibit significant plaque and tangle accumulation (Schmechel *et al.* 1993; West *et al.* 1994). The extracellular plaques are formed by the aggregation of Aβ peptides, a 40-42 amino acid peptide produced from the proteolytic cleavage of amyloid precursor protein (APP) by beta-secretase and the gamma-secretase intramembrane protease complex. NFTs are formed following hyperphosphorylation of the microtubule-associated protein tau,

causing it to self-aggregate (Bancher *et al.* 1989). As a result, neuronal microtubule organization becomes compromised due to the inability of tau to bind and stabilize them.

1.2 – Causes of Alzheimer's disease

Although the exact mechanism driving AD progression is currently unknown, the production and accumulation of Aβ peptides and plaques within the brain are strongly suggested to be the primary causative factor in AD pathogenesis and development. This view, known as the amyloid hypothesis, has garnered much support within the scientific community. First, individuals possessing mutations in *APP*, *PSEN1*, or *PSEN2* develop early-onset familial AD (Chartier-Harlin *et al.* 1991; Levy-Lahad *et al.* 1995). Mutations in *APP* favour proteolytic processing of APP by beta and gamma secretases, and have been demonstrated to promote Aβ peptide formation and accumulation *in vivo* (Scheuner et al. 1996). Mutations in *PSEN1* and *PSEN2* also result in increased Aβ peptide production *in vivo* (Scheuner *et al*. 1996). In addition, inheritance of the apolipoprotein E (APOE) ε4 allele has been identified as a major risk factor for the development of late-onset AD, where up to 80% of individuals suffering from the disease possess at least one copy of APOEε4 (Mahley *et al.* 2006). The APOE family is a class of apolipoproteins that promote the proteolytic degradation or clearance of Aβ peptide, however, the APOEε4 isoform is not nearly as effective at accomplishing this task (Jiang *et al.* 2008). Therefore, individuals possessing one or more copies of the APOEε4 allele exhibit increased risk of Aβ plaque accumulation in the brain compared to those who have not inherited it (Jiang *et al.* 2008). Furthermore, mutations in *Triggering receptor expressed on myeloid cells 2* (*TREM2*), a gene encoding Ig superfamily receptors involved in inflammatory responses, have been associated with compromised microglia-mediated clearance of Aβ plaques from the human brain, increasing the likelihood of developing late onset AD (Guerreiro *et al.* 2013; Jonsson, *et al.* 2013).

Investigations by De Felice *et al.* (2007) have shown that, in rat hippocampal neurons and neuroblastoma cells, soluble Aβ oligomers promote tau phosphorylation at specific epitopes demonstrated to be hyperphosphorylated in AD. Additionally, studies in which mice overexpress mutant APP (mAPP) and tau exhibit NFT accumulation in the cortex

and hippocampus, whereas mice overexpressing mutant tau alone do not (Lewis *et al.* 2001). Therefore, these studies suggest that Aβ promotes the formation and deposition of NFTs in brain regions affected by AD. Taken together, these findings provide compelling evidence supporting Aβ production and accumulation as the primary driving force for AD pathogenesis and progression.

1.3 – APP Processing and Aβ Peptide Formation

APP is a highly conserved, widely expressed transmembrane protein present in both vertebrate and invertebrate species. APP is highly localized to neuronal membranes, including the endoplasmic reticulum (ER) and Golgi complex, the plasma membrane, as well as mitochondrial membranes (Hartmann *et al.* 1997). Although the physiological role of APP is not well characterized, recent studies suggest that it may regulate synapse formation and function, neuroplasticity, and hormonal regulation (Priller *et al.* 2006). Interestingly, only the vertebrate form of the APP protein contains the pathological 40 – 42 peptide fragment sequence associated with AD (Tharp and Sarkar 2013). Like many other proteins, APP is subject to a host of post-translational modifications, including phosphorylation, glycosylation, but also proteolytic cleavage, which can result in the generation of Aβ peptides. Proteolytic processing of APP is accomplished by secretases, and the resulting peptides can be classified as either amyloidogenic, which results in the formation of the neurotoxic Aβ peptide, or non-amyloidogenic. In non-amyloidogenic processing, alpha secretases prevent pathological Aβ production by cleaving within the APP fragment that normally gives rise to Aβ peptide (De Strooper and Annaert 2000; Selkoe *et al.* 1998). This yields a non-pathological, soluble p3 peptide that can be readily cleared by the brain (Selkoe *et al.* 1998). However, in amyloidogenic processing, APP is sequentially cleaved by beta and gamma secretases, the latter of which forms a complex with PSEN1 and PSEN2 to fulfill its function. Cleavage of the extracellular APP domain by beta secretases yields a membrane-bound amyloidogenic C-terminal fragment (C99), which is subsequently processed by the gamma secretase protease complex, releasing the pathogenic Aβ peptide (Selkoe *et al.* 1998). The Aβ peptide formed is typically 36 – 43 amino acids in length, with the $Aβ_{40}$ and $Aβ_{42}$ isoforms being the most prevalent (Hartmann *et al.* 1997). Aβ peptides, in particular the $Aβ_{42}$ isoform, readily self-

aggregate, forming soluble oligomers and insoluble fibrils and plaques, all of which are neurotoxic and promote progression of AD pathology.

1.4 – Oxidative Stress in the AD Brain

In addition to the Aβ plaque and NFT accumulation typical of AD pathology, many studies have also observed that the AD brain displays markers of increased oxidative stress and damage (Zhao and Zhao 2013; Butterfield *et al.* 2001; Markesbery 1997). Numerous studies have suggested that Aβ accumulation in the AD brain promotes an imbalance between the production and clearance of reactive oxygen species (ROS) by antioxidant defense mechanisms (Butterfield *et al.* 2001; Markesbery 1997). ROS are an oxygen-containing class of chemically reactive molecules typically formed as byproducts of normal cellular metabolism. Common forms of ROS found in cells include superoxide radicals ($O_2^{\bullet}$) and hydroxyl radicals ($OH^{\bullet}$), the latter of which is derived from hydrogen peroxide (H_2O_2). Despite having beneficial physiological roles, including maintaining cellular homeostasis and acting as second messengers in cell signaling, ROS, at high concentrations, can cause extensive damage to cellular structures and molecules, including lipids, nucleic acids, and proteins, thereby impairing function and promoting disease states (Devasagayam *et al.* 2004; Markesbery 1997; Halliwell 1987; Halliwell and Cross 1994). Lipids, principle components of cellular membranes, readily undergo ROS-induced peroxidation that can compromise membrane integrity and function (Butterfield *et al.* 2001; Markesbery 1997). Oxidative damage to nucleic acids can promote mutagenesis and carcinogenesis, while protein oxidation can negatively impact enzyme structure, and therefore function (Butterfield *et al*. 2001; Markesbery 1997). Antioxidant defense systems protect against oxidative damage by neutralizing potentially harmful ROS. These systems include manganese superoxide dismutase (Mn-SOD), catalases, peroxiredoxins, and glutathione peroxidases.

The human brain possesses several characteristics that make it extremely susceptible to oxidative damage, which is readily apparent in AD. First, the brain derives approximately 95% of its energy, in the form of ATP, from the oxygen-dependent metabolism of glucose in mitochondria (Chandrasekaran *et al.* 1994). This process, also known as

oxidative phosphorylation (OXPHOS), depends upon the transfer of electrons between protein complexes embedded in the inner mitochondrial membrane (IMM), to generate a proton (H^+) gradient across the IMM, which is used to drive ATP production. In addition, OXPHOS is also a major potential source of intracellular ROS production. In the AD brain, mitochondrial electron transfer is impaired due to decreased ETC protein complex activity, resulting in the accumulation of H^+ in the mitochondrial intermembrane space (IMS), and thus, an increased mitochondrial membrane potential ($\Delta\psi_m$) across the IMM (Parker *et al.* 1994; Lin and Beal 2006; Chandrasekaran *et al.* 1994; Manczak *et al.* 2006). These conditions increase the likelihood of electron off-loading from the ETC to react with available oxygen, forming ROS and promoting widespread oxidative damage in the brain (Lin and Beal 2006; Manczak *et al.* 2006). Oxidative damage observed in the AD brain includes significantly increased nuclear and mitochondrial DNA oxidation, lipid peroxidation, as well as protein oxidation (Markesbery 1997; Butterfield *et al.* 2001). Second, studies demonstrate that the brain has significantly reduced levels of antioxidant defenses, such as SOD and catalase activity, compared to other tissues (Lovell *et al.* 1998; Behl 1997; Marcus *et al.* 1998). In AD, this inability to effectively detoxify ROS may result in increased oxidative damage and eventual neuronal cell death in the brain (Behl 1997; Marcus *et al.* 1998). Third, the brain has a naturally high lipid content, specifically polyunsaturated fats (PUFAs), which are readily oxidizable by ROS (Butterfield *et al.* 2001; Markesbery 1997). In AD, elevated concentrations of ROS in the brain induce the peroxidation of PUFAs and subsequent formation of 4-hydroxynonenal (4-HNE), a highly reactive byproduct that promotes oxidative damage and neurotoxicity (Butterfield *et al.* 2001; Markesbery and Lovell 1998). 4-HNE has been shown to inhibit glycolysis, as well as nucleic acid and protein synthesis. 4-HNE is also involved in protein oxidation. For example, treatment of rat hippocampal neurons with Aβ has proven to significantly increase levels of 4-HNE, which directly impairs Na^+/K^+ ATPase activity and increases intracellular levels of calcium. This disruption of calcium homeostasis promoted further ROS production and cytotoxicity, resulting in neuronal cell death (Mark *et al.* 1997). Furthermore, the activity of glutathione S-transferase, an antioxidant enzyme that mediates detoxification of 4-HNE, is significantly decreased in all brain regions of AD patients compared to controls (Lovell *et al.* 1998).

Thus, the brain has a large capacity for the production of ROS, and is also highly susceptible to ROS-induced damage due to its physiology and metabolism. Together, these observations provide support for the Oxidative Stress Hypothesis of AD, which suggests that the accumulation of oxidative damage in the brain over time contributes to the progressive neurodegeneration characteristic of the disease. Interestingly, mounting evidence suggests that the observed increase in oxidative damage in the AD brain may be directly attributable to Aβ accumulation and mitochondrial dysfunction.

1.5 – Aβ Accumulation in the Brain is Associated with Increased Oxidative Stress

For the past two decades, studies have presented growing evidence that Aβ may exert its toxic effect, in part, by elevating oxidative stress in the brain. Investigations by Behl *et al.* (1994) demonstrate that treatment of primary and clonal cell lines, derived from the mammalian central nervous system (CNS), with exogenous Aβ is neurotoxic and induces significant intracellular accumulation of H_2O_2 in a concentration and time-dependent manner. Treatment of these cell lines with Aβ in the presence of various enzymatic and non-enzymatic antioxidants rescues cell viability and reduces intracellular levels of H_2O_2, while treatment with increasing concentrations of H_2O_2 alone mimics the detrimental effect of Aβ on cell survival (Behl *et al.* 1992; Behl *et al.* 1994). Interestingly, clonal cell lines that were continually selected for resistance to Aβ, are also resistant to H_2O_2 toxicity (Behl *et al.* 1994). Later *in vivo* studies corroborated these findings by demonstrating that the production of H_2O_2, and other ROS, are directly correlated with levels of soluble Aβ peptides (Manczak *et al.* 2006; McLellan *et al.* 2003; Xie *et al.* 2013). In a similar fashion to cell culture models, Aβ-induced increases in oxidative stress results in neuronal degeneration of the transgenic AD mouse brain *in vivo* (Manczak *et al.* 2006; McLellan *et al.* 2003; Xie *et al.* 2013). These works suggest that, both *in vitro* and *in vivo*, the neurotoxic effect of Aβ may be mediated by H_2O_2 or one of its derivatives. Furthermore, other studies have shown that H_2O_2 may mediate its toxic effect via its conversion to $OH^{\bullet}$. H_2O_2, in the presence of iron or copper ions, readily oxidizes iron (II) to iron (III) with the production of $OH^{\bullet}$ as a byproduct, a process known as the Fenton reaction (Halliwell and Gutteridge 1984). Interestingly, both oxidation states of iron have been shown to specifically localize with Aβ plaques and

NFTs in the human AD brain (Smith *et al.* 1997). $OH^{\bullet}$, one of the most reactive forms of ROS, readily promotes oxidative damage within cells, including protein and nucleic acid oxidation as well as lipid peroxidation (Halliwell and Cross 1994; Halliwell and Gutteridge 1990). Moreover, reaction of $OH^{\bullet}$ with other biomolecules has the potential to produce additional ROS, potentiating further oxidative damage (Halliwell and Gutteridge 1990). Due to its extremely short half-life (approximately 10^{-9} seconds) and high rate of reaction (rate constant $\cong 10^{9} - 10^{10}$ $M^{-1}sec^{-1}$), $OH^{\bullet}$ is difficult to detoxify and rapidly reacts with biological molecules, making it one of the most dangerous and potentially devastating form of oxygen free radicals (Sies 1993; Halliwell and Cross 1994).

H_2O_2 accumulation in AD has been suggested to promote further Aβ production by potentially increasing the activity of β- and γ-secretases *in vitro* and *in vivo*. Investigations by Bourne *et al.* (2007) revealed that H_2O_2 activates nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor involved in stress response and cell survival, in neuronal cell populations. NF-κB is also activated in response to exogenous Aβ exposure (Behl *et al.* 1994). The promoter of the human β-secretase/β-site APP cleaving enzyme (BACE1) gene contains a NF-κB binding site, and studies in which NF-κB subunit p65 is overexpressed resulted in increased activity of β-secretase as well as increased Aβ production in human neuroblastoma cell lines (Chen *et al.* 2012). Alternatively, in a study by Tamagno *et al*. (2005), 4-HNE, the reactive byproduct formed from ROS-induced lipid peroxidation, increases the expression and activity of β-secretase as well, resulting in increased Aβ accumulation. In addition, H_2O_2 enhances γ-secretase-mediated cleavage of C99 in human neuroblastoma SH-SY5Y cells by promoting the phosphorylation and activation of c-Jun N-terminal kinase (JNK), a stress-activated enzyme that modifies the activity of other proteins via phosphorylation (Shen *et al.* 2008). Although active JNK (p-JNK) appears to be required for H_2O_2-induced increases in γ-secretase activity and C99 cleavage, the underlying molecular mechanism by which this occurs remains to be determined (Shen *et al.* 2008). Interestingly, p-JNK was observed to co-localize in brain regions surrounding Aβ plaques in the AD transgenic mouse model Tg2576 (Shen *et al.* 2008). Moreover, hippocampal

and cortical brain tissues from AD patients express higher levels of p-JNK compared to controls (Shen *et al.* 2008). Together, these findings suggest that Aβ-induced increases in H_2O_2 and other ROS induce the oxidative damage and neurotoxicity associated with AD, but may also increase the activity of β- and γ-secretases, further potentiating Aβ production in a positive feedback loop. Therefore, these studies provide strong evidence that Aβ may mediate its neurotoxic effect through ROS.

1.6– Aβ-induced Mitochondrial Dysfunction and Oxidative Stress

Although it only accounts for approximately 2% of total body weight, the human brain requires large amounts of energy to function, utilizing as much as 20 – 25% of total body metabolism (Mink *et al.* 1981). Glucose is the primary metabolite processed by the brain to yield usable energy in the form of ATP, however lactate and ketone bodies can also be metabolized. Specifically, glucose is converted into pyruvate in the cytosol via a series of enzyme-catalyzed reactions, a process known as glycolysis. Glycolysis also yields the electron carrier NADH and ATP, as byproducts. Pyruvate is then imported into the mitochondrial matrix and converted to acetyl coenzyme A (acetyl-CoA), via the activity of the pyruvate dehydrogenase (PDH) complex, with concomitant NADH production. Acetyl-CoA is further processed via the Krebs cycle to CO_2, yielding the electron carriers NADH and $FADH_2$. The majority of glucose-derived ATP, required for proper neuronal function, is produced via OXPHOS. OXPHOS is dependent upon the transfer of electrons, donated by NADH and $FADH_2$, between protein complexes embedded in the IMM, which compose the mitochondrial ETC. This electron transfer generates a H^+ gradient across the IMM, increasing the $\Delta\psi_m$ as a result. The H^+ gradient is used by the enzyme ATP synthase to produce enough ATP to meet a neuron's energy requirements.

In addition to ATP production, mitochondria are also a major source of endogenous ROS in neuronal cells and are also important regulators of apoptosis. During normal OXPHOS, a small percentage of electrons may leak from the ETC, univalently reducing oxygen and forming $O_2^{\bullet}$ as a byproduct. In neuronal mitochondria, Mn-SOD neutralizes $O_2^{\bullet}$ by converting it to O_2 and H_2O_2. Mitochondrial dysfunction, characterized by decreased activity of the ETC protein complexes, results in increased electron leakage

and ROS production, promoting oxidation of mitochondrial proteins, DNA, and lipids (Butterfield *et al.* 2001; Markesbery 1997). Increasing oxidative damage within the mitochondria eventually results in the release of cytochrome c, a component of the mitochondrial ETC, to the cytoplasm, where it can initiate apoptosis-mediated neuronal cell death by promoting capase-9 activity.

Previous studies have suggested that Aβ may play a role in promoting increased mitochondrial dysfunction and ROS production in AD, although the exact mechanism by which this is accomplished has not been well characterized. For example, *in vivo* studies of transgenic mice expressing mutant APP (tg-mAPP) demonstrate gradual accumulation of Aβ in the mitochondrial matrix of neurons derived from the cerebral cortex (Manczak *et al.* 2006). Interestingly, this accumulation is closely associated with decreased activity of coenzyme Q-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV), two major protein complexes of the mitochondrial ETC (Manczak *et al.* 2006). Further investigations suggest that this mitochondrial dysfunction may be partly mediated by the interaction of Aβ and amyloid-beta peptide binding alcohol dehydrogenase (ABAD). ABAD, primarily localized within the mitochondria of neurons, oxidizes a wide variety of alcohol substrates (Chen and Yan 2007). ABAD is upregulated in AD-afflicted brain regions of tg-mAPP mice, including the hippocampus and cortex, and readily binds Aβ peptides and oligomers (Lustbader *et al.* 2004; Chen and Yan 2007; He *et al.* 2002; Yan *et al.* 1997). Studies in cultured neurons derived from transgenic mice expressing mAPP and ABAD (tg-mAPP/ABAD) by Takuma *et al.* (2005) suggest that elevated levels of mitochondrial-derived ROS, specifically $O_2^{\bullet}$ and H_2O_2, are due to decreased enzymatic activity of ETC complex IV, and is dependent on the interaction between Aβ and ABAD. In addition, these neurons also display increased caspase-3-like activity, suggesting that this Aβ-ABAD interaction, by promoting mitochondrial dysfunction, induces enough oxidative damage to produce a cytotoxic environment to neurons, resulting in apoptosis-mediated cell death (Takuma *et al.* 2005). Interference of Aβ-ABAD binding, however, prevents increased mitochondrial ROS production and related oxidative damage (Takuma *et al.* 2005).

1.7 – Resistance to Aβ Toxicity: The Warburg Effect

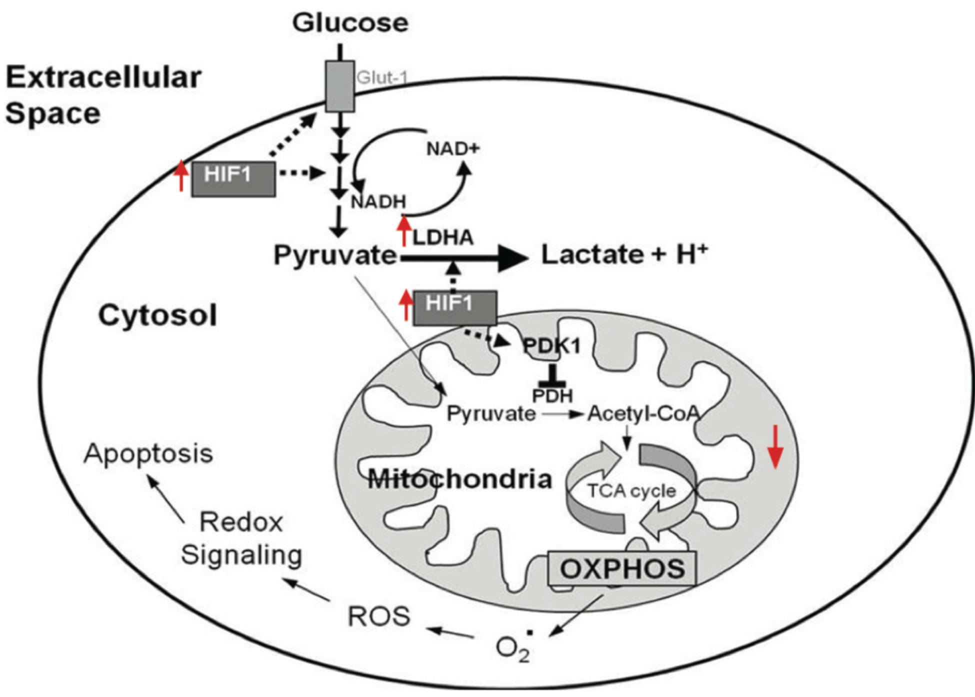
Despite the abundant amount of evidence supporting a strong association between Aβ accumulation and ROS-mediated neuronal toxicity, it has been observed that nearly 30% of elderly individuals demonstrate a significant degree of Aβ plaque accumulation in various brain regions, yet they do not exhibit the characteristic neuronal cell death or cognitive impairment associated with AD (Price and Morris 1999; Bouras *et al.* 1994). Intriguingly, this suggests that the neurons of these individuals may have an innate resistance mechanism that protects against progressive Aβ accumulation and the neurotoxic environment that it creates.

In order to better understand how Aβ-resistance arises, past investigations have continuously treated Aβ-sensitive neuronal cell lines with toxic levels of Aβ and isolated any emergent surviving populations as Aβ-resistant clonal cell lines. These stable Aβ-resistant cell lines demonstrated a characteristic increase in glucose uptake and metabolism, an extreme sensitivity to glucose starvation, and preferentially utilized glycolysis over mitochondrial respiration as a primary form of ATP production, even under normal oxygen conditions (Soucek *et al.* 2003). This phenomenon, known as Aerobic Glycolysis, or the Warburg Effect, was first studied and characterized in cancer cell models and is partially mediated through the activity of hypoxia inducible factor-1 (HIF-1) (Figure 1) (Soucek *et al.* 2003; Semenza *et al.* 2007).

HIF-1 is a heterodimeric protein, composed of HIF-1α and HIF-1β subunits that function in transcriptional activation and nuclear translocation, respectively (Wang and Semenza 1995; Jiang *et al.* 1996). HIF-1α binds HIF-1β to form the fully functional HIF-1 heterodimer under hypoxic conditions, however, at higher oxygen concentrations, HIF-1α is hydroxylated by prolyl hydroxylases, ubiquitinated and then degraded via the proteasome (Maxwell *et al.* 1999). In cancer cells, HIF-1α is stabilized, allowing HIF-1 to act as a transcription factor to induce the expression of many genes involved in cellular metabolism, including pyruvate dehydrogenase kinase 1 (PDK1) (Kim *et al.* 2006; Semenza *et al.* 2007). PDK1 functions by phosphorylating and inhibiting PDH, the

Figure 1. Proposed model of Aerobic Glycolysis (The Warburg Effect). Increased HIF-1 activity in Aβ-resistant neuronal cells results in upregulation of glucose transporters and glycolytic enzymes, thereby increasing glucose conversion to pyruvate in the cytosol. Furthermore, HIF-1 activity promotes the reduction of pyruvate to lactate by increasing the activity of lactate dehydrogenase A (LDHA). HIF-1 also increases activity of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inhibits pyruvate dehydrogenase (PDH), inhibiting conversion of pyruvate to acetyl coenzyme A (acetyl-CoA) in the mitochondrial matrix. Lack of availability of acetyl-CoA prevents the generation of reducing equivalents required for oxidative phosphorylation across the IMM. Decreased activity of electron transport may lower the probability of reactive oxygen species (ROS) generation in the mitochondria, thereby conferring resistance to Aβ-mediated toxicity. Dashed lines represent HIF-1-mediated upregulation of enzymatic activity. Reproduced from ©Newington *et al.* 2011.

Figure 1



Newington et al. 2011

mitochondrial matrix-localized enzyme complex that catalyzes the decarboxylation of pyruvate to acetyl-CoA. Inhibition of PDH reduces the availability of acetyl-CoA, the substrate utilized by the Krebs cycle, to generate the electron carriers NADH and $FADH_2$, which are ultimately used to drive ATP production via OXPHOS in the mitochondria. Interestingly, HIF-1 also increases expression of many glucose transporters and glycolytic enzymes, including glucose transporter-1 (Glut1), aldolase A, and enolase 1 (Semenza *et al.* 1996). In addition, HIF-1 promotes the expression of lactate dehydrogenase isoform A (LDHA), an enzyme that catalyzes the conversion of pyruvate to lactate, with the concomitant oxidation of NADH to NAD^+ (Semenza *et al.* 1996). LDHA, together with the lactate dehydrogenase isoform B (LDHB) enzyme, form the tetrameric holoenzyme lactate dehydrogenase (LDH). It is currently unknown whether or not HIF-1 regulates *LDHB* expression, although future studies could be conducted to determine if HIF-1 binds the LDHB promoter. In contrast to LDHA, LDHB catalyzes the conversion of lactate to pyruvate coupled with the reduction of NAD^+ to NADH. Both *LDHA* and *LDHB* are nuclear-encoded and located on chromosome 11 and 12, respectively. The relative number of LDHA or LDHB enzymatic subunits composing LDH determines the overall activity of the holoenzyme. For instance, there are 5 distinct LDH holoenzymes determined by the LDHB to LDHA ratio: LDH1 (4:0), LDH2 (3:1), LDH3 (2:2), LDH4 (1:3), and LDH5 (0:4). Therefore, through the activity of HIF-1, lactate production is increased and mitochondrial respiration is decreased in cancer cells, suggesting this cell type is highly dependent on glycolysis to produce ATP, in order to maintain normal energy requirements.

Previous investigations revealed that Aβ-resistant neuronal cell populations may adopt a Warburg Effect-like metabolism, demonstrating increased HIF-1, PDK1, and LDHA activity in comparison to Aβ-sensitive controls (Soucek *et al.* 2003; Newington *et al.* 2011). While knockdown of PDK1 or LDHA in Aβ-resistant PC12 cells induces re-sensitization to Aβ toxicity (Newington *et al.* 2011), overexpression of PDK1 or LDHA in Aβ-sensitive neuronal cells has been shown to confer resistance to toxic levels of exogenous Aβ and H_2O_2, both of which induce apoptosis-mediated cell death (Newington *et al.* 2012). Furthermore, PDK1 or LDHA overexpressing cells display decreased

oxygen consumption and $\Delta\psi_m$, but maintain ATP levels comparable to cells expressing low levels of PDK1 or LDHA (Newington *et al.* 2012). In addition, Aβ-sensitive cells overexpressing PDK1 or LDHA exhibit significantly decreased mitochondrial ROS levels following treatment with exogenous Aβ, compared to controls (Newington *et al.* 2012). Taken together, these data suggest that PDK1 or LDHA overexpression may confer Aβ resistance in mammalian neuronal cell populations by inhibiting mitochondrial respiration and ROS production, while maintaining normal ATP levels through the glycolytic pathway. Thus aerobic glycolysis may provide a general resistance mechanism to Aβ and other toxins in neurons.

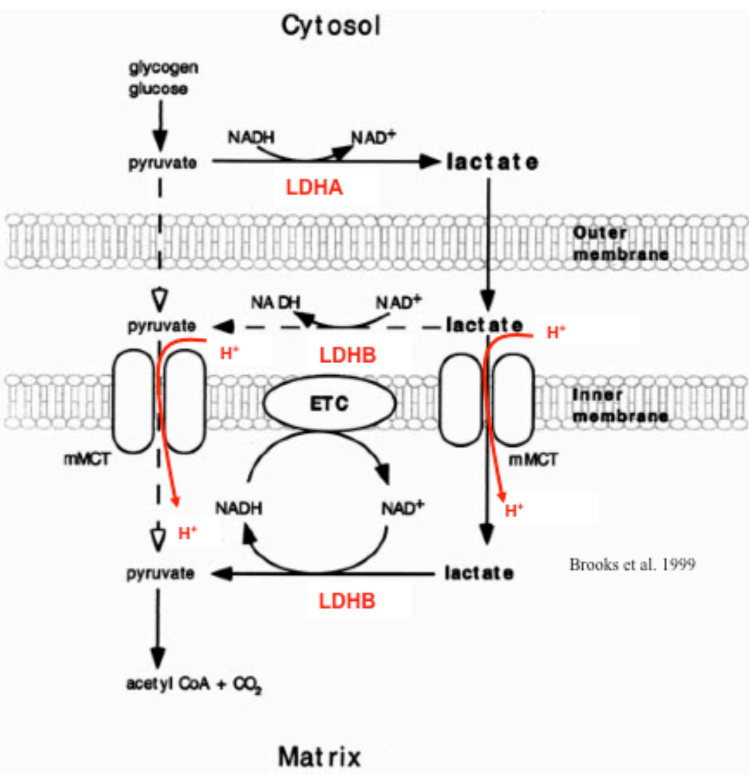
1.8– Lactate Metabolism and The Intracellular Lactate Shuttle Hypothesis

Although increased LDHA activity appears to be important for imparting resistance to Aβ toxicity in neuronal cells, the role of lactate and its metabolism have not been investigated in this regard. Typically, lactate has been considered a metabolic waste product that becomes cytotoxic at high concentrations and is removed from neuronal cells via monocarboxylate transporters (MCT) 1, 2, and 4. However, lactate may play a vital role for proper neuronal function, as a number of studies have found that lactate is preferred over glucose as an energy substrate in neurons (Bouzier-Sore *et al.* 2003; Wyss *et al.* 2011). Metabolism of lactate is achieved primarily through the activity of LDH.

Brain metabolism of lactate has been previously examined from the perspective of the Intracellular Lactate Shuttle (ILS) Hypothesis, which suggests that LDHA and LDHB may be localized in different subcellular compartments (Santos de Oliveira Cruz *et al.* 2012; Brooks *et al.* 1999) (Figure 2). Specifically, the ILS Hypothesis posits that, in the cytosol, LDHA catalyzes the reduction of pyruvate to lactate. Lactate is then transported across the outer mitochondrial membrane (OMM), and is oxidized back to pyruvate, via the catalytic activity of LDHB, in the IMS. Pyruvate can then be co-transported with H^+ from the IMS to the mitochondrial matrix via monocarboxylate transporters (MCTs) or the mitochondrial pyruvate carrier (MPC) embedded in the IMM. Alternatively, lactate may be co-transported with H^+ directly to the mitochondrial matrix by MCTs, where it is

Figure 2. Schematic of the Intracellular Lactate Shuttle Hypothesis. Lactate is a monocarboxylate transported into the mitochondrial intermembrane space. Cytosol-localized pyruvate is reduced to lactate, which is imported into the mitochondrial matrix via MCT transporters. Matrix-localized LDHB promotes the oxidation of lactate to pyruvate, which then enters the TCA cycle to yield reducing equivalents for OXPHOS. Lactate may also be converted to pyruvate in the intermembrane space. The effect of altered NADH/NAD^+ ratios on OXPHOS following increased LDHB activity is currently unknown. Solid lines represent a proposed pathway by which lactate or pyruvate may be imported into the mitochondria, while dashed lines represent an alternate pathway. Modified from ©Brooks *et al*. 1999.

Figure 2



then oxidized to pyruvate by LDHB within the mitochondrial matrix. PDH then catalyzes the conversion of pyruvate to acetyl-CoA, which is then processed by the TCA cycle and OXPHOS to generate ATP. Interestingly, this co-transport of H^+ with either pyruvate or lactate may help protect against oxidative stress by partly dissipating the $\Delta\psi_m$ and reducing the likelihood of mitochondrial ROS production. Recent investigations by Hashimoto *et al.* (2008) support the notion of mitochondrial-localized oxidation of lactate via a mitochondrial lactate oxidation complex (mLOC), composed of cytochrome oxidase, MCT1/2, and LDHB, in the IMM of rat neuronal cells. This mLOC readily mediates the conversion of lactate to pyruvate in the IMS (Hashimoto *et al.* 2008). Although this suggests that neurons may possess intracellular machinery allowing them to metabolize lactate, the impact of mitochondrial-localized LDHB on mitochondrial activity is not well characterized. It should be noted, however, that the intracellular localization of LDHA and LDHB, in either the cytosol or mitochondria, remains controversial. Furthermore, despite evidence suggesting that lactate metabolism plays a vital role in neuronal function, the mechanism by which it may mediate Aβ-resistance remains to be determined.

1.9 – Rationale, Hypothesis, and Research Outline

Resistance to Aβ toxicity is observed in neuronal cell populations that adopt a Warburg Effect-like metabolism, where glucose uptake and glycolysis are upregulated but mitochondrial respiration is diminished (Newington *et al.* 2011; Newington *et al.* 2012). As a result, neuronal cells are able to maintain normal levels of ATP, while mitochondrial ROS production and oxidative stress are limited (Newington *et al.* 2011; Newington *et al.* 2012). In addition, LDHA is another important enzyme that helps mediate this resistance, as reduced expression of this enzyme in cultured neurons results in re-sensitization to Aβ (Newington *et al.* 2011; Newington *et al.* 2012). These findings suggest that the conversion of pyruvate to lactate may play a role in conferring Aβ-resistance. Although LDHB activity catalyzes the reverse reaction, which produces pyruvate and NADH from lactate and NAD^+, its role has not been well characterized in AD models. A recent investigation by Ciavardelli *et al*. (2010), examining differential expression of LDHB in whole brain extracts of control and transgenic AD mice, reveals

significantly diminished expression of LDHB in the latter. Interestingly, LDHB expression was significantly upregulated in the cerebellum, a brain region unaffected by Aβ accumulation (Ciavardelli *et al.* 2010). Based on these results, the authors speculate that LDHB may be protective against AD pathology in brain tissue. However, the role of LDHB in attenuating Aβ toxicity in neurons has yet to be determined.

Intriguingly, both pyruvate and NADH have been implicated in reducing oxidative stress. First, NADH may fulfill an important role in glutathione and thioredoxin antioxidant defense mechanisms in neuronal mitochondria by mediating the regeneration of NADPH (Alvarez *et al.* 2003; Miranda-Vizuete *et al.* 2000; Vogel *et al.* 1999). In these pathways, NADPH is a key substrate that fuels a series of reduction-oxidation reactions that ultimately detoxify H_2O_2. NADPH regeneration is accomplished by $NADP^+$ transhydrogenase, an IMM-localized enzyme that utilizes the H^+ gradient across the IMM to drive electron transfer from NADH to $NADP^+$ (Rydström 2006; Vogel *et al.* 1999). Interestingly, $NADP^+$ transhydrogenase-mediated NADPH regeneration and glutathione antioxidant accumulation have been observed in rat forebrain mitochondria (Vogel *et al.* 1999). Thioredoxin enzymes, which are dependent on NADPH for antioxidant activity, are also protective in mammalian neuronal cell lines (Miranda-Vizuete *et al.* 2000). Second, in addition to being a central metabolic substrate, pyruvate has been shown to function as a potent antioxidant and can directly neutralize H_2O_2 and other peroxides via a non-enzymatic decarboxylation reaction (Constantopoulos and Barranger 1984; Vlessis *et al.* 1990). Studies by Desagher *et al.* (1997) demonstrated that pyruvate protects against H_2O_2-induced neurotoxicity in mouse primary cortical neurons by undergoing non-enzymatic decarboxylation in the presence of H_2O_2. Interestingly, pyruvate was neuroprotective at relatively low concentrations, whereas high concentrations were ineffective (Desagher *et al.* 1997). In addition, lactate was not neuroprotective against H_2O_2 (Desagher *et al.* 1997). Futhermore, Alvarez *et al.* (2003) showed that pyruvate protects against Aβ toxicity and reduces caspase-3 activation by limiting mitochondrial ROS accumulation and oxidative damage in rat primary cortical neurons. Importantly, this protection was dependent upon the import of pyruvate into the mitochondria (Alvarez *et al.* 2003). Therefore, LDHB may mediate resistance to Aβ toxicity in neurons by

supplying substrates with the capacity to directly or indirectly limit Aβ–induced increases in $\Delta\psi_m$ and mitochondrial ROS levels. This in turn, may reduce overall oxidative damage and protect against neurotoxicity.

Investigations by Soucek *et al.* (2003) demonstrated that Aβ-resistant PC12 cells may exhibit increased production of pyruvate, compared to Aβ-sensitive cells, due to upregulated glycolytic activity. Although pyruvate functions as a potent antioxidant in neuronal mitochondria exposed to Aβ (Alvarez *et al.* 2003), the importance of MPC activity, which facilitates the transport of pyruvate into the mitochondrial matrix, on neuronal viability has not been previously investigated. In addition, MCTs have an important role in the transport of pyruvate and lactate, preventing the cytotoxic accumulation in the latter. Aβ-resistant PC12 cells may therefore be more dependent on MCT activity, due to increased production of lactate. However, the effect of MCT inhibition on Aβ-resistant neuronal cell viability remains undetermined.

Therefore, the role of LDHB in aerobic glycolysis-mediated resistance to Aβ will be the primary focus of this investigation. In addition, the importance of the MPC as well as MCT1, 2, and 4 on viability will be evaluated in Aβ-resistant neuronal cells. This study will utilize an Aβ-sensitive PC12 cell line (PC12-P) and two Aβ-resistant cell lines (PC12-R1 & R7) as model systems. **I hypothesize that LDHB expression and activity are upregulated in mitochondria of Aβ-resistant PC12 cells, compared to the Aβ-sensitive parental cell line, resulting in decreased mitochondrial $\Delta\psi_m$, ROS levels, and cell death. Additionally, compared to the sensitive cell line, I predict that Aβ-resistant PC12 cell lines will be more sensitive to the chemical inhibition of MCTs as well as the MPC.** LDHB's role will be evaluated using plasmid shRNA knockdown technologies in PC12 cells. Experiments will be performed to ascertain whether knockdown of LDHB in PC12 cells will (1) promote increased levels of mitochondrial ROS, (2) induce increased $\Delta\psi_m$, and (3) result in decreased cell viability when exposed to toxic concentrations of Aβ. In addition, I will seek to elucidate whether LDHA and/or LDHB are localized to the mitochondria of PC12 cells.

Chapter 2: Materials and Methods

2.1 – Cell Culture

This investigation uses the immortalized, Aβ-sensitive PC12 nerve cell line (PC12-P) and its two Aβ-resistant derivatives (PC12-R1 & PC12-R7), provided by Dr. David Schubert (The Salk Institute, La Jolla, CA). These cell lines are subclones of the original PC12 clonal cell line, which itself, is derived from a rat pheochromocytoma. Studies by Greene and Tischler (1976) demonstrate that the PC12 cell line possesses inherent sympathetic neuronal properties including synthesis and secretion of catecholamine neurotransmitters, as well as becoming post-mitotic and forming neuronal-like processes following treatment with nerve growth factor, making it a useful model system for the study of neuropathologies. All PC12 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Biowhittaker, Walkersville, MD, USA), supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories Inc., Etobicoke, ON, Canada), 5% horse serum (HS) (PAA Laboratories Inc., Etobicoke, ON, Canada), and 1% penicillin/streptomycin (Biowhittaker, Walkersville, MD, USA). PC12 cells were incubated at 37°C with 5% CO_2. For Aβ toxicity studies, PC12 cells were cultured in DMEM containing half the level of serum, to ensure optimal sensitivity to Aβ peptide, as serum metabolites may be protective against the neurotoxic effect of Aβ.

2.2 – Construction of HuSH pRS shLDHB Vector & Derivation of LDHB-knockdown PC12 Cell Lines

To generate a rat *LDHB* knockdown vector derived from the HuSH pRS plasmid (OriGene, Rockville, MD), the coding sequence of rat *LDHB* was obtained from the National Center for Biotechnology Information database. Using this information, a forward primer (Invitrogen, Carlsbad, CA, USA) corresponding to the U6 promoter of the pRS vector and a reverse primer encoding *LDHB* shRNA were generated by Eurofins MWG Operon. The forward primer was 5' - GGCGAATTCCCCAGTGGAAAGACG - 3'. The reverse primer was 5' – AGAGGATCCAAAAAAGCCACTGGGTTGGAAACCACGATGATGGTGACC TCGAGCACACCATCATCGTGGTTTCCAACCCAGTGGCGCGTCCTTTCCACAAG

ATATATAAAC – 3'. Using the forward and reverse primers, as well as the U6 promoter within the HuSH pRS plasmid vector (OriGene, Rockville, MD) as a template, an amplicon encoding the U6 promoter followed by LDHB shRNA was generated through a polymerase chain reaction (PCR) – based strategy. The amplified sequence was digested with *Eco*RI and *Bam*HI restriction enzymes (New England BioLabs, Ipswich, MA, USA), as the *Eco*RI and *Bam*HI restriction sites were incorporated into the forward and reverse primers respectively. The digested PCR product was then ligated into a pRS vector, previously digested with *Eco*RI and *Bam*HI, yielding the HuSH pRS shLDHB vector.

For the purposes of my investigation, PC12-R1 and R7 cells were seeded in 60 mm cell culture dishes (BD BioSciences) and allowed to achieve approximately 70-80% confluency. Cells were transfected with either a pRS scrambled shRNA vector (control) or the pRS shLDHB plasmid. In accordance with standard transfection protocol, exactly 5μg of plasmid was added to 8μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 1mL of Opti-MEM I transfection medium (Invitrogen, Carlsbad, CA, USA), and added to PC12 cells which were then incubated at 37°C (5% CO_2, 95% O_2) for 6 hours. Transfection medium was replaced with normal DMEM and serum and allowed to recover at 37°C overnight. To ensure stable expression of either the pRS scrambled or pRS shLDHB vector, transfected cells were placed in DMEM containing 2μg/mL puromycin, as both vectors encode a puromycin resistance gene. Surviving clonal populations of PC12 cells transfected with the pRS shLDHB vector were screened for reduced LDHB protein expression, and compared against PC12 cells transfected with pRS scrambled vector, by immunoblot analysis.

2.3 – Immunoblot Analysis

To assess the expression levels of proteins of interest in the PC12 cell lines, cells were washed twice with 1x Dulbecco's Phosphate Buffered Saline (DPBS) (Biowhittaker, Walkersville, MD, USA), and harvested with lysis buffer (2% SDS, 50 mM Tris pH=7.5, 1 mM PMSF). Whole cell lysates were then sonicated and centrifuged at 4°C for 10 minutes at 13200 rpm. Protein levels were quantified by Lowry assay using a colourimetric DC™ Protein Assay Kit (BioRad, Hercules, CA, USA). Protein extracts were then reduced in 5X loading buffer containing 100 mM dithiothreitol (DTT) and 2% beta-

mercaptoethanol (BME) and boiled for 5 minutes. Protein samples were resolved on a 12% polyacrylamide gel by SDS-PAGE using a Mini-PROTEAN® gel electrophoresis apparatus (BioRad, Hercules, CA, USA), and then transferred onto polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA, USA) by overnight electroblotting at 4°C. PVDF membrane was washed 3x for 5 minutes in Tris-buffered saline with 1% Tween-20 (TBST), incubated in blocking solution (3% bovine serum albumin, 1% blotting milk) for at least 1 hour at room temperature, then washed with TBST another 3x for 5 minutes. Each membrane was then probed with a single primary antibody overnight at 4°C. The following antibodies were used in this investigation: polyclonal rabbit anti-LDHA 2012S (Cell Signaling, Danvers, MA, USA), monoclonal mouse anti-LDHB AF60H11 (AbFrontier, Seoul, South Korea), monoclonal mouse anti-Pyruvate Dehydrogenase E1-alpha subunit [8D10E6] ab110334 (Abcam, Cambridge, England, UK), monoclonal mouse anti-β-Actin A5441 (Sigma-Aldrich, St. Louis, MO, USA), polyclonal rabbit anti-Voltage Dependent Anion Channel (VDAC) (Cell Signaling, Danvers, MA, USA), monoclonal mouse anti-HA.11 16B12 (Covance), polyclonal rabbit anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Imgenex), polyclonal rabbit anti-monocarboxylate transporter 1 (MCT1) (Millipore, Etobicoke, ON, Canada), polyclonal rabbit anti-monocarboxylate transporter 2 (MCT2) (Millipore, Etobicoke, ON, Canada), polyclonal rabbit anti-monocarboxylate transporter 4 (MCT4) (Millipore, Etobicoke, ON, Canada). After overnight incubation with the primary antibody, the PVDF membrane was washed 3x for 10 minutes in TBST and incubated in blocking solution (3% BSA, 1% blotting milk, 1:10000 secondary antibody) containing an appropriate secondary antibody at room temperature for at least 1 hour. The following secondary antibodies were used in this investigation: blotting grade goat anti-rabbit IgG (H+L) Horseradish Peroxidase Conjugate 170-6515 (BioRad, Hercules, CA, USA) and blotting grade goat anti-mouse IgG (H+L) Horseradish Peroxidase Conjugate 170-6516 (BioRad, Hercules, CA, USA). Membranes were washed with TBST 3x for 5 minutes and developed using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Protein signal was detected by Molecular Imager® ChemiDoc™ XRS System (BioRad, Hercules, CA, USA).

2.4 – Trypan Blue Exclusion Test

The effects of Aβ on viability of PC12-P, R1-Scram, R7-Scram, R1-shLDHB7, and R7-shLDHB6 cells were assessed by trypan blue (VWR) dye exclusion test. PC12 cells were seeded at $1x10^5$ cells/well (PC12-P) or $2.5x10^4$ cells/well (PC12-R1-Scram/R7-Scram/R1-shLDHB7/R7-shLDHB6) in triplicate in 12-well dishes. Cells were allowed to adhere overnight at 37°C. The following day, cells were treated with 20μM $A\beta_{(25-35)}$ and incubated for 48 hours. Following this treatment, cell media (containing non-adherent, dead cells) from each well (1mL) was collected and placed in separate 15mL conical tubes (BD Biosciences). Exactly 100μL of pre-warmed TrypLE Express (Invitrogen, Carlsbad, CA, USA) was added to live, adherent cells in each well, then incubated for 5 minutes at 37°C to allow cells to detach from the plates. The collected cell media was added back to each respective well and placed back in separate 15mL conical tubes, yielding a suspension containing both live and dead cells. Conical tubes were centrifuged at 500x g for 5 minutes, followed by the removal of the supernatant, yielding a pellet of live and dead cells that were then suspended in 100μL of media. Exactly 100μL of trypan blue was added to each 100μL of cell suspension, mixed, and then loaded into a haemocytometer for counting. Cells that excluded trypan blue (white) are viable, whereas those that are unable to exclude the dye (blue) are dead. Cell viability was determined as a percentage based on the number of viable cells divided by the total number of cells per treatment.

2.5 – Mitochondrial Isolation and Proteinase K Protection Assay

The subcellular localization of LDHA and LDHB isoforms within neurons is highly debated among the scientific community, however, recent studies suggest both isoforms may localize within the mitochondria of rat neuronal cells (Hashimoto *et al.* 2008). The localization of LDHA and LDHB in the context of neuronal Aβ-resistance has not been previously investigated. Therefore, this study sought to determine if LDHA or LDHB isoforms are localized within the mitochondria of Aβ-resistant PC12 cells, via a proteinase K protection assay, which has been used in past investigations to remove potential cytosolic contaminants from crude mitochondrial isolates (Hitosugi *et al.* 2011). Proteinase K is a highly reactive, yet stable proteolytic enzyme with broad substrate

specificity, while Triton X-100 is a non-ionic detergent that permeabilizes biological membranes. Therefore, treatment of mitochondria with proteinase K alone results in the digestion of extramitochondrial proteins, while any intramitochondrial proteins are protected from digestion due to the mitochondrial double membrane. However, treatment of mitochondria with Triton X-100 compromises mitochondrial membrane integrity, allowing proteinase K to digest both extra- and intramitochondrial proteins.

To determine whether LDHA and/or LDHB proteins are predominantly localized in the mitochondria, subcellular fractionation of Aβ-resistant PC12 cells was performed. Mitochondria and cytosolic fractions were first isolated from PC12-R1 and R7 cells using a Mitochondria Isolation Kit for Mammalian Cells (Thermo Scientific, Rockford, IL, USA). Isolated mitochondrial pellets were then resuspended in Hepes Buffer (20 mM Hepes, 0.28M Sucrose, 1 mM EDTA, 5 mM $CaCl_2$) and subjected to the following treatments: 1) Untreated (Control), 2) 10μg/mL proteinase K incubated at 37°C for 3 hours, and 3) 10μg/mL proteinase K + 1% Triton X-100 incubated at 37°C for 3 hours. Following incubation, proteinase K activity was inhibited with the protease inhibitor PMSF (5 mM final concentration), and mitochondria were lysed with 2% SDS. Mitochondrial lysates were then resolved by SDS-PAGE followed by immunoblot analysis.

2.6 – Mitochondrial ROS Quantification in PC12 cells following Aβ exposure

Visualization of mitochondrial ROS levels in PC12 cells was accomplished using the fluorescent dye MitoTracker Red CM-H_2XRos (MTR) (Invitrogen, Carlsbad, CA, USA). MTR is a reduced dye that fluoresces upon its oxidation within mitochondria. Therefore, increased fluorescence intensity may be indicative of elevated ROS levels. Cells were seeded in 35 mm plastic cell culture dishes (pre-treated with 50μg/mL poly-D-lysine) at $1x10^6$ cells/dish (PC12-P) or $1x10^5$ cells/dish (PC12-R1/R7) and incubated at 37°C overnight. PC12 cells were then treated with 20μM $A\beta_{(25-35)}$ peptide for 48 hours. Following treatment, media was aspirated, replaced with media containing 100nM MTR, and incubated at 37°C for 20 minutes. Cells were then washed in pre-warmed DPBS containing 10μg/mL Hoescht stain for an additional 20 minutes, washed with DPBS, and placed in phenol red-free DMEM for visualization by fluorescence microscopy (Zeiss-

AxioObserver, 40x objective). Images were taken from four random fields of view for each dish using a Q Imaging (Retiga 1300 monochrome 10-bit) camera with Q Capture software. Fluorescent intensity of each image was quantified with ImageJ software.

2.7 – Analysis of mitochondrial membrane potential in PC12 cells following Aβ exposure

Mitochondrial membrane potential is generated by mitochondrial electron transport activity, which facilitates increased H^+ transport from the mitochondrial matrix to the IMS. In PC12 cells, $\Delta\psi_m$ was evaluated using the fluorescent dye tetramethylrhodamine methyl ester (TMRM) (Invitrogen, Carlsbad, CA, USA). TMRM is a lipophilic, red fluorescent dye that is readily sequestered within the negatively charged matrix of active mitochondria. Dissipation of $\Delta\psi_m$ induces leakage of TMRM from the matrix, and therefore, decreased fluorescent intensity. Cells were seeded in 35 mm plastic cell culture dishes (pre-treated with 50µg/mL poly-D-lysine) at $1x10^6$ cells/dish (PC12-P) or $1x10^5$ cells/dish (PC12-R1/R7) and incubated at 37°C overnight. PC12 cells were then treated with 20µM $A\beta_{(25-35)}$ peptide for 48 hours. Following treatment, media was aspirated, replaced with media containing 200nM TMRM, and incubated at 37°C for 20 minutes. Cells were then washed in pre-warmed DPBS containing 10µg/mL Hoescht stain for an additional 20 minutes, washed with DPBS, and placed in phenol red-free DMEM for visualization by fluorescence microscopy as previously described. Fluorescent intensity of each image was quantified with ImageJ software.

2.8 – MTT Assay

Inhibition of MCTs and the MPC were accomplished chemically using the general MCT inhibitor, α-cyano-4-hydroxycinnamic acid (CHC), and the specific MPC inhibitor, UK-5099. The effect of UK-5099 and CHC on the viability of PC12-P, R1, and R7 cells was assessed using an MTT colourimetric assay, in which only viable cells are able reduce the tetrazolium dye MTT, forming purple formazan crystals that can be subsequently solubilized. Non-viable cells are unable to produce these crystals. Therefore, the extent of purple colouring is indicative of the degree of viable cells. UK-5099 and CHC are soluble in dimethyl sulfoxide (DMSO). Each cell line was seeded in quadruplicate in a

96-well cell culture plate at 10,000 cells/well (PC12-P) or 4000 cells/well (PC12-R1/R7) in 100μL of phenol red-free DMEM and incubated overnight at 37°C. For assessing the effect of UK-5099, media was removed and replaced with phenol red-free DMEM treated with 500μM or 1000μM UK-5099 dissolved in DMSO (1% final concentration). For assessing the effect of CHC, media was removed and replaced with phenol red-free DMEM treated with 10 mM or 20 mM UK-5099 dissolved in DMSO (0.5% final concentration). PC12 cells were then incubated at 37°C for 24 and 48 hours. Following treatment, 10μL of 2.5 mg/mL MTT solution was added to each well and cells were incubated at 37 °C for 6 hours. After incubation, 100μL of MTT stop mix solution was added to each well, the 96-well plate was rocked overnight at room temperature, and absorbance at 570 nm was measured, following subtraction of background absorbance at 690 nm, using a Tecan M1000 plate reader.

2.9 – Statistical Analyses

Cell viability data, MitoTracker® Red CM-H2XRos data measuring mitochondrial ROS, and TMRM data measuring mitochondrial membrane potential were evaluated by One-way ANOVA analysis followed by post-hoc Tukey's test. p-values < 0.05 were considered significant.

Chapter 3: Results

3.1 – Basal levels of LDHB are elevated in Aβ-resistant PC12 cells

Previous research has revealed that increased LDHA activity in PC12 cells promotes resistance to Aβ toxicity (Newington *et al.* 2011). However, the potential role of LDHB in mediating Aβ resistance in PC12 cells has not been previously explored. Therefore, a logical first step was to examine basal LDHB levels in PC12 cell lines. Whole cell lysates from PC12-P, R1, and R7 cells treated with or without 20 μM $A\beta_{(25-35)}$ for 48 hours were analyzed by immunoblot analysis using a primary antibody specific for LDHB (Figure 3). LDHB levels exhibited an approximate 8-fold elevation in both Aβ-resistant PC12 cell lines, compared to the sensitive parental cell line, and levels were unaffected by treatment with Aβ.

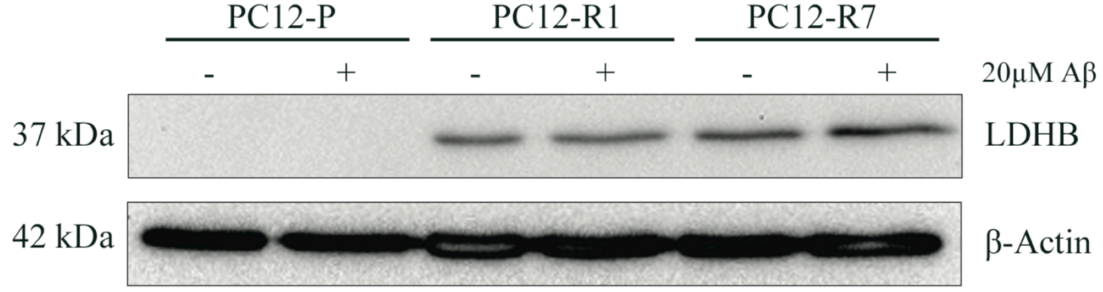
3.2 – LDHA and LDHB are localized within the mitochondria of Aβ-resistant PC12 cells

Mitochondria of PC12-R1 and R7 cells were isolated and incubated with proteinase K (10 μg/mL) in the absence or presence of Triton X-100 (1%) for 3 hours at 37 °C (Figure 4). This assay has been used previously in past studies to rule out the possibility of cytosolic protein contaminants in crude mitochondrial isolates (Hitosugi *et al.* 2011). Mitochondrial lysates were analyzed by immunoblot analysis, using primary antibodies specific for either LDHA or LDHB isoforms. Under control conditions, LDHA and LDHB were localized within the mitochondria of both resistant PC12 cell lines. LDHA and LDHB isoforms were partially protected from digestion when mitochondria are treated with proteinase K alone, but were digested when treated with proteinase K in the presence of Triton X-100. Additionally, mitochondrial lysates under each treatment were probed with antibodies specific for GAPDH, VDAC, and PDH-E1α to assess the purity of mitochondrial fractions and to ensure proper activity of proteinase K and Triton X-100. These data suggest that both the LDHA and LDHB isoforms were localized within the mitochondria of Aβ-resistant PC12 cells.

Figure 3. LDHB expression is increased in Aβ-resistant PC12 clonal cell lines. A) Immunoblot analysis of PC12-P, R1, and R7 whole cell lysates reveals elevated LDHB protein levels in both Aβ-resistant PC12 clonal cell lines, compared to the parental cell line. Treatment with 20 μM Aβ for 48 hours did not appear to affect LDHB levels. B) Densitometric analysis reveals an approximate 8-fold elevation of LDHB levels in resistant PC12 cell lines treated with or without Aβ, compared to the parental cell line, in which LDHB levels were barely detectable. An anti- β-actin antibody was used as a loading control. These results were representative of 3 independent experiments.

Figure 3

A)



B)

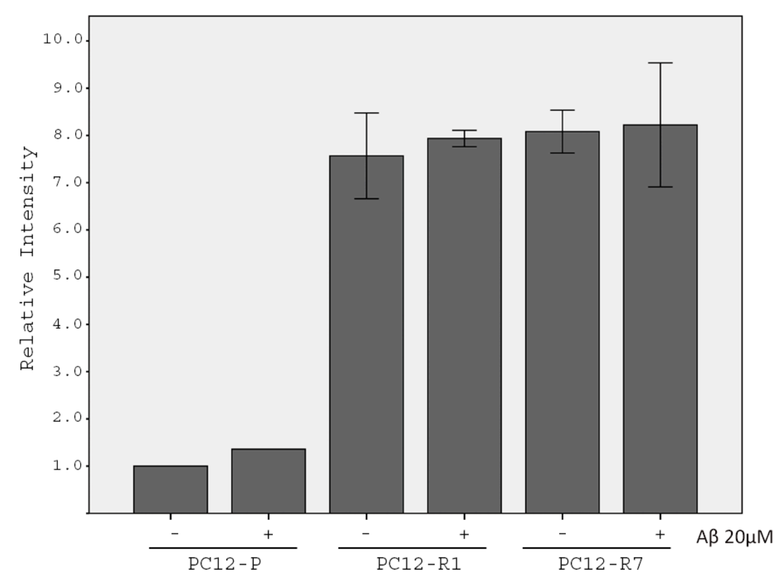
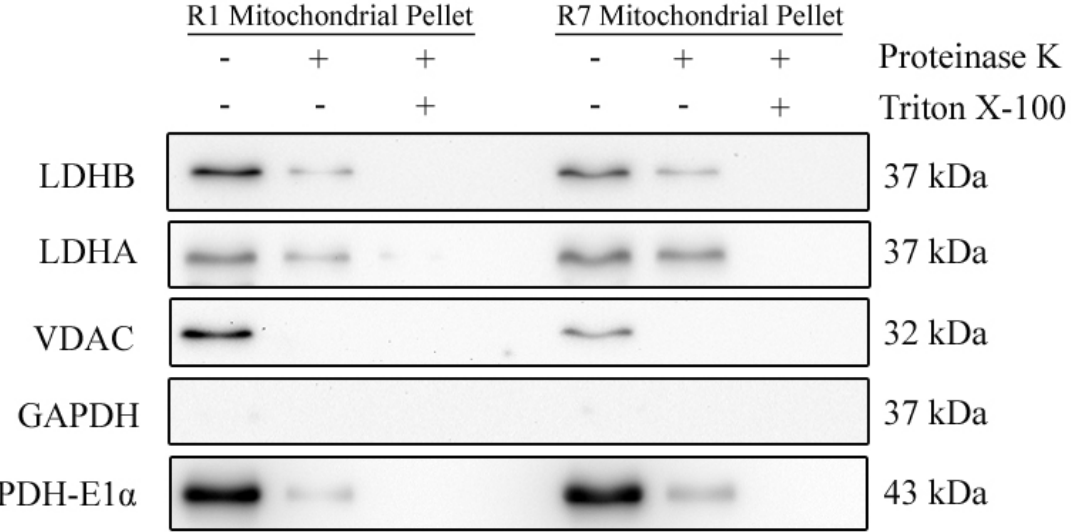


Figure 4. LDHA and LDHB isoforms are localized within the mitochondria of Aβ-resistant PC12 clonal cell lines. Immunoblot analysis of PC12-R1 and R7 mitochondrial pellets treated with or without proteinase K (10 μg/mL) in the presence or absence of Triton X-100 (1%) for 3 hours. LDHA and LDHB levels were partially protected in R1 and R7 mitochondrial pellets treated with proteinase K alone, but were almost completely absent when treated with proteinase K + Triton X-100, compared to control conditions. In addition, the blot was reprobed with an anti-GAPDH antibody to assess any potential cytosolic contamination of the mitochondrial fraction. Furthermore, the blot was also probed with anti-VDAC and anti-PDHE1α antibodies to assess the purity of mitochondrial isolates and proper functionality of proteinase K and Triton X-100. These results were representative of 3 independent experiments.

Figure 4



3.3 – Derivation of LDHB knockdown in PC12 clonal cell lines

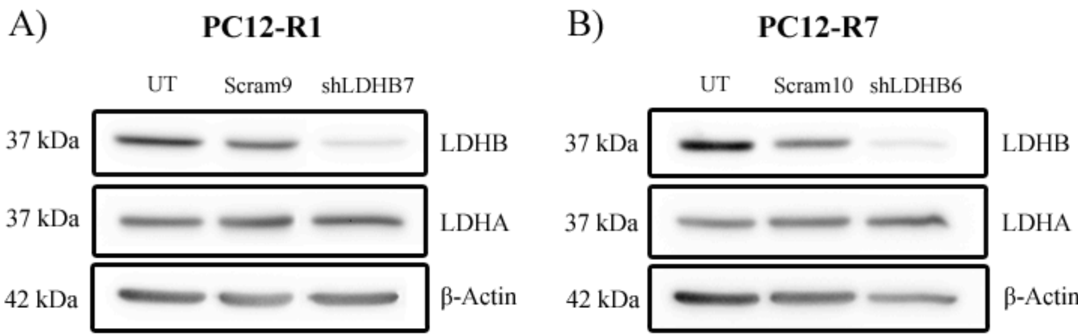
To determine if LDHB is important in mediating resistance to Aβ toxicity, LDHB levels were knocked down in Aβ-resistant PC12 cells using shRNA that specifically targeted the *LDHB* transcript. PC12-R1 and R7 cells were transfected with the HuSH pRS(shLDHB) vector and selected with puromycin (2 μg/mL). Whole cell lysates from surviving clones were subsequently screened by immunoblotting, using primary antibodies specific to either the LDHB or LDHA isoforms. R1 and R7 clonal cells stably expressing the pRS(shLDHB) vector (clones 7 and 6, respectively) exhibited reduced LDHB levels compared to untransfected (UT) or pRS(Scram)-transfected R1 and R7 cells (Figure 5A and 5B). LDHA levels remained constant across UT, pRS(Scram)-, and pRS(shLDHB)-transfected R1 and R7 clonal cell lines demonstrating the specificity of the shRNA to target only the LDHB isoform. Densitometric analyses revealed LDHB levels were significantly reduced ($p < 0.001$) by nearly 60% in R1-shLDHB7 and R7-shLDHB6 cells compared to R1-Scram and R7-Scram cells, respectively, while LDHA levels were not significantly altered (Figure 5C and 5D). Together, these data indicated that the HuSH pRS(shLDHB) vector construct was stably expressed in Aβ-resistant PC12 cells and specifically reduced LDHB levels without affecting levels of LDHA.

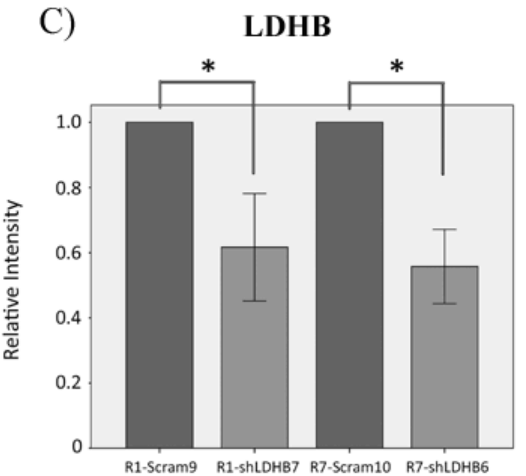
3.4 – LDHB knockdown re-sensitizes resistant PC12 cells to Aβ toxicity

The effect of LDHB knockdown on PC12 cell viability, following treatment with 20 μM Aβ for 48 hours, was evaluated by trypan blue exclusion assay (Figure 6). Cell viability was significantly reduced ($p < 0.001$) to approximately 60% in PC12-P, R1-shLDHB7, and R7-shLDHB6 cells treated with Aβ, compared to parental cells treated without Aβ. Conversely, R1-Scram or R7-Scram cells maintained high cell viability under both control and Aβ-treated conditions.

Figure 5. Derivation of LDHB knockdown PC12 clonal cell lines. Immunoblot analysis of A) PC12-R1 and B) R7 cells untransfected (UT) or stably transfected with either pRS (Scrambled) vector or a pRS vector containing rat LDHB shRNA revealed a clonal cell line (clone 7 and 6, respectively) with significantly reduced LDHB protein expression levels (*, $p < 0.001$). LDHA protein levels remained constant across treatment groups. C) Densitometric analyses of LDHB and D) LDHA protein levels among treatment groups. Data presented are the mean ± SEM of 3 independent experiments.

Figure 5





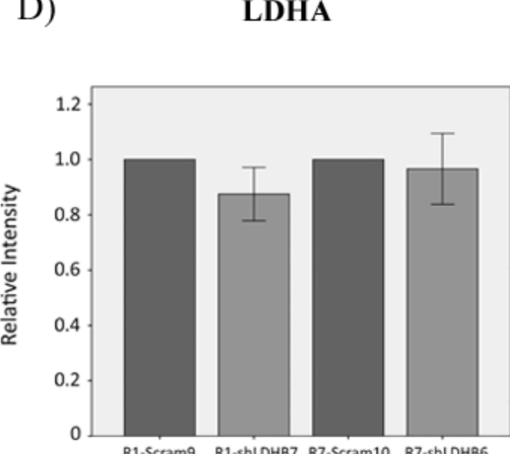
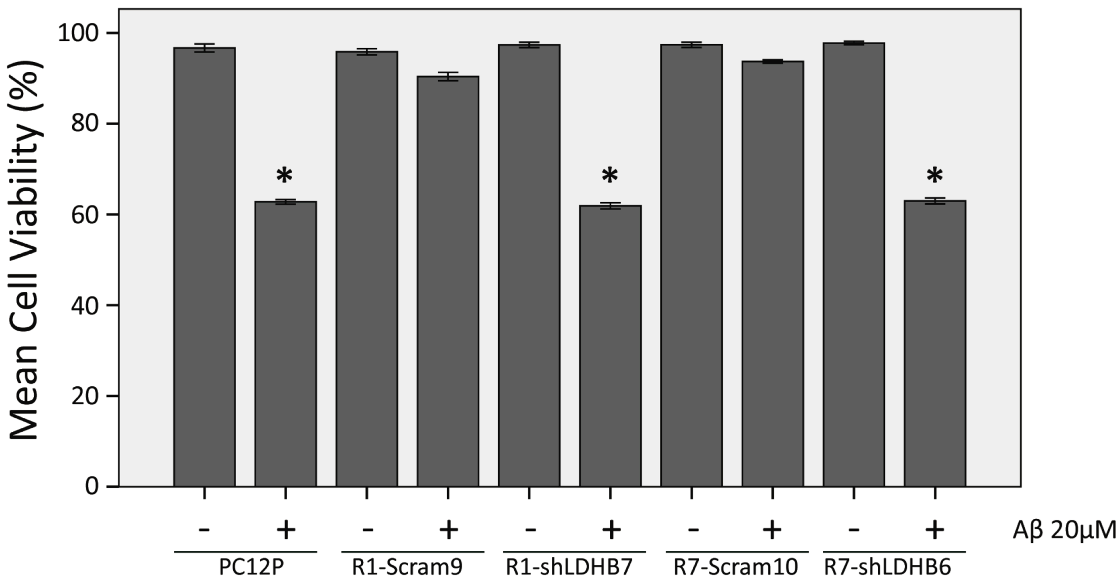


Figure 6. Knockdown of LDHB expression results in re-sensitization to Aβ toxicity in resistant PC12 cells. Cell viability of PC12 parental and resistant LDHB knockdown clonal cell lines treated with Aβ (20 μM) for 48 hours was significantly decreased compared to parental cells treated without Aβ (*, $p < 0.001$). Data presented are the mean of three independent experiments. Viability of all treatment groups were compared against the control group, in which PC12-P cells were not treated with Aβ.

Figure 6



3.5 – LDHB knockdown increases mitochondrial ROS levels in the presence of Aβ

To measure the impact of LDHB knockdown on mitochondrial ROS levels, live PC12 cells were stained with MitoTracker Red CM-H_2XRos (MTR) following treatment with 20 μM Aβ for 48 hours. PC12 cells were then visualized by fluorescence microscopy and images were taken at 400x magnification (Figure 7A). Quantification of fluorescence intensity revealed a significant increase of approximately 50% ($p < 0.05$) in PC12-P and R1-shLDHB7 cells treated with Aβ, compared to parental cells not treated with Aβ (Figure 7B). Fluorescence intensity appeared elevated, but not significantly, in R7-shLDHB6 cells treated with Aβ compared to parental cells not treated with Aβ. In addition, no significant difference in fluorescence intensity was observed in R1-Scram and R7-Scram cells under control and Aβ treatments, compared to parental cells treated without Aβ.

3.6 – LDHB knockdown increases mitochondrial membrane potential in the presence of Aβ

To evaluate the effect of LDHB knockdown on $\Delta\psi_m$, live PC12 cells were stained with TMRM following exposure to 20 μM Aβ for 48 hours. PC12 cells were visualized by fluorescence microscopy and images were taken at 400x magnification (Figure 8A). Quantification analysis revealed fluorescence intensity was significantly elevated ($p < 0.05$) in parental (~60%), R1-shLDHB7 (~30%) and R7-shLDHB6 (~60%) cells treated with Aβ, compared to parental cells treated without Aβ (Figure 8B). Fluorescence intensity was not significantly different in Aβ- and control-treated R1-Scram or R7-Scram cells, compared to parental cells treated without Aβ.

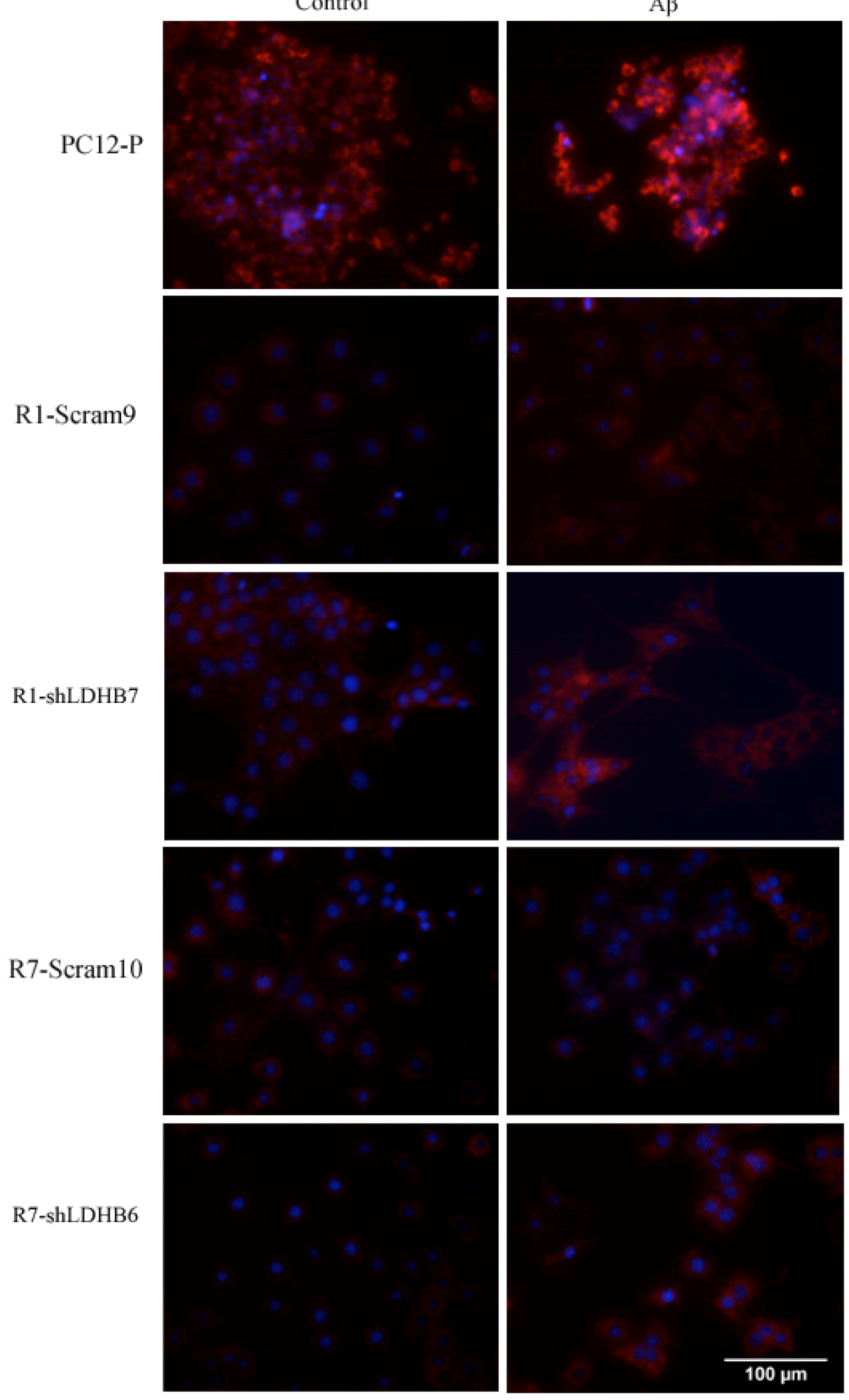
3.7 – Basal levels of MCT1, 2, and 4 are elevated in Aβ-sensitive PC12 cells

Lactate is transported across the plasma membrane and other intracellular membranes via a family of monocarboxylate (MCT) transporters. To determine if MCT levels were altered in resistant versus sensitive cells, MCT1, 2, and 4 protein expression was assessed in PC12-P, R1, and R7 cells, treated with or without 20 μM Aβ for 48 hours by immunoblotting using primary antibodies specific for either MCT1, 2, or 4 (Figure 9).

Figure 7. LDHB knockdown in Aβ-resistant PC12 cells results in increased mitochondrial reactive oxygen species levels. A) Mitochondrial reactive oxygen species (ROS) levels were measured in PC12 cells following staining with the red fluorescent dye MitoTracker Red CM-H_2XRos (MTR). Parental and Aβ-resistant PC12 clonal cell lines with knocked down LDHB levels exhibited elevated fluorescence intensity following exposure to Aβ (20 μM) for 48 hours compared to parental cells treated without Aβ. Nuclei were stained with Hoescht stain (blue) and images were visualized by fluorescence microscopy at 400x magnification. B) Quantification of MTR fluorescent images revealed that fluorescence intensity was significantly increased in Aβ-treated parental and R1-shLDHB7 cells compared to parental cells treated without Aβ (*, $p < 0.05$). MTR fluorescence intensity appeared elevated in Aβ-treated R7-shLDHB6 cells, but not significantly different compared to parental cells treated without Aβ. Data presented are the mean ± SEM of three independent experiments.

Figure 7

A)



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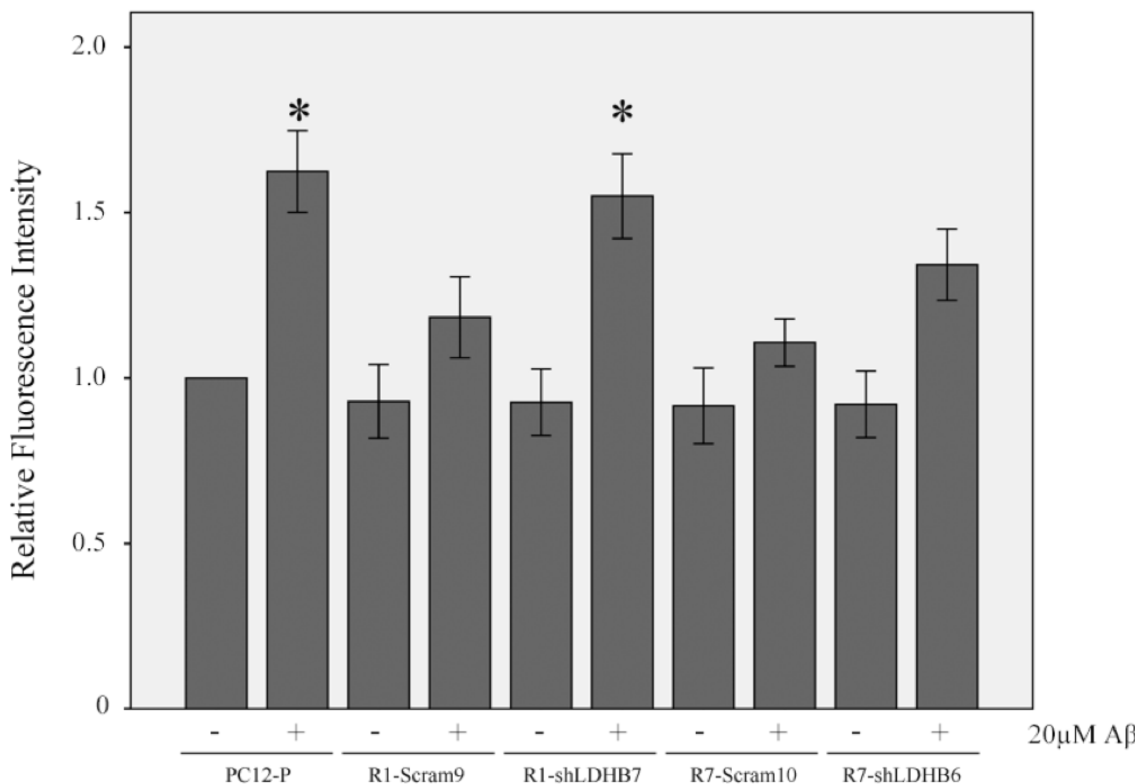
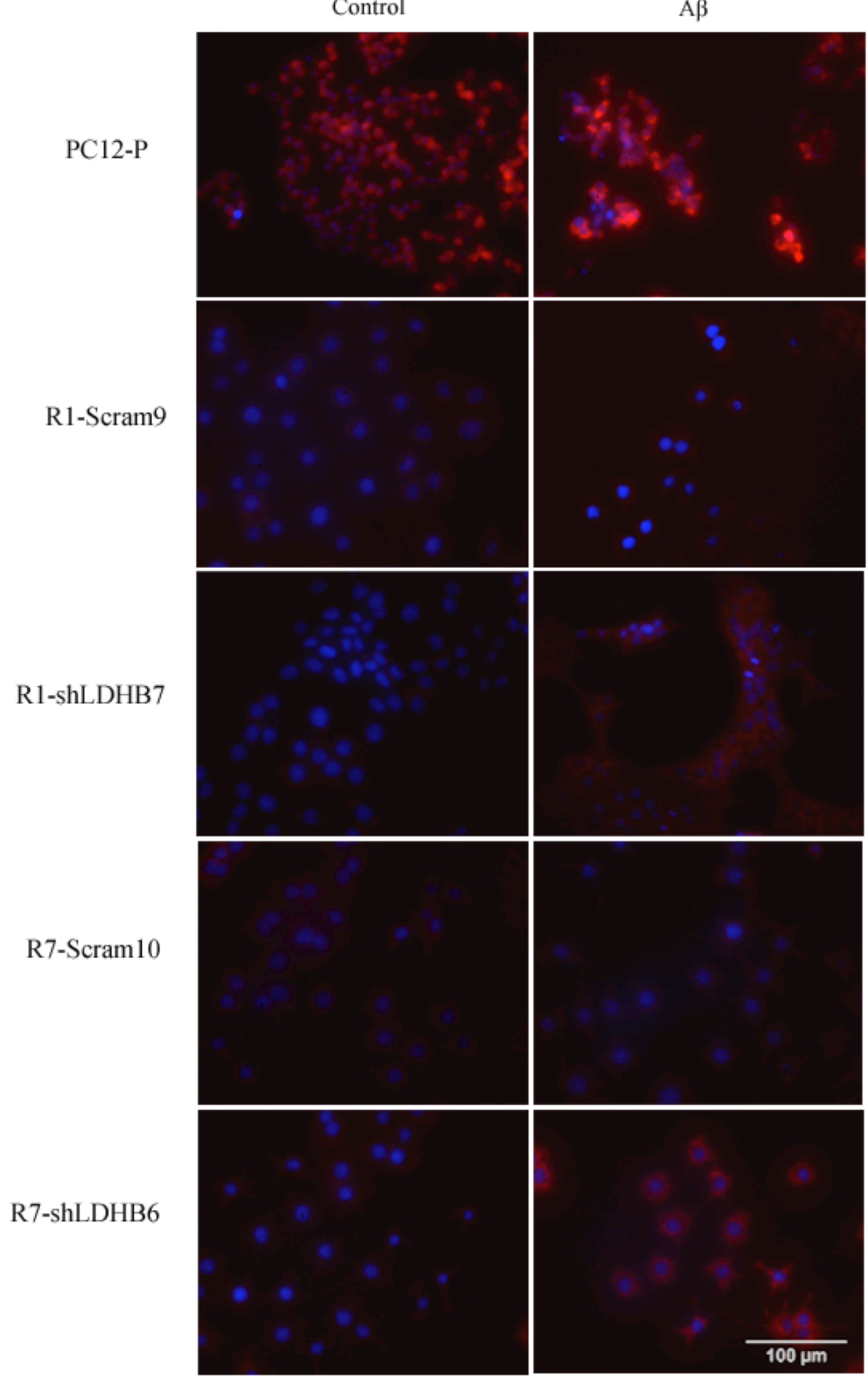


Figure 8. Knockdown of LDHB protein expression results in increased mitochondrial membrane potential in resistant PC12 cells treated with Aβ. A) Mitochondrial membrane potential ($\Delta\psi_m$) was measured in PC12 cells following staining with the red fluorescent dye TMRM. Parental and Aβ-resistant PC12 clonal cell lines with knocked down LDHB levels exhibited elevated fluorescence intensity following exposure to Aβ (20 μM) for 48 hours compared to parental cells treated without Aβ. Nuclei were stained with Hoescht stain (blue) and visualized by fluorescence microscopy at 400x magnification. B) Quantification of TMRM fluorescent images revealed that fluorescence intensity was significantly increased in Aβ-treated PC12-P and LDHB knockdown PC12-R1 and R7 cell lines compared with parental cells in the absence of Aβ treatment (*, $p < 0.05$). Data presented are the mean ± SEM of three independent experiments.

Figure 8

A)



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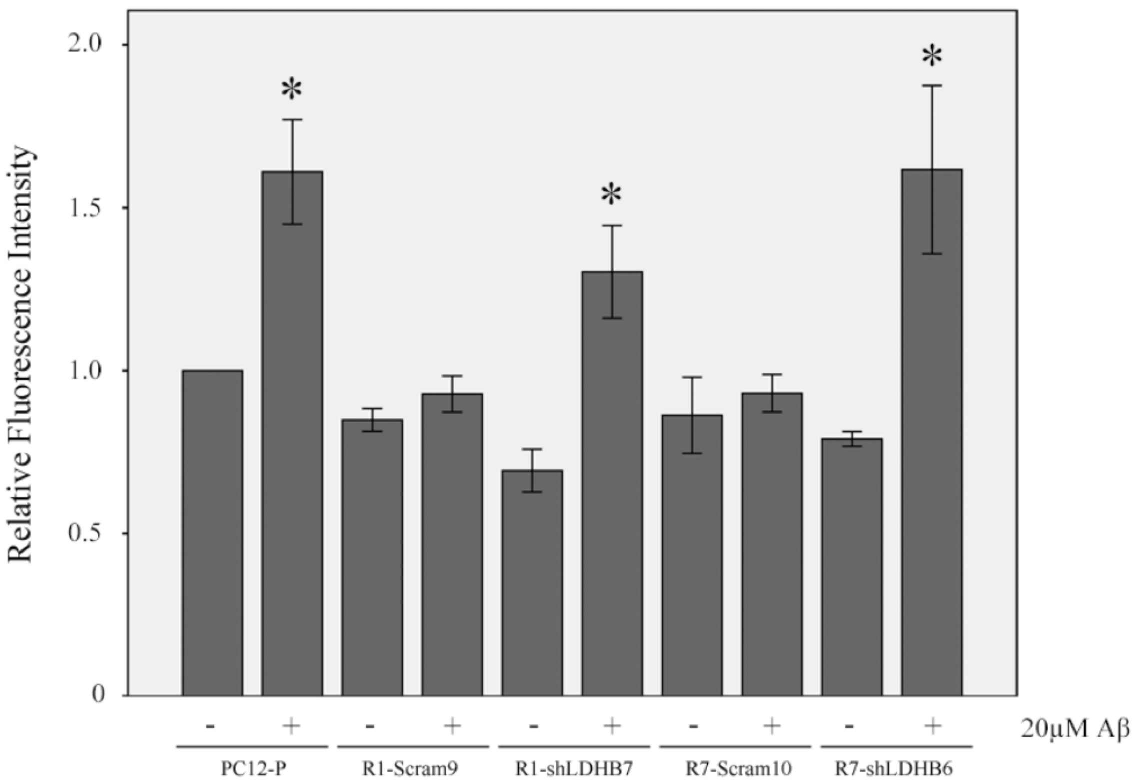
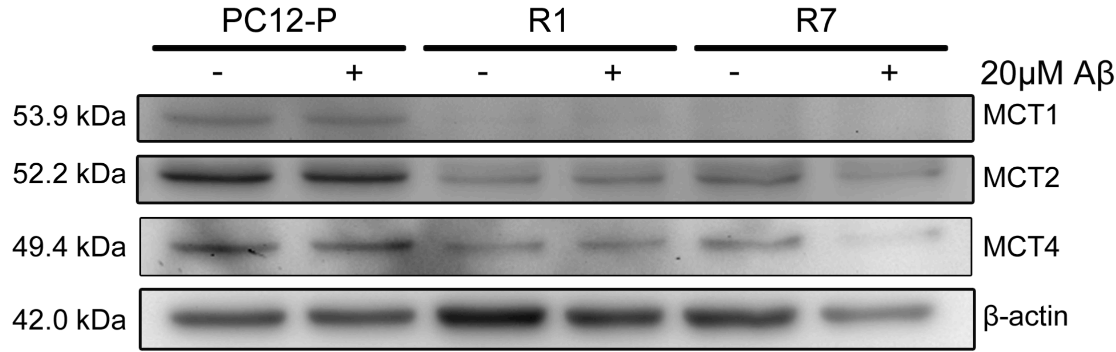


Figure 9. MCT1, 2, and 4 levels are decreased in Aβ-resistant PC12 clonal cell lines. Immunoblot analysis of PC12-P, R1, and R7 whole cell lysates revealed decreased MCT 1, 2, and 4 protein levels in both Aβ-resistant PC12 clonal cell lines, compared to the parental cell line. Treatment with 20 μM Aβ for 48 hours did not appear to affect LDHB levels. An anti- β-actin antibody was used as a loading control. These results were representative of 3 independent experiments.

Figure 9



MCT1, 2, and 4 protein levels were elevated in PC12-P cells, compared to both Aβ-resistant PC12 cell lines, and were unaffected by Aβ treatment.

3.8 – CHC-induced MCT inhibition results in decreased viability in Aβ-resistant PC12 cells

As previously discussed, accumulation of toxic levels of lactate is prevented by transport of this metabolite across biological membranes via MCTs. Thus, the effect of chemical inhibition of MCTs was examined in Aβ-sensitive and resistant PC12 cell lines. PC12-P, R1, and R7 cells were treated with CHC (10 mM and 20 mM) for 24 or 48 hours (Figure 10). Cell viability was measured by a MTT colourimetric assay. Treatment of PC12-P cells with CHC (10 mM and 20 mM) resulted in a significant reduction in cell viability (*, $p < 0.05$), compared to parental cells treated without CHC, for both time-points. In contrast, cell viability was not significantly reduced in R1 or R7 cells, when treated for either 24 or 48 hours with 10 mM CHC, compared to untreated parental cells. Although R1 or R7 cells exhibited increased cytotoxicity following 20 mM CHC exposure, both resistant cell lines displayed higher viability compared to the parental cell line treated under the same conditions and same time.

3.9 – UK-5099-mediated inhibition of the mitochondrial pyruvate carrier results in decreased viability in PC12 cells

As previously mentioned, pyruvate levels in Aβ-resistant PC12 cells may be elevated due to increased glycolytic activity (Soucek *et al.* 2003). Since pyruvate acts as an important antioxidant and energy substrate in neuronal mitochondria, the importance of the MPC, which imports cytosolic pyruvate into the mitochondria, on PC12 cell viability was investigated. The effect of MPC inhibition on PC12-P, R1, and R7 cell viability was evaluated following treatment with 500 or 1000 µM UK-5099 for 48 hours (Figure 11). Cell viability was measured by a MTT colourimetric assay. A 48-hour treatment of PC12-P, R1, and R7 cells with either concentration of UK-5099 resulted in a significant reduction (approximately 60 – 80%) in cell viability (*, $p < 0.05$), compared to parental cells not treated with UK-5099. These findings indicate that both Aβ-sensitive and resistant cells are dependent on pyruvate transport into the mitochondria for survival.

Figure 10. Chemical inhibition of MCT1, 2, and 4 results in decreased viability in Aβ-resistant PC12 cells. Viability of PC12-P cells was significantly reduced (*, $p < 0.05$) when treated with 10 mM or 20 mM CHC, compared to untreated parental cells, following a 24 or 48-hour incubation period. PC12-R1 and R7 cell viability was not significantly altered following treatment with 10 mM CHC, compared to parental cells treated without CHC, for both 24 and 48-hour time-points. For both time-points, viability of resistant cell lines was significantly decreased ($^{\#}$, $p < 0.05$) following 20 mM CHC exposure, compared to untreated parental cells, but higher than parental cells treated under the same conditions and same time-points. Data presented are the mean ± SEM of three independent experiments.

Figure 10

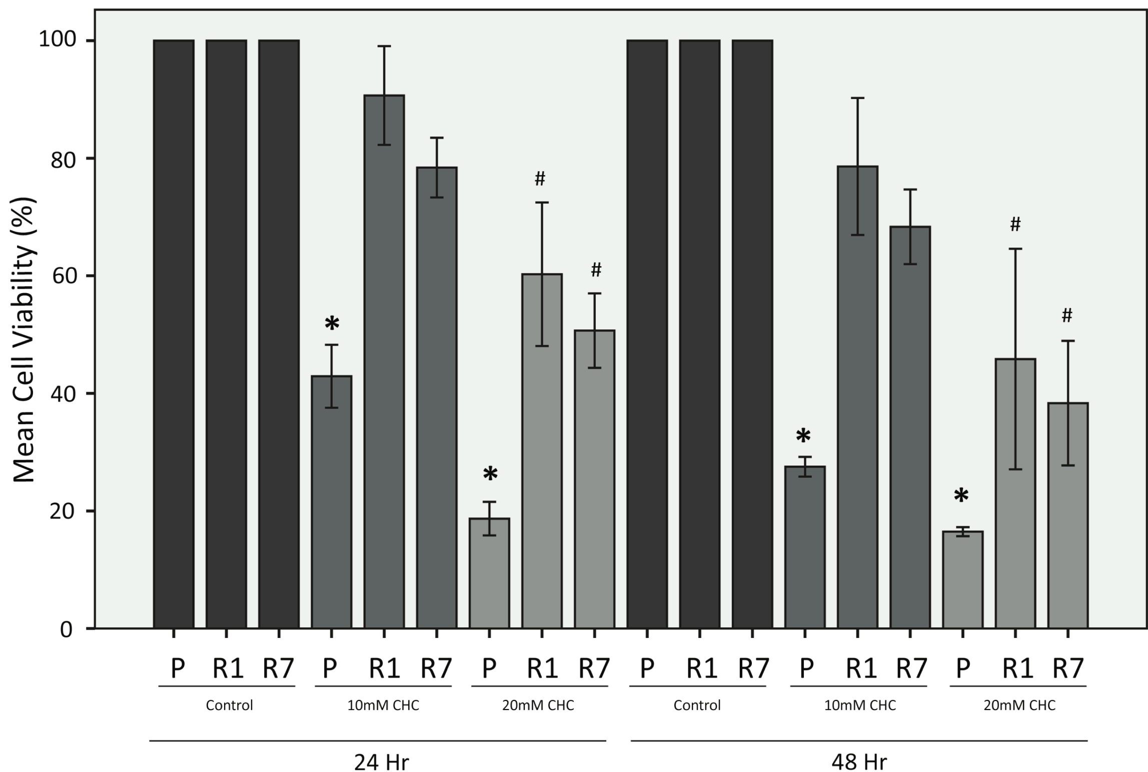
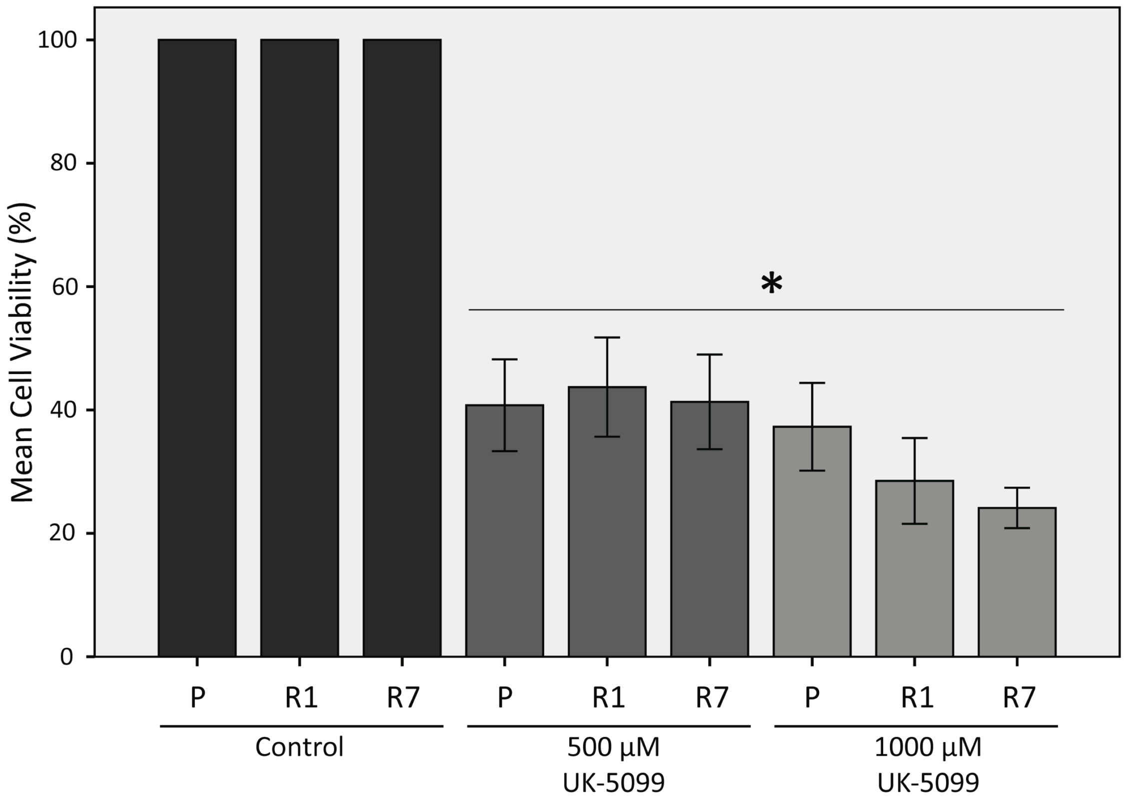


Figure 11. UK-5099–mediated mitochondrial pyruvate carrier inhibition results in decreased viability in PC12 cells. Viability of PC12-P, R1, and R7 cells was significantly reduced (*, $p < 0.05$) when treated with 500 μM or 1000 μM UK-5099 for 48 hours, compared to parental cells treated without UK-5099. Data presented are the mean ± SEM of three independent experiments.

Figure 11



Chapter 4: Discussion & Future Research

4.1 – Overview

Although past studies have established LDHA activity as an important factor in neuronal protection against Aβ toxicity, this investigation sought to characterize LDHB's potential role in mediating resistance to Aβ. In addition, the subcellular compartmentalization of LDHA and LDHB remains highly disputed by the scientific community, although a recent study indicated that both LDH isoforms are localized to the mitochondria of rat hippocampal and cortical neurons (Hashimoto *et al.* 2008). Therefore, mitochondrial localization of the specific LDH isoforms in PC12 cells was also examined in this investigation. The findings of this study suggest that LDHB may be important in reducing oxidative stress and mediating resistance to Aβ neurotoxicity. Furthermore, both LDHB and LDHA localize within the mitochondria of Aβ-resistant neuronal cells. Finally, general monocarboxylate transporter inhibition is tolerated in Aβ-resistant cells, but inhibition of the mitochondrial pyruvate carrier causes a decline in viability in both Aβ-sensitive and resistant PC12 cell lines.

4.2 – LDHB and LDHA are localized in the mitochondria of Aβ-resistant PC12 cells

Preliminary results of this investigation revealed that LDHB levels are elevated in Aβ-resistant versus sensitive PC12 cells (Figure 3). Interestingly, past studies by Newington *et al*. (2011) revealed that LDHA activity is also increased in Aβ-resistant versus sensitive PC12 cells. Although LDHB and LDHA have antagonistic functions, these observations may be partially addressed by the aforementioned ILS hypothesis (Figure 2). Differential compartmentalization of LDHB and LDHA in the cytosol or mitochondria, as proposed by this hypothesis, may allow each isoform to fulfill their respective functions, without interfering with one another's activities. The mitochondrial Proteinase K Protection Assay confirmed that both LDHB and LDHA are localized within the mitochondria of resistant PC12 cells. These results support the proposed hypothesis of this study, as well as previous work, in which LDHB and LDHA were suggested to localize within rat neuronal mitochondria, both *in vitro* and *in vivo* (Alante *et al.* 2007, Hashimoto *et al.* 2008). Despite being localized within mitochondria, the

relative levels and activities of LDHB and LDHA associated with the OMM, IMS, IMM, and mitochondrial matrix, have not been well determined. Therefore, mitochondrial subfractionation studies should be performed to investigate any differential compartmentalization of LDHB and LDHA in neuronal mitochondria. In addition, examining LDHB and LDHA localization in PC12 cells by immunofluorescence microscopy using isoform-specific antibodies would provide strong evidence to support the results of this study, and thus merits further research. Furthermore, additional studies should be conducted to ascertain the mitochondrial sublocalization of the LDH isoenzymes (LDH1-5). Identification of the LDH isoenzymes may be accomplished by gel electrophoretic zymography.

4.3 – LDHB mediates Aβ-resistance by reducing ROS levels and oxidative damage-induced neurotoxicity

Aβ may promote its neurotoxic effect by increasing mitochondrial ROS levels and $\Delta\psi_m$, leading to widespread oxidative damage in sensitive cells. Comparative analysis of PC12-P, R1, and R7 whole cell lysates revealed that LDHB levels are elevated in Aβ-resistant versus sensitive cell lines (Figure 3), providing preliminary evidence that LDHB may play an important role in mediating resistance to Aβ in PC12 cells. In addition, an increase in MTR and TMRM fluorescent intensity, as well as a decrease in cell viability, was observed in PC12-P and pRS(shLDHB)-expressing R1 and R7 cells when treated with Aβ (20μM) for 48 hours, compared to control-treated parental cells (Figures 6, 7, 8). In contrast, PC12-R1 and R7 control cells under the same treatment conditions did not exhibit increased MTR and TMRM fluorescent intensity, or decreased cell viability, compared to parental cells treated without Aβ. Therefore, these findings indicate that LDHB may protect against Aβ-induced oxidative damage and neurotoxicity, as knockdown of LDHB in resistant PC12 cells promotes increased $\Delta\psi_m$ and mitochondrial ROS levels, as well as re-sensitization to Aβ toxicity. As previously discussed, LDHB activity yields pyruvate and NADH, both of which limit increases in $\Delta\psi_m$, neutralize mitochondrial ROS, and prevent oxidative stress-related neurotoxicity (Alvarez *et al.* 2003; Miranda-Vizuete *et al.* 2000; Vogel *et al.* 1999; Constantopoulos and Barranger 1984; Vlessis *et al.* 1990; Desagher *et al.* 1997). Taken together with the present

findings, this suggests that LDHB knockdown in resistant PC12 cells may reduce available levels of pyruvate and NADH, compromising their ability to neutralize potentially harmful ROS and increasing the likelihood of oxidative damage-related neurotoxicity. Collectively, these data support the proposed hypothesis and suggest LDHB is protective against Aβ-induced oxidative stress and neurotoxicity, however the exact mechanism by which this occurs is not fully elucidated.

Further investigations are required to better characterize the role of LDHB in mediating resistance against Aβ-induced neurotoxicity. For example, LDHB activity in the PC12-P, R1, and R7 cell lines should be assessed to complement the present findings, which primarily focused on overall LDHB levels. Since LDHB catalyzes the conversion of lactate to pyruvate using NAD^+ as a co-substrate, LDHB activity may be evaluated by measuring the reduction of NAD^+ to NADH. Additional studies should also examine the activity and levels of LDH1-5 in the PC12 clonal cell lines. Additionally, the effect of LDHB overexpression on mitochondrial ROS levels, $\Delta\psi_m$, and viability, in PC12 sensitive cells treated with Aβ, should also be investigated. Furthermore, it should be determined whether or not Aβ-resistant PC12 cells exhibit increased $NADP^+$ transhydrogenase levels and activity, compared to parental cells, to determine if NADH-mediated regeneration of NADPH may be an important factor in limiting mitochondrial ROS levels.

4.4 – Impairment of monocarboxylate transport decreases PC12 cell viability

Since the Aβ-resistant PC12 cell lines presumably need to export higher volumes of lactate, it was expected that they would be more sensitive to MCT inhibition than the parental cell line. Contrary to these expectations, the findings of this study indicate that MCT1, 2, and 4 levels are lower in the PC12-R1 and R7 cell lines compared to the parental cell line. CHC-mediated MCT inhibition is also tolerated in both Aβ-resistant PC12 cell lines, but not in the PC12-P cell line. Taken together with studies by Newington *et al.* (2011), which demonstrate extracellular lactate accumulation in PC12 cells, these results suggest the existence of a currently unknown alternate lactate transporter (ALT) that may facilitate lactate export in Aβ-resistant cell lines. In addition,

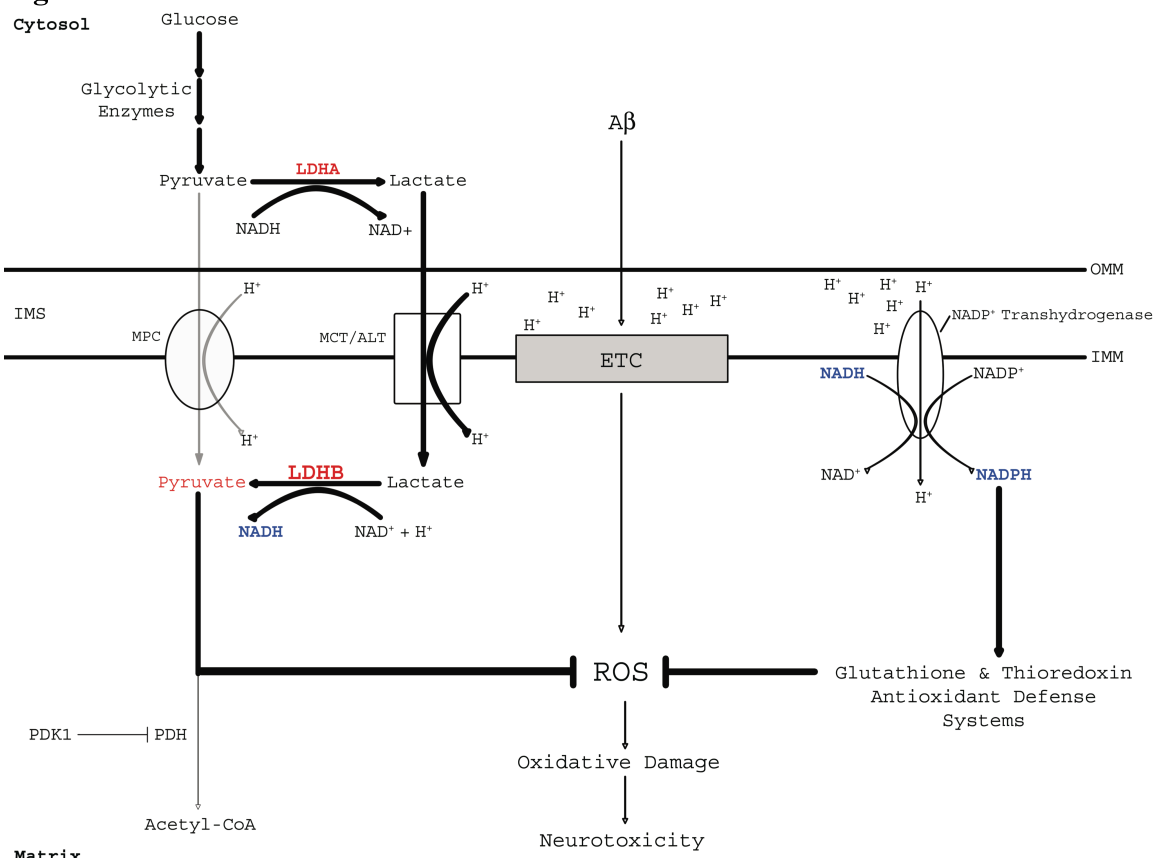
to evaluate the importance of pyruvate import into the mitochondria, MPC activity was chemically inhibited by UK-5099 in PC12 cells. Interestingly, PC12-P, R1, and R7 cells were intolerant of UK-5099–mediated inhibition of the MPC. This suggests that Aβ-sensitive and resistant cell lines are reliant on pyruvate import into the mitochondria. To further complement these findings, MPC, as well as MCT1, 2, and 4 activities, should be examined in the PC12-P, R1, and R7 cell lines treated with and without Aβ. Additionally, the effect of monocarboxylate transport inhibition on PC12 cell viability should be examined using inducible knockdown vector constructs specific for MCT1, 2, 4, as well as the MPC. Furthermore, Aβ-resistant PC12 cells exhibit elevated extracellular levels of lactate (Newington *et al.* 2011). To confirm the possible existence of an ALT, extracellular lactate levels should be measured from Aβ-resistant PC12 cells in which MCT activity is chemically or genetically inhibited.

4.5 – Conclusions

This study provides novel insight into the role of lactate metabolism on neuronal Aβ resistance. Past research indicates that increased LDHA activity, as observed in Aβ-resistant neuronal cell lines, is protective against Aβ-induced oxidative stress and neurotoxicity. However, LDHB's role in protecting against Aβ toxicity has never been previously explored and was therefore the focus of this work. Using Aβ-sensitive and resistant PC12 clonal cell lines as an experimental model, this investigation demonstrated, for the first time, that LDHB is important in mediating resistance to Aβ-induced neurotoxicity in PC12 cells by potentially limiting $\Delta\psi_m$ and levels of mitochondrial ROS. A proposed model outlining LDHB's neuroprotective role against Aβ-induced oxidative damage is outlined below (Figure 12). In addition, this study provides confirmation that both LDHA and LDHB are localized within the mitochondria of Aβ-resistant PC12 cells. Interestingly, this work also indicates that although Aβ-resistant cells are tolerant of general monocarboxylate transporter inhibition and sensitive cells are not, all PC12 clonal cell lines are reliant on MPC activity. Although further study is warranted, this investigation lays the foundation for understanding how the transport and metabolism of lactate and its derivatives may be protective against the neurotoxic effect of Aβ.

Figure 12. Proposed mechanism by which LDHB protects against Aβ-induced oxidative damage in resistant PC12 cells. Amyloid β (Aβ) promotes mitochondrial electron transport chain (ETC) dysfunction and H^+ accumulation in the mitochondrial intermembrane space (IMS), resulting in increased production of reactive oxygen species (ROS). Increasing ROS levels subsequently promote widespread oxidative damage and neurotoxicity. Aβ-resistant PC12 cells exhibit increased activity of glycolytic enzymes as well as lactate dehydrogenase A (LDHA), lactate dehydrogenase B (LDHB), and pyruvate dehydrogenase kinase 1 (PDK1). Elevated levels of glycolytic enzymes promote the conversion of glucose to pyruvate in the cytosol. Increased LDHA activity then promotes the reduction of pyruvate to lactate, coupled with the oxidation of NADH to NAD^+. Co-transport of lactate with H^+ into the mitochondrial matrix is facilitated by either a monocarboxylate transporter (MCT) or currently unknown, alternate lactate transporter (ALT) and may help dissipate the H^+ gradient across the inner mitochondrial membrane (IMM). Increased LDHB levels in the matrix facilitate oxidation of lactate to pyruvate, coupled with the reduction of NAD^+ to NADH. Alternatively, cytosol-localized pyruvate may be co-transported with H^+ into the mitochondrial matrix via the MPC. Additionally, increased PDK1 activity promotes the phosphorylation and inhibition of pyruvate dehydrogenase (PDH), limiting the conversion of pyruvate to acetyl-CoA. Therefore, mitochondrial matrix-localized pyruvate may directly neutralize potentially harmful ROS via a non-enzymatic decarboxylation reaction. Furthermore, $NADP^+$ transhydrogenase may utilize NADH and IMS-localized H^+ to drive the regeneration of NADPH, a key substrate of the glutathione and thioredoxin antioxidant defense mechanisms. Thus, LDHB promotes increased levels of pyruvate and NADH in the mitochondrial matrix, which may provide direct and indirect mechanisms to detoxify Aβ-derived ROS, resulting in reduced oxidative damage and neurotoxicity.

Figure 12


Cytosol
Glucose
Glycolytic Enzymes
Pyruvate
LDHA
Lactate
NADH
NAD+
Aβ
OMM
IMS
H+
MPC
MCT/ALT
ETC
NADP+ Transhydrogenase
IMM
NADH
NADP+
NAD+
NADPH
Pyruvate
LDHB
Lactate
NADH
NAD+ + H+
ROS
Glutathione & Thioredoxin Antioxidant Defense Systems
PDK1
PDH
Oxidative Damage
Acetyl-CoA
Neurotoxicity
Matrix

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