Submitted for partial fulfillment of Doctor of Philosophy (PhD)

ANALYSING THE BIOLOGICAL FUNCTION OF PS2V: AN ABERRANT SPLICING PHENOMENON OR EVOLUTIONARILY CONSERVED MECHANISM IN ALZHEIMER'S DISEASE

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LIST OF PUBLICATIONS CONTRIBUTED TO DURING PH.D. CANDIDATURE

The Guinea pig as a model for sporadic Alzheimer's disease (AD): the impact of cholesterol intake on expression of AD-related genes.
Mathew J. Sharman, Seyyed Hani Moussavi Nik, Mengqi M. Chen, Daniel Ong, Linda Wijaya, Simon M. Laws, Kevin Taddei, Morgan Newman, Michael Lardelli, Ralph N. Martins, Giuseppe Verdile.
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BMC Research Notes, 2014

Alzheimer's disease-related peptide PS2V plays ancient, conserved roles in stimulation of γ-secretase activity and suppression of the unfolded protein response under hypoxia.
Seyyed Hani Moussavi-Nik, Morgan Newman, Lachlan Wilson, Esmaeil Ebrahimie, Simon Wells, Mark Van Der Hoek, Giuseppe Verdile, Ralph N. Martins and Michael Lardelli.
Human Molecular Genetics, 2014 (Under review)

A zebrafish homologue of Alzheimer's disease-associated PRESENILIN isoform PS2V regulates inflammatory and other responses to hypoxic stress.
Esmaeil Ebrahimie, Seyyed Hani Moussavi-Nik, Morgan Newman, Mark Van Der Hoek, and Michael Lardelli**.**
Human Molecular Genetics, 2014 (Under review)

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with pathologies such as neuron loss, glial cell proliferation, extracellular deposition of senile plaques from the accumulation of amyloid beta (Aβ) peptides and deposition of intracellular neurofibrillary tangles. Aβ is generated from the cleavage of the Amyloid Precursor Protein (AβPP) by two different types of aspartyl proteases, β- and γ-secretase. The majorities of AD cases are sporadic and have a late onset. Mutations in genes encoding APP, PRESENILIN1 and *2* (*PSEN1* and *PSEN2*) cause an autosomal dominant inherited form of the disease with an early onset known as familial AD. In some sporadic cases an aberrant splice variant of *PSEN2* named PS2V is formed that can be found in inclusion bodies in the brain. PS2V results from the binding of the High Mobility Group A1a (HMGA1a) protein close to the splice donor site of exon 5 of *PSEN2*. HMGA1a is widely expressed during embryo development but not in adults. Its expression can be induced in adult neurons by hypoxia/oxidative stress and it is commonly reactivated in many types of cancer.

Zebrafish embryos have a unique combination of characteristics that allows genetic manipulation and analysis of molecular pathways implicated in neurodegenerative diseases. The embryos are numerous, macroscopic, external to the mother and transparent making them easy to inject and observe. Changes in different aspects of their rapid development can be used as bioassays to assess gene activity. In **chapter II** of this thesis, we present evidence from a number of different assays that acute exposure to hypoxia or chemical mimicry of hypoxia increases oxidative stress in zebrafish brain tissue. We demonstrated that intracellular ROS levels are significantly increased in zebrafish brains exposed to actual hypoxia or chemical mimicry of hypoxia using NaN_3 hypoxia. In **chapter III** of this thesis we examine the evolutionary conservation of PS2V and investigate its effect on gene expression profiles, γ- secretase activity. In this chapter we show evidence for an important role of PRESENILIN genes in cellular responses to low oxygen (hypoxia). The PS2V splicing isoform of human *PSEN2* transcripts is generated under hypoxic conditions through induction of HMGA1a that binds to exon 5 sequence in transcripts. We show that an orthologue of the PS2V isoform, PS1IV, exists in the zebrafish. The novel splice product of zebrafish *psen1*, "PS1IV" codes for a much smaller peptide then PS2V but, nevertheless is capable of boosting γ-secretase activity. In **chapter IV** we utilised microarray to analysis the function of PS1IV in modulation of a wide variety of gene products. We show that that production of this PS1IV is accompanied with activation of stress response genes such as interleukin 1 Beta (*IL1B),* tyrosine hydroxylase *(TH)*, and myelin expression factor (*MYEF)* which leads to triggering apoptosis and autophagy. We also demonstrate that PS1IV is an important contributor in signaling pathways associated with AD.

In **chapter V** we investigated the guinea pig, *Cavia porcellus*, as a model for Alzheimer's disease (AD), both in terms of the conservation of genes involved in AD and the regulatory responses of these to a known AD risk factor - high cholesterol intake. We demonstrate that PS2V formation is up-regulated by hypoxia and a high-cholesterol diet while, consistent with observations in humans, Aβ concentrations are raised in some brain regions but not others. We have previously identified two paralogues (co-orthologues) of *MAPT* in zebrafish, denoted *mapta* and *maptb* and have shown that both genes are expressed in the developing central nervous system. In **chapter VI** we extend our examination of expression of the zebrafish tau co-orthologues to study their response to actual hypoxia and chemical mimicry of hypoxia in explanted adult fish brains. We observed increases in the overall levels of both *mapta* and *maptb* transcripts due to specific increases in the levels of *mapta* 6R and *maptb* 4R transcript isoforms. This is consistent with dramatically decreased levels of transcripts of the zebrafish orthologue of the human *TRA2B* gene that codes for a splicing factor proposed to regulate alternative splicing of *MAPT* transcripts.

Chapter I

LITERATURE REVIEW

1.1Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases and the most common form of dementia in the industrial world, accounting for 60-80% of cases, although there is growing awareness that AD is often mixed with other dementia cases [1]. AD is divided into two different subtypes: Late onset AD (LOAD) and early onset familial AD (FAD). LOAD occurs beyond the age of 65 and represents 95% of all AD cases. LOAD lacks a clear genetic etiology. However, the remaining 5% of cases are due to the autosomally inherited FAD [2, 3].

Clinically, AD is defined by a slow progressive loss of cognitive functions, memory loss and behavioral changes, which ultimately lead to dementia and death [4, 5]. The neurodegeneration in AD is characterized initially by synaptic injury followed by neuronal loss [6]. Neuropathologically, AD is characterized by the aggregation and deposition of misfolded proteins, especially aggregated β-amyloid (Aβ) peptide in the form of extracellular senile plaques and hyperphosphorylated tau (τ) proteins in the form of intracellular neurofibrillary tangles (NFTs). These pathognomonic changes are generally followed by abundant microvascular damage, including vascular amyloid deposits, and noticeable inflammation of the affected brain regions [7].

Alternative splicing of pre-mRNA is a common strategy for the regulation of gene expression in eukaryotes. Variation in the selection of the alternative splice sites leads to the generation of various protein isoforms from a single gene, generally in response to tissue specific, physiologically or developmentally regulated states [8]. Misregulation of this process is also a major source of splicing defects in disease related genes [9]. *PRESENILIN2* (*PSEN2*) is one of the known AD related genes. Under certain conditions (hypoxia/oxidative stress) [10] *PSEN2* transcripts undergo alternative splicing to generate a truncated form of PSEN2 protein, PS2V [11, 12]. PS2V is a diagnostic feature of sporadic Alzheimer's disease. It accumulates in the hippocampus of sporadic AD patients as visible PS2V bodies. This alternative splicing of *PSEN2* transcripts is controlled by *HIGH MOBILITY GROUP A PROTEIN 1a* (*HMGA1a*), which binds to a specific, sequences in exon 5, causing exclusion of exon 5 and fusion of exons 4 and 6 [13, 14].

Alzheimer's disease and risk factors

The two most common forms of dementia are Alzheimer's disease (AD) and vascular dementia (VaD), with AD being more prevalent. Patients with AD experience symptoms including cognitive alternations, memory loss and behavioral changes [5, 15]. VaD, the second most common form of dementia, generally occurs from accumulation of damage to the vascular system. An unreliable vascular system causes reduced cereberal blood flow (CBF) and deprives the brain of oxygen and nutrients which can lead to cell death. A hallmark for VaD is atherosclerosis pathologies [16].

Sporadic forms of AD generally affect patients later in life, with onset of sporadic AD occurring usually between the ages of 60 and 70 [17]. Sporadic AD makes up the majority of AD cases. Less than 5 % of AD patients have a genetically linked familial form of AD (FAD). FAD patients generally have an earlier onset of the disease. FAD is associated with mutations in several genes, including *APP, PSEN1* and *PSEN2* [18]. Mutations in 3 genes, *AMYLOID BETA PRECURSOR PROTEIN* (*APP*), located on chromosome 21, *PRESENILIN1* (*PSEN1*) located in chromosome 14, and *PRESENILIN2* (*PSEN2*) located on chromosome 1 are the main dominant genetic abnormalities seen in AD cases [11, 19]. The majority of the mutations are seen in the *PSEN1* gene with over 180 mutations described to date when compared to 30 and 14 mutations in *APP* and *PSEN2* respectively [15, 20].

A common feature of AD is the characteristic senile plaques observed in the brain tissue of the cortex, hippocampus and amygdala [21]. Theses plaques are essentially aggregates of amyloid β (Aβ) [22]. Aβ peptide generation occurs through proteolysis of APP by means of β, and γ secretases [23, 24]. These secretases generally process APP through two different pathways to produce various peptides, including Aβ [25, 26]. The non amyloidogenic pathway occurs when a membrane associated α-secretase cleaves within the Aβ domain in APP (there by preventing Aβ formation) and an intramembrane cleavage occurs by the γ-secretase complex to produce a shortened peptide called p3 [26] and a cytoplasmic fragment identified as the APP intracellular domain (AICD) [27] (**Figure 1**). While, the amyloidogenic pathway cleaves APP at the Aβ domain by β-secretase [28]. This is followed by γ-secretase cleavage to generate the a range of Aβ peptides, predominantly $A\beta_{40}$ and $A\beta_{42}$ peptides, as well as AICD [26] (**Figure 1**). Several lines of evidence support the notion that the pathogenesis of AD is related to progressive accumulation of Aβ protein which is the product of APP proteolysis [29].

Abnormal accumulation of Aβ is the result of an imbalance between the levels of Aβ production, aggregation and clearance. Clearance Aβ from the neuronal cells is mediated by proteolytic enzymes such as neprilysin [30], chaperone molecules such as apoE [31], lysosomal pathways (e.g. autophagy) [32] and non-lysosomal pathways (e.g. via the proteasome) [33].

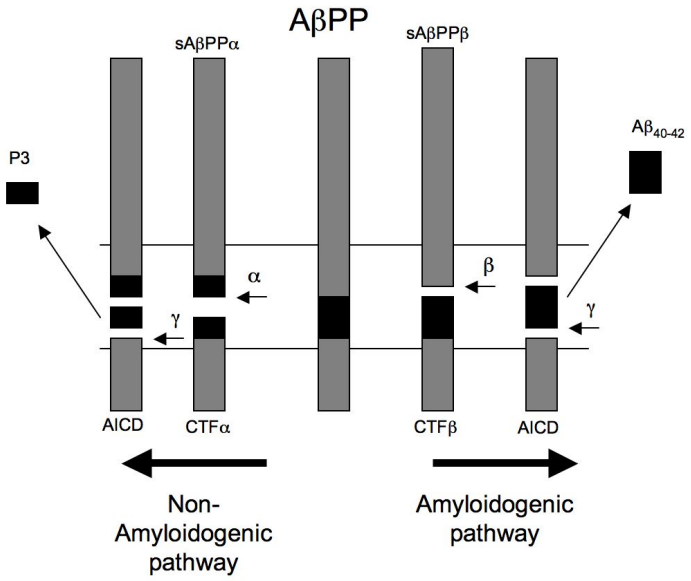

Figure 1: APP Processing: α-secretase and γ-secretase produce non-plaque forming p3, while β-secretase and γ-secretase produce amyloid plaque-forming Aβ. The different regions of the APP protein are indicated.

Risk factors for cardiovascular disease (i.e. high cholesterol, hypertension, and atherosclerosis), and diabetes have shown to increase the risk of developing sporadic form of AD (reviewed in [34]). The rate to which these factors are involved in AD may be influenced by genetic factors, for example alleles of Apolipoprotein E (ApoE) which affect AD and vascular disease [35]. ApoE is a plasma cholesterol transport protein, the gene for which is found on chromosome 19 [36, 37]. The mechanism by which ApoE is involved in AD is not fully understood. Overwhelming evidence indicate that ApoE has isoform specific capability as a chaperone molecules for Aβ and influences Aβ metabolism, deposition, toxicity, fibril formation, and clearance from the brain [38, 39]. This is supported by evidence suggesting that elevated levels of plasma cholesterol increase Aβ assembly in the brains of humans and transgenic mice [40].

Non-genetic Risk Factors of Alzheimer's disease

Vascular Disease: A Critical Risk Factor for AD

It has been clearly stated that vascular and metabolic dysfunctions leads to AD pathology and might indicate early pathologies [41-43]. In normal physiological conditions blood flow through the vasculature mediates the neuronal activity and nutritional requirements [43]. Vascular dysfunctions compromise the transport of glucose and oxygen to the brain [44]. Vascular disruption in AD is recognized by abnormalities in vascular architecture including lower capillary density, vessel curvature resulting in narrowed blood vessels, reduced blood flow and poor arterial responsiveness [45, 46]. The cerebrovascular dysfunction often precedes the onset of cognitive impairment, suggesting a role in the mechanisms of the dementia [47-49].

Data from several studies suggested that there is an interaction between Aβ deposition and vascular dysfunction in neuronal degeneration [49]. Mouse models of AD, in which mutated amyloid precursor protein (APP) is overexpressed to increase Aβ levels, have a profound dysregulation of the cerebral circulation [36, 40]. Vascular dysfunction and abnormal cerebral blood flow was also observed when $A\beta_{42}$ was applied systemically [15]. Additionally, increases in APP activity and Aβ production have been observed in the hippocampus of rodents after ischemia, a non-specific response to cerebral brain trauma [50, 51]. In people without dementia, severe coronary artery disease has been linked to increased senile plaque counts. Hypertension has also been linked to increased plaques and neurofibrillary tangle densities, suggesting that AD pathology may be increased by systemic and cerebral vascular disease [52].

Aβ clearance is via the perivascular macrophages, perivasculture [53, 54] and microglia [42]. This clearance is regulated, by RAGE (receptor for advanced glycation end products) and LPR (lipoprotein receptor-related protein) receptors moving Aβ into and out of the brain [46, 55, 56]. Disruption of this clearance across the blood brain barrier has been shown to increase cerebral amyloid angiopathy (CAA) and neuritic plaques [57].

Metabolic Dysfunction

Metabolic abnormalities have also been seen to be involved in AD, and could possibly interact with vascular dysfunction. The drop in glucose in the brain is serious, as glucose is the brain's desirable and first source of nutrition [58]. Decrease in glucose usage is a well-known pathological characteristic of AD [58, 59], which is observed in the early stages of the disease in patients [60, 61]. In the early stages of AD, glucose usage deficiencies are greater than oxygen/blood flow deficit and at a later stage of AD the changes in these factors become similar [47, 62].

Aβ might also be involved directly with the metabolic dysfunction in glucose homeostasis [62]. For example, in several studies it has been observed that overexpression of Aβ in transgenic mice leads to decrease in glucose usage [63]. Additionally, Aβ changes the characteristic of insulin resistance, including decreased glucose usage [64], a decrease in insulin receptors [65] and also promotes insulin resistance [66]. Insulin resistance and poor glucose usage elevate Aβ production and decreases the clearances of Aβ. Insulin resistance reduces Aβ clearance by decreasing the expression and activity of insulin degrading enzyme (IDE), an enzyme involved in Aβ clearance [67, 68]. Disruption of *IDE* expression can result in increased Aβ plaque load in AD models [68]. Additionally, the IDE knockout mouse model exhibits increased insulin levels, glucose intolerance and increased Aβ levels [49].

Insulin resistance is the main feature of diabetes and increasing evidence supports that insulin resistance leads to AD pathology [66]. Insulin resistance in AD has been termed as type 3 diabetes [69], which is characterized with a low number of insulin receptors, low brain insulin levels [66, 70] and reduced glucose homeostasis [62]. Additionally, it

has been observed that reduction in insulin and its receptors contributes to the conversion of mild cognitive impairment (MCI, prelude to AD) to AD as well as increase in AD severity [67, 70, 71]. Furthermore, insulin resistance contributes to increased oxidative stress and mitochondrial dysfunction [72, 73].

Cholesterol

Epidemiological evidence has identified a link between plasma cholesterol levels and AD development [74, 75]. High levels of cholesterol are associated with Aβ deposition and increased risk of developing AD [75, 76]. Epidemiological data has linked high cholesterol levels with increased Aβ production and AD progression. This has been supported by a number of *in vivo* and *in vitro* studies [77, 78]. For example, animals fed a high cholesterol diet showed increased Aβ accumulation and BACE1 activity. In contrast treatment with cholesterol lowering drugs resulted in lower Aβ levels [77-79]. This was shown in a study by Notkola *et al.* when patients taking the cholesterol lowering drug, statin, were found to have a lower incidence of AD [75]. However, recent studies examining statin usage have produced mixed results.

The effect of cholesterol on Aβ production has also been studied in *in vivo.* Staining the hippocampal and frontal cortex for Aβ immunoreactivity in rabbits given high cholesterol diets showed an increase in levels of Aβ in the brain. When these animals were returned to normal conditions, the strenght of the staining was decreased and Aβ levels in the brain decreased as well [80, 81]. In addition, Refolo *et al.* demonstrated that diets with high cholesterol applied to rabbits and AD mouse models, increase Aβ levels [79].

Increasing evidence supports the hypothesis that amyloidogenic processing of APP occurs in cholesterol rich lipid rafts, whereas non-amyloidogenic processing of APP occurs mainly in other regions of the membrane [82-84]. Therefore, altering cellular cholesterol levels regulates processing of APP through these two pathways [82, 83]. The activity of β-secretase and γ-secretase seems to be dependent on the composition of the lipids in the membrane [85-87].

Several *in vitro* studies have shown that elevated levels of cholesterol affects α- secretase and β-secretase activity, which results in the reduction of soluble APP (sAPPα) and an increase in $A\beta_{40}$ and $A\beta_{42}$. A decrease in cholesterol levels can boost α-secretase activity and the production of sAPPα while decreasing the generation of $A\beta_{40}$ and $A\beta_{42}$ [78, 88, 89]. The influence of cholesterol on γ-secretase activity has shown a variety of different results. While several studies have shown that γ-secretase activity relies on lipid rafts but it is not cholesterol dependent. On the other hand several groups have reported that cholesterol can regulate enzyme activity [83, 90, 91]. Recently it has been proposed that high levels of cholesterol alter plasma membrane lipid structure and block membrane fluidity, thereby preventing the cleavage of APP by α-secretase and inhibiting the formation of sAPPα. In return this increases the interaction between APP and BACE1 and the production of Aβ [89]. This displacement of APP within the plasma membrane

and the increase and decrease in lipid rafts in the plasma membrane contributes to neuronal degeneration because sAPPα is neuroprotective. sAPPα has been reported to play a key role as a trophic factor, and also helps to decrease intracellular calcium levels, and protect the cells from hypoglycemic damage and glutamate toxicity [88, 92].

Oxidative stress

Oxidative stress is the result of an imbalance between oxidants and antioxidants. The brain is especially vulnerable to oxidative stress because of its high oxygen utilization (which accounts for 20-25% of total body consumption), the high concentration of unsaturated lipids in the neuronal membrane, the high levels of transition metals and its relative absence of antioxidant systems compared to other organs [93, 94]. Under normal physiological conditions, ROS damage is handled by a structured array of antioxidant systems that provides an effective line of defense. On the other hand, in age-related neurodegenerative disease, for example AD, the balance between ROS and antioxidant defense is altered, resulting in assorted forms of molecular and cellular damage [94].

There is well-documented evidence that brain tissue in AD patients is exposed to oxidative stress during the course of the disease. Evidence of oxidative stress in AD is characterized through high levels of oxidized proteins, advanced glycation end products (AGEs), lipid peroxidation end products, and formation of toxic species, such as peroxides, alcohols, aldehydes, free carbonyls and oxidative modifications in nuclear and mitochondrial DNA [95-101]. Reduced plasma antioxidant levels and changes in antioxidant enzyme activities are reported in MCI patients and patients at early stages of AD [102-104]. This suggests a systemic imbalance between ROS generation and antioxidant defense system in the plasma of AD patients and this is supported by increases in DNA, lipid and protein oxidation products found in blood and cerebrospinal fluid (CSF) acquired from AD patients in contrast with controls [105-107].

Considerable evidence has suggested that accumulation of Aβ in the brain might be a protective response to oxidative stress [108, 109]. In some studies Aβ is proposed to act as a neurotoxin in AD, inducing oxidative stress and impairing mitochondria function [110, 111]. Oxidative stress in return prompts Aβ deposition [112].

Generally most highly reactive oxidants (including many radicals) interact with practically all-biological molecules, including DNA, RNA, proteins, lipids, carbohydrates and antioxidants. Less reactive species, such as peroxides (e.g. H_2O_2 lipid- or protein peroxides) are more selective in the targets that they damage, with alterations to particular molecules at specific sites.

Brain tissue has high levels of phospholipids, which are vital for the processes of neural transmission. Phospholipids in the CNS are made up of high levels of polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid and arachidonic acid. In oxidative stress conditions, PUFA are the first to be attacked because of its conjugated double bonds, which results in the increase of lipid peroxidation. Consequently, because of

increase in the formation of free radicals, there is a decrease in the amount of PUFA in the brain in AD [113].

Lipid hydroperoxides, produced as a result of lipid peroxidation. These unstable products are then non-enzymatically decomposed to different products, such as aldehydes malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), ketones, epoxides and hydrocarbons. Several studies have reported an increase in the levels of MDA and 4-HNE in AD and mild cognative impairment (MCI) [114-117]. Aldehydes that are produced during lipid peroxidation of PUFA can be released from their production sites and be used as markers of oxidative stress.

Isoprostanes are also products of lipid peroxidation. They are prostaglandin-like molecules produced from PUFAs that possesses, including arachidonic and docosahexaenoic acid. Isoprostanes can by formed peroxidation of phospholipids non-enzymatically. Their measurement is possibly the most reliable assay for measuring lipid peroxidation produced by oxidative stress. F2-isoprostanes (F2-IsoPs) are produced from arachidonic acid through esterification of phospholipids. In AD, high levels of F2-IsoPs were observed in cerebrospinal fluid (CSF) [118, 119].

F4-isoprostanes (F4-IsoPs) are compounds with similar composition to isoprostanes, and are produced by l peroxidation of docosahexaenoic acid, which predominantly is seen in the brain. The possession of six double bands means that docosahexaenoic acids are more vulnerable to free radical attack than arachidonic acid. Thus, measurement of its peroxidative products is an vital marker of oxidative damage in the brain and neurodegenerative diseases. F4-IsoPs levels are increased in CSF of AD compared with controls [120].

Hypoxia and its role in Alzheimer's disease

Access to oxygen is vital for almost every organism. At limited oxygen levels, cells react by changing their cellular metabolism to reduce oxygen consumption, decreasing the production of free oxygen radicals. In cells under hypoxic conditions, oxidative phosphorylation is no longer used to produce energy. Cells rely on glycolysis as the first source of ATP production [121, 122]. At the tissue level, adaptation to low levels of oxygen lies in regulating physiological variables in the body to increase oxygen supply to vital organs. For example, under hypoxic conditions there is an increase in vascularization and *de novo* angiogenesis by regulation of the angiogenic factor VASCULAR ENDOTHELIALGROWTH FACTOR A (VEGFA), and an increase in the capacity of the blood to carry oxygen by increasing the expression of erythropoietin (EPO) [121]. Hypoxia induces the transcription of regulatory genes that promote oxygen delivery and anaerobic metabolism while suppressing major energy requiring processes and inhibiting growth and development in animals ranging from invertebrates to mammals [123]. There are a number of oxygen-sensing pathways which assist in hypoxia

tolerance by activating transcription and inhibiting translation of proteins involved in the energy and nutrition sensor mTOR, the unfolded protein response that activates the endoplasmic reticulum stress response, nuclear factor (NF)-κB transcriptional response and the transcriptional response regulated by HYPOXIA INDUCIBLE FACTOR (HIF), which are key features of the cellular response to hypoxia. In normal physiology, hypoxic conditions play a role in blocking adipogenic differentiation and, in the process of wound healing through tissue reorganization [121].

HIF is a heterodimeric transcription factor composed of two subunits, HIF-α and aryl hydrocarbon receptor nuclear translocator (ARNT also called HIF-β). These factors bind through two Per-ARNT-Sim (PAS) domains, bind to DNA through their N-terminal basic helix-loop-helix (bHLH) domains and activate transcription via their C-terminal transcription transactivation domains (TADs). In mammals, *HIF-α* expression is regulated by cellular oxygen concentration whereas *ARNT* is expressed constitutively. Under normal physiological conditions at oxygen concentrations above 5%, prolyl hydrolase domain protein families (PHD1-3) hydroxylate two proline residues of HIF-α. This hydroxylation enables binding of the von Hippel-Lindau tumor suppressor protein (vhl), which is the recognition subunit of the E3 ubiquitin-protein ligase, and targets HIF-α for ubiquitylation and proteosomal degradation [124, 125]. Additionally, hydroxylation of asparagine residues in the TAD domains of HIF by factor inhibiting HIF (FIH) blocks the binding of transcriptional co-activators, for example CBP/p300. In contrast, under low oxygen conditions (at oxygen concentrations less than 5%) prolyl and asparaginyl hydroxylation is decreased which results in HIF-α accumulation as well as dimerization with ARNT. This complex is then translocated to the nucleus, where it binds to DNA and interacts with transcriptional coactivators. The HIF complex binds to hypoxia responsive elements (HRE) to mediate the transcription of over 200 genes in response to hypoxia [126].

Hypoxic conditions in cells and tissues can be a direct result of hypoperfusion which may play an important role in AD pathogenesis [123]. Recent studies in postmortem samples of human AD brains have shown that APP levels increased after mild and severe brain ischemia [127]. In addition, in several studies it has been observed that chronic hypoperfusion or focal ischemia insults in animal models contributes to increased accumulation of APP in brain cells which may lead to enhanced Aβ deposition in AD [128]. Recently, it was observed in humans and mice that the promoter of *BACE1* gene (that cleaves APP to produce Aβ) possess' the HIF binding sites and these hypoxia response elements (HREs) are physiologically functional in transcriptional modulation of *BACE1* gene expression both *in vitro* and *in vivo* [129, 130]. Studies done under hypoxic conditions have shown that endogenous BACE1 and Aβ levels are increased in SH-SY5Y cells. More interestingly, Sun *et al.* found that when APP23 transgenic mice (mice overexpressing human APP with the Swedish double mutation) were subjected to low oxygen levels they produced more and larger amyloid plaques [129].

A number of metallopeptidases have been suggested for the role of amyloid-degrading ensymes, such as neprlysin (NEP), insulin degrading enzyme (IDE) and endothelin-converting enzyme (ECE). Recent studies using ischemia rat models described a considerable decrease in the level of cerebral NEP and ECE-1 in the rat brain after global transient ischemia [131]. In *in vitro* studies Fisk *et al.* showed that hypoxia decreased the levels of NEP protein and mRNA as well as its activity in human NB7 neuroblastoma cells and rat primary cortical neurons [132]. Arterial hypoperfusion and abnormal cerebral blood flow (CBF) may notably impact the clearance of Aβ from the brain. In recent studies on AD mouse models and AD patients indicate that serum response factor (SRF) and myocardin (MYCOD), two transcription factors that are critical for CBF regulation, may decrease the expression of LRP-1, which responsible for clearing Aβ from the blood and brain barrier (BBB) [133].

Recent studies also have shown that chronic hypoxia, in addition to promoting the production of Aβ, also potentiated depolarization-evoked, calcium dependent neurotransmitter release because of an increase in the calcium influx [134, 135]. This was confirmed in PC12 cells, which showed that hypoxia increased the whole-cell calcium current. This effect is entirely due to selective and functional upregulation of L-type (Cav1) calcium channels [136]. Increase in L-type channels is due to post-transcriptional trafficking, such that at any given time a high number of calcium channels are present and active in the plasma membrane even though the total number of cell channel content was not changed [137]. However, selective inhibitors of β- or γ-secretase can inhibit the effects of hypoxia indicating an absolute role for Aβ in this process [137].

Genetic Risk Factors of Alzheimer's disease

Early-onset Familial Alzheimer's disease (FAD)

From a genetic point view AD is divided into two different groups: early-onset familial cases generally described by dominant Mendelian inheritance (Familial Alzheimer's disease (FAD)), and sporadic late-onset (≥60 years), with no known mode of transmission (Late onset "sporadic" Alzheimer's disease (SAD)). Familial form of AD cases strikes under the age of 60 years while the sporadic AD is influenced by genetic dysfunctions combined with lifestyle factors. FAD is mostly caused by dominant missense mutations in *APP, PSEN1* and *PSEN2* [138].

In 1984, Glenner and Wong [139] found a 4.2 kDa polypeptide called the beta-amyloid protein (Aβ) from fibrils seen in cerebrovascular amyloidosis and in the senile plaques linked with AD. The gene that encodes this protein was later located on chromosome 21 [140, 141]. *In vitro* studies also showed that the Aβ peptide is expressed as a much bigger protein named APP. In 1991 Goate *et al*. described a mutation (missense) in APP (London mutation; V717I) in families with AD [138].

Since the first reported mutation in APP [142], additional mutations in *APP* have been found. These mutations are seen in the γ-secretase cleavage sites or the transmembrane domain, which are present on exons 16 and 17. Mutations close to the β- and γ- secretase cleavage sites increases the production of total Aβ or a change the $A\beta_{1\text{-}40}$ $A\beta_{1\text{-}42}$ ratio towards formation of the more toxic $A\beta_{1\text{-}42}$ peptide. Although, mutations inside the Aβ domain can result into formation of Aβ with increased tendency to aggregate [143, 144]. To date, over 30 different APP missense mutations have been recognized and most of them of these are pathogenic. These mutations in most cases result in autosomal dominant FAD[145].

To date, only two recessive mutations in APP have been found. These mutations are: a trinucleotide deletion E693Δ in a family from japan with reduced $A\beta_{40}$ and $A\beta_{42}$ peptide formation, and no changes in their ratio [146] and A673V in another family. These mutations have been found to cause AD in the homozygous state. In Down syndrome, AD is due to an overexpression effect. This due to an extra chromosome 21 providing three APP copies [147]. The mutation A673T, near to the β-secretase cleavage site in APP, was found to be protective against AD. *In vitro* studies showed a ~40% reduction in the production of Aβ [148].
A year later after the discovery of the first APP mutation, a second AD linked region on chromosome 14 was found (14q.24). As two other research teams independently found the 14q24.3 loci in their studies, the 14q24.3 gene was identified as a major loci for FAD [149]. In 1995, this gene was identified as *PRESENIIN1* (*PSEN1*) [150]. Missense mutations in *PSEN1* are the major cause of FAD and are responsible for 18-50% of FAD cases [149]. More than 180 mutations in PSEN1 have been reported [145]. They are generally missense mutations, which result in amino acid changes throughout PSEN1 protein and cause a relative increase in the ratio of $A\beta_{42}$ peptides. Mutations in *PSEN1* cause the most potent forms of AD with complete penetrance and disease onset occurring as early as 30 years of age. The PSEN1 L166P mutation has an unusual age of onset in adolescence and *in vitro* studies suggest that this mutation induces abnormal levels of $A\beta_{42}$ formation as well as dysfunction in notch signaling [151].

In 1995, a gene in chromosome 1 was found in a German family with AD [152]. The gene was later named *PRESENILIN2* (*PSEN2*) [153]. Missense mutations in the *PSEN2* gene rarely cause FAD, at least in Caucasian populations. The age of onset for disease caused as a result of mutations in PSEN2 is 45-88 years and generally older than some families with *PSEN1* mutations [2]. Missense mutations in the *PSEN2* gene may be of lower penetrance than in the *PSEN1* gene [154]. The age of onset is highly different among patients with *PSEN2* mutations in the same family, in contrast with *PSEN1* where the age at onset is quite similar among affected family members.

Three years after their discovery in 1995, the *PSENs* were seen to be essential for the formation of Aβ from APP [155]. Wolfe et al. (1999) showed that the *PSENs* act as aspartyl proteases that carry out γ-secretase cleavage of APP to produce Aβ. γ-Secretase can generate a variety of Aβ species ranging in size from 37 to 46 amino acids [156]. Whether, FAD mutations appear in APP near the γ-secretase cleavage site or in the PSENs, the vast majority of them serve to increase the ratio of $A\beta_{42}$:$A\beta_{40}$ [157]. To date there has been, 40, 197, and 25 mutations reported in the *APP, PSEN1* and *PSEN2* genes, respectively. A list of all reported mutations is maintained in the Alzheimer Disease Mutation Database (http://www.molgen.ua.ac.be/ADmutations).

Apart from *APP* and *PRSENILINS* endeavors to find genes involved in FAD have resulted in identification of pathogenic mutations in other genes. A mutation in a family with FAD from the Holland was linked to chromosome 7 near the PAX transcription activation domain interacting protein gene (*PAXIP1*) [158]. Interestingly, a missense mutation, D90N was reported in *PSENEN2* located on chromosome 19, encoding one of γ-secretase subunits pen-2, which leads to AD pathology [159].

Late onset "sporadic" Alzheimer's disease (LOAD)

The genetic basis of late onset sporadic Alzheimer's disease (LOAD) seems much more unknown than the EOAD cases. LOAD, is mostly a result of the dysfunction of multiple genes and the interaction between them and environmental factors. As a result, it is harder to find new LOAD locus, especially because attempts to replicate often yields unclear results [138]. Most LOAD cases are sporadic with no family history of the disease, however, a gene for LOAD with recessive inheritance was identified recently by a genome-wide studies and the gene maps to locus 8p22-p21.2 [160].

To date the only genetic risk factor for LOAD is the ε4 allele of the apolipoprotein E gene (*APOE*) on chromosome 19q13 [161]. *APOE* is a component of several lipoproteins, such as high and very low-density lipoproteins, and chylomicrons. APOE plays an important role in lipid and cholesterol transport in the body. It is also a ligand for low density lipoprotein (LDL) receptors and mediates the binding, internalization, and catabolism of lipoproteins in cells [36]. In addition to its function in lipid and cholesterol metabolism, APOE has a role in mediating synaptogenesis, synaptic plasticity, and neuron inflammation [162]. *APOE* is highly expressed in the brain and its receptors in the brain include LDL receptors on normal astrocytes and the LDL receptor related protein seen in normal neurons as well as the senile plaques and seems to regulate the effects of APOE in the brain [163].

The three alleles of APOE are linked to specific amino acids at residues 112 and 158 (ε2: Cys112/Cys158; ε3: Cys112/Arg158; ε4: Arg112/ Arg158) [161]. *APOE* ε3 is the most common allele, whereas ε4 and ε2 have allele frequencies of 14% and 7%. The ε4-allele has been shown to increase AD risk by around four fold when inherited in one copy and

by more than 10-fold for two doses of the allele, while, the ε2 allele of *APOE* has a "protective" effect [164].

Genome-wide association studies

Genome-wide association study (GWAS) is the most common strategy for finding new AD gene candidates [7]. The GWAS studies require the genotyping of ancestral polymorphisms that commonly take place in less than 5% of the population. The results seen by the GWAS-derived genes are tiny, and they present only an ~0.10- to 0.15-fold increase or decrease in AD risk in carriers versus non-carriers of the identified alleles, as compared with a 4- to 15-fold increase in AD risk owing to the inheritance of *APOEε4* [165].

The first GWAS studies found GRB2-associated binding protein 2 (*GAB2*) as gene of interest. *GAB2* has been suggested to affect tau phosphorylation and to regulate Aβ production by interacting with growth factor receptor-bound protein 2 (*GRB2*), which then binds *APP* and the *PSENs* [166, 167]. GWAS studies also has led to the discovery of new AD genes including ataxin 1 (*ATXN1*), siglec 3 (*CD33*), and an uncharacterized locus on chromosome 14 (GWA_14q31.2) [168]. *ATXN1* can generate an enlarged polyglutamine repeat that causes spinocerebellar ataxia type 1. In correlation with AD, *ATXN1* was shown to affect Aβ levels by regulating β-secretase levels and cleavage of APP [169]. *CD33* is a member of the sialic acid–binding, immunoglobulin-like lectins. These proteins increase cell–cell interactions that mediate the innate immune system, including inflammation [170].

In 2009, Harold *et al*. (2009) and Lambert *et al*. (2009) reported the discovery of three new AD genes: clusterin (*CLU*), complement component (3b/4b) receptor 1 (*CR1*), and phosphatidylinositol binding clathrin assembly protein (*PICALM*) in two large case controlled GWASs [171, 172]. At the molecular level, clusterin (*CLU*) has been shown to take part in Aβ transport from plasma to brain and also Aβ fibrillization [173, 174]. *PICALM* is involved in clathrin-mediated endocytosis [175], which might be essential for APP to be cleaved by γ-secretase into Aβ [176]. *CR1* is the receptor for complement C3b, an important protein involved in inflammation, that is switched on as part of the CNS's innate immune system in AD [177], and might be able to defend against Aβ-induced neurotoxicity [178].

In 2010, GWAS reported the existence of additional AD genetic risk factors. Among these genes were bridging integrator 1 (*BIN1*), which it association with AD has been previously studied [172, 179]. *BIN1* is ubiquitously expressed in the CNS and plays a role in receptor mediated endocytosis, which has been proposed to induce APP processing and Aβ production or Aβ clearance from brain [180]. In 2011, four more Alzheimer genes were identified: *CD2AP, MS4A6A/ MS4A4E, EPHA1*, and *ABCA7* from GWASs studies [181, 182].

GWAS are designed to identify frequent variants (with a minor allele frequency >5%) that are associated with risk of complex diseases, including AD. Identification of less frequent (1-5% MAF) and rare (<1% MAF) variants is ideally done using sequencing techniques, which attempt to characterize every single base pair in each sequenced individual. It appears likely that rare variants associated with risk of disease are particularly located in the coding regions of genes and may have larger effect size than the more common variants. Recent studies have indicated that an increase of rare variants may be central to some of LOAD cases, altering disease risk or clinical expression [183].

In 2012, a whole genome sequencing effort followed by genotype propagation in an Icelandic population identified a very rare variant associated with a lower risk of late onset-AD and cognitive decline. The A allele from the rs63750847 SNP is responsible for an alanine to threonine change at position 673 in APP, which results in the inhibition of β-site cleavage of APP and leads to a decrease in Aβ production, *in vitro* [148]. This mutation is unique in two ways: it is the first mutation in *APP* to be associated with late-onset AD and it is the first protective mutation described in *APP*. Although, the existence of this mutation in LOAD is interesting in term of pathophysiology and identifying therapeutic targets, this result needs confirmation and so far, the variant has been very hard to find outside Iceland, as exemplified by the observation of one very old carrier (age of death 104.8 years old) with little Aβ brain pathology in a Finnish population and absence of the variant in two large Asian studies from Singapore and China [184-186].

Following similar methodology, the same team identified and replicated a rare variant in the *TREM2* gene. The variation, rs75932628, results in an arginine to histidine substitution at position 47 (R47H), and conferred an increased risk leading to an earlier age of onset in LOAD patients [187, 188]. Furthermore, brain expressions of *TREM2* were observed in a mouse model of AD and were seen within granules in the cytoplasm of neurons and also around amyloid plaques, in microglia. R47H was also found to modulate risk of early-onset AD in a French population [189]. Additionally, R47H was replicated in a Spanish population from the USA [190], a Columbian family study with frontotemporal and AD dementia [191], a Belgian study [192], and an African-American sample [193]. Several studies have also reported that variants in *TREM2* have also been associated with risk of frontotemporal dementia [192]. Studies by Benitez *et al*., reported an association of R47H with risk of Parkinson disease (PD) [194]. These results indicate that TREM2 could mediate neurodegeneration in general through a role in cleaning extracellular debris and regulation of inflammation [194].

Additionally, several other studies using various sequencing approaches have characterized rare variants that seem to correlate with risk of AD in genes such as phospholipase D3 (*PLD3*) [195], the cholesterol and phospholipid regulator, ATP-binding cassette transporter (*ABCA1*) [196], nicastrin [197], and the Disintegrin and metalloproteinase domain-containing protein 10, *ADAM10*, which is an α-secretase [198].

Presenilins

The contribution of the PRESENILINs to disease pathology is not surprising given the crucial role they play in γ-secretase proteolytic activity, cell signaling, tau phosphorylation, oxidative stress, calcium homeostasis and autophagy, factors pivotal in neurodegeneration and, specifically, AD pathogenesis. The majority of FAD mutations are within the *PRESENILIN* genes, with over 185 of them being identified in PSEN1. These mutations, all are dominantly inherited and are almost uniformly missense mutations. Given that the bulk of all known FAD gene mutations exist in PRESENILIN genes, and multiple molecular events considered to be critical in AD pathology can be linked to PRESENILIN function, this implies that PRESENILIN dysfunction may play a critical role in both familial and sporadic AD (**Figure 2**). However, to date there is no convincing, unifying explanation as to how PRESENILIN function differs in the brains of normal, aged individuals and those who develop sporadic AD.

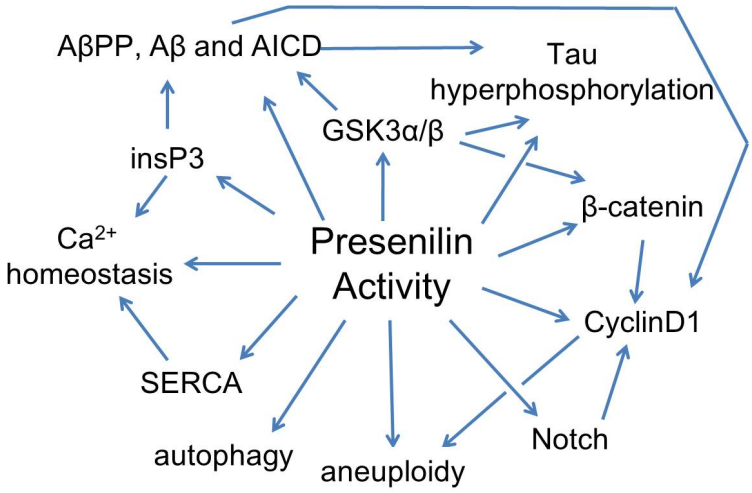

Figure 2: PRESENILIN proteins interact directly with many molecular processes thought to be involved in AD pathology. The PRESENILINs directly interact with APP, Tau, GSK3alpha/**β**, **β**-catenin and autophagy. PRESENILINs also influence calcium ion homeostasis. They regulate proteins controlling the cell cycle and are involved in chromosome segregation during mitosis. They facilitate a great number of cell signaling pathways through **γ**-secretase activity and also have highly conserved non **γ**-secretase functions involving microtubule/cytoskeletion function. There is limited knowledge about these functions and so possible, non-**γ**-secretase effects of the numerous AD mutations in the PRESENILINs are rarely considered.

The *PRESENIIN1* (*PSEN1*) gene in humans is located on chromosome 14 (14q24.3) and was originally discovered by genetic analysis of Alzheimer's disease patients with autosomal dominant trait. The closely related *PRESENILIN2* (*PSEN2*) gene is located on chromosome 1 (1q42.2) and was detected later on by sequence homology. Human *PSEN1* and *PSEN2* have different patterns of expression. Whilst *PSEN1* is transcribed similarly throughout the brain and in peripheral tissue, *PSEN2* is transcribed at low levels in the brain, apart from the corpus collosim and some peripheral tissues including the pancreas, heart and skeletal muscle where it is highly expressed. In brain tissue *PSEN*s have been found to be expressed in neurons and glial cells. Within cells PSENs are known to be

mainly localized in intracellular membranes such as the endoplasmic reticulum (ER), nuclear envelope, the mitochondria and the Golgi apparatus but there are also reports of PSENs on the cell surface [199], and in centrosomes and kinetochores [200]. PSENs have also been reported to be in the mitochondria associated membranes (MAM) of the ER. MAM is a specific compartment involved in the synthesis and transfer of phospholipids between ER and mitochondria. During neuronal development, *PSENs* are expressed in the early stages of differentiation and are localized to the cell body [201]. Both *PSEN1* and *PSEN2* initially exist as a holoprotein of approximately 50kDa with nine transmembrane domains (**Figure 3**) [156, 202].

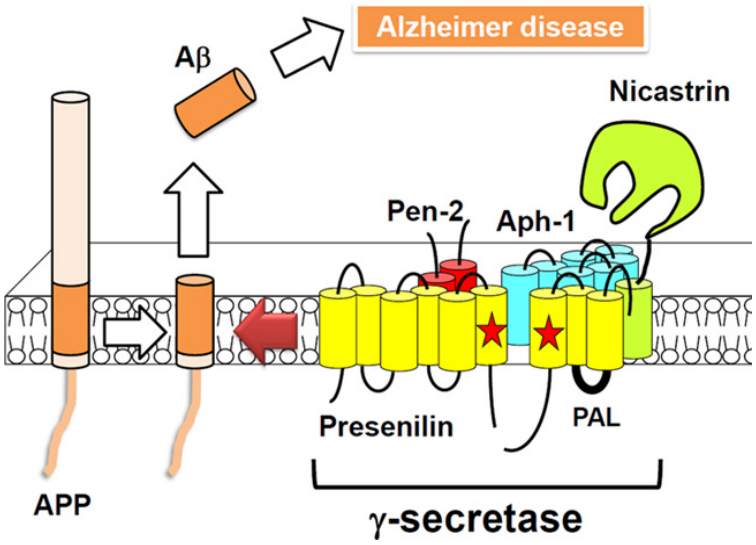

Figure 3: The four members of the γ-secretase complex within the cellular membrane. γ-Secretase executes the intramembrane proteolysis of several single membrane-spanning proteins, including APP (Diagram taken from Tomita and Takeshi (2013) [203]).

Cleavage within the hydrophilic loop by protease produces an N-terminal 28 kDa fragment and a C-terminal fragment of about 17kDa. These two fragments remain tightly associated with each other; have a long half-life together [204]. The hydrophobic amino acid sequences in the TM domains are highly evolutionarily conserved among various PSEN homologs. The cytoplasmic loop domain is the region of least conservation in PSENs [199]. The role for PSENs in APP processing was proposed when PSEN FAD mutations were obscured to cause an increase in the relative amount of $A\beta_{42}$:$A\beta_{40}$ indicating an altered processing at the γ-secretase stage. The increase in the ratio of the more aggregation-prone $A\beta_{42}$ is thought to trigger the disease process.

The formation of the γ-secretase complex is initiated when a sub-complex containing the highly hydrophobic APH-1 and immature glycosylated forms of NCT directly interact to form an intermediate scaffold for the assembling complex. The formation of this intermediate is governed by the ER retention factor, retrieval to endoplasmic reticulum 1 protein (*RER1p*), which can inhibit APH-1 binding to NCT. It has been suggested that PSEN (1 or 2) may directly bind to the APH1-NCT scaffold followed by PSENEN. It has also been postulated that a sub-complex of PSEN-PSENEN may bind to the APH1-NCT

complex to form a stable γ-secretase complex. Experiments utilizing chimaeric PSEN1 have shown that the 'NFGVVGM' motif within TMD4 of PSEN1 is necessary for binding to PSENEN (PEN-2), while the proximal two-thirds of TMD1, as well as the full sequence of PSENEN C-terminal domain, is needed to bind to PSEN1. Upon the binding of PSENEN, the PSEN TM6-TM7 loop 21 domain is positioned into the transmembrane channel to allow endoproteoylsis to occur thereby activating the complex for cleavage of other substrates (**Figure 3).**

Splicing of pre-mRNAs is an essential regulatory step in expression of all mutli-exon genes, broadening the information content of eukaryotic genomes (reviewed in [205]). Throughout this multistep process, introns are spliced out and exons are ligated producing the mature mRNA. In this process, the spliceosome, a large RNA/protein complex, is involved. This nanomachine is made up of five small ribonucleoprotein particles (snRNPs) U1, U2, U4, U5 and U6, and a large number of splicing-associated protein factors (reviewed in [205]). The selection of alternatively spliced exons results in generation of different protein isoforms from the same gene. Various splicing defects have been reported in AD related genes, for example, splicing variants lacking exons 4 and 9 in *PSEN1* have been reported in familial Alzheimer's disease [206, 207]. In this thesis, the main focus is a splice variant seen in the sporadic form of Alzheimer's disease (PS2V).

Other splice variants in PSENs have been seen including alternative splicing of exon 4 in *PSEN1* which introduces 4 additional amino acids (+VRSQ). This splice variant has not been reported in the *PSEN2* gene, which may be because the N-terminus of *PSEN1* and *PSEN2* are highly divergent. This splice variant is expressed ubiquitously. However a decrease in +VRSQ transcripts is seen in both sporadic and familial AD, which may be related to higher formation of $A\beta_{42}$ by –VRSQ [208]. In addition, another splice variant of *PSEN1* was detected in patients with frontal temporal dementia (FTD). In this case, exon 8 is deleted which resulted in accumulation of the γ-secretase substrates, AβPP C-terminal fragment and Notch, but whether it affects Aβ levels is not understood [209].

PS2V formation as a response to hypoxia

Several laboratories have observed a novel *PSEN2* transcript splice variant that lacks exon 5, PS2V, in brain tissues from sporadic AD patients [12, 210]. PS2V has also been observed in neuroblastoma cells under hypoxic/oxidative stress conditions. This suggests that hypoxia and oxidative stress disrupts the normal splicing of *PSEN2* mRNA, which then leads to production of PS2V [14]. Additionally, a *trans*-acting factor responsible for the regulation of *PSEN2* exon 5 splicing under hypoxic stress was detected. This splicing factor was identified as HMGA1a, which binds to specific sequences in exon 5 of the *PSEN2* transcripts [14]. In recent studies, it has been observed that HMGA1a binds to the U1 SnRNP through its 70k protein. Under hypoxia the HMGA1a-U1 SnRNP complex

interferes with the normal binding of U1 SnRNP to the adjacent 5′ splice site of exon 5 in *PSEN2* transcripts. This results in exclusion of exon 5 in *PSEN2* pre-mRNA. The exclusion of exon 5 creates a frame shift resulting in a premature stop codon in exon 6. PS2V encodes the N-terminal portion of PSEN2 from Met1 to Leu119 and an additional 5 amino acids (SSMAG) at its C-terminal (**Figure 4**) [12, 13, 211]. It has been reported that a stem loop structure upstream in exon 5 in the *PSEN2* gene may play a role in exon 5 skipping [211].

The PS2V transcript encodes truncated protein, which form intracellular inclusion bodies known as PS2V bodies and are generally observed in the pyramidal cells of the cerebral cortex and hippocampus of sporadic AD patients [11]. Expression of PS2V was not observed to have any effect in tau protein phosphorylation, however, PS2V proteins can change the conformation of tau proteins [212]. PS2V-expressing neuroblastoma cells were also seen to be susceptible to ER stress. PS2V prevents the unfolded protein response (UPR) in the ER from increasing, which results in aggregation and condensation of tau protein and other faulty proteins inside the cell [212]. Secretion of Aβ species was also seen to increase in neuroblastoma cells expressing PS2V when compared with controls. This increase in Aβ species might be due to disruption of the UPR in ER stresses [212].

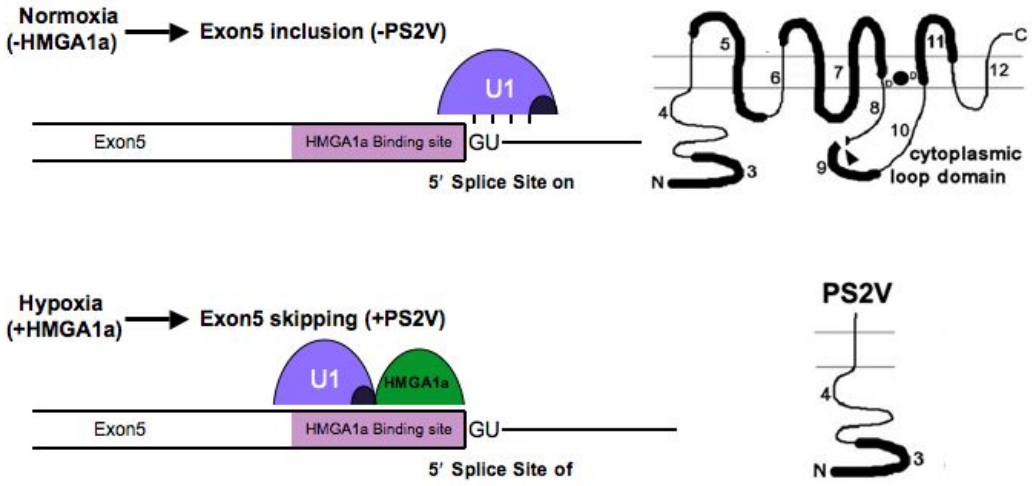

Figure 4: A molecular model for the mechanism of hypoxia-induced PS2V generation. Hypoxia induces overexpression of HMGA1a protein that subsequently leads to the generation of PS2V. HMGA1a binds to the target site adjacent to the 5′ splice site and it recruits U1 snRNP through U1-70K protein. The aberrant U1 snRNP–HMGA1a complex causes inactivation of the 5′ spice site and subsequent exon 5 skipping.

HMGA superfamily

The high mobility group proteins (HMG) are one of the non-histone chromosomal proteins. These proteins are nuclear proteins with high electrophoretic mobility on polyacrylamide gels. They are comprised of charged amino acid residues and have a molecular weight less than 30kDa [213-215]. HMG proteins act as architectural elements modifying DNA structure and creating new formations that enhance different DNA-

dependent activities, for example transcription, replication, recombination and repair [10, 214].

The HMG protein super family consists of three subfamilies: HMGA, HMGB and HMGN also known as HMGI/Y, HMG1/2 and HMG14/17. Each subfamily has its own specific protein sequence and functional sequence motif; the "AT-hook" for the HMGA family [10, 214], the "HMG box" for the HMGB family and the "nucleosomal binding domain" for the HMGN family [14, 214, 216]. These motifs of the HMG proteins have also been seen in other nuclear proteins called HMG-motif proteins. The difference between these HMG-motif proteins and the HMG archetypal proteins is that HMG-motif proteins show cell type-specific expression, are not ubiquitous and bind to DNA in a sequence-specific manner, whereas the archetypal HMG proteins are expressed in all cells, are mostly ubiquitous and do not bind to DNA in a sequence-dependent fashion [214].

In higher eukaryotes and especially mammals, the HMGA family is comprised of two functional members, HMGA1 and HMGA2. Using chromosome-mapping studies the locations of these genes have been found to be on chromosome 6 (6p21) for HMGA1 and chromosome 12 (12q14-15) for HMGA2. The *HMGA1* gene consists of eight exons and extends over a 10kb region whereas *HMGA2* only has five exons and extends over a 160 kb region. Alternative splicing of *HMGA1*gene transcripts creates three mRNAs encoding HMGA1a (107 amino acids), HMGA1b (96 amino acids) and HMGA1c (179 amino acids). The HMGA1a and HMGA1b isoforms vary by 11 amino acids found only in HMGA1a (**Figure 5**). The HMGA1c isoform is generated from the HMGA1 gene by use of noncanonical splice donor and acceptor sites, resulting in a frame shift creating two proteins that have the same first 65 residues but differ afterward [214].

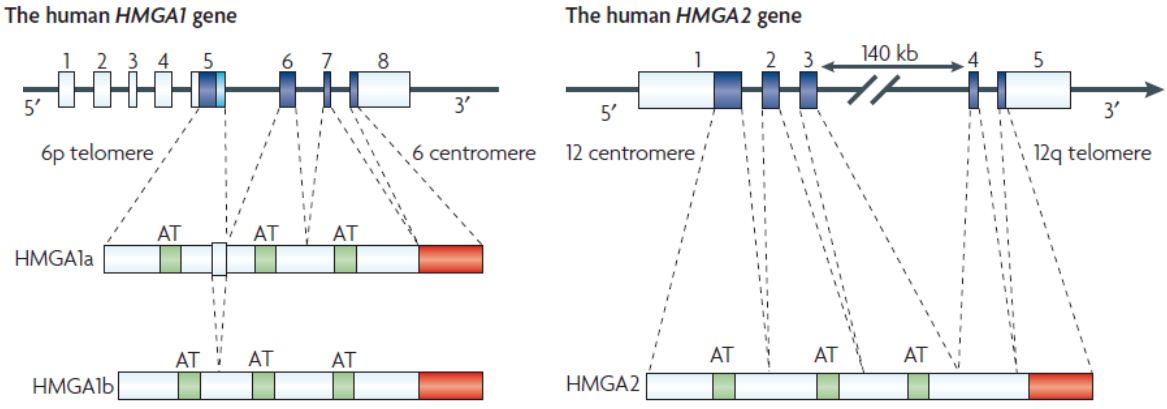

Figure 5: Nomenclature and general characteristics of the HMGA genes and proteins. (Diagram taken from Fusco and Fedele (2007))

From analysis of the amino acid sequence of the HMGA protein family, it has been reported that HMGA2 proteins have high amino acid identity with HMGA1a and HMGA1b including the conserved DNA binding domains. In the C-terminal of the

HMGA proteins, except for HMGA1c, there is a high amount of negatively charged acidic amino acid residues. The "AT-hook" motif consists of positively charged residues with the invariant repeated sequence of Arg-Gly-Arg-Pro (R-G-R-P) [213], followed by other positively charged amino acids, typically Arg and Lys. The binding site on DNA stretches between four to six base pairs, the equivalent of half a turn of double helix. "AT-hooks" allow HMGA proteins to recognize and bind to AT-rich sequences in the minor groove of B-form DNA [214, 217-219].

When two or more AT-hooks bind to DNA the interaction between HMGA protein and DNA is strengthened. The acidic tail (C-terminal) of the protein is important in the regulation of protein-protein interaction and may increase transcription factor activity. Recently, it was reported that HMGA can bind to specific types of structures formed by non AT-rich DNA sequences [214].

HMGA proteins undergo certain post-translational modifications such as phosphorylation, acetylation and methylation. Typically these biochemical modifications significantly change the binding potential of the HMGA proteins to both DNA and protein, which results in different biological functions. HMGAs are some of the most phosphorylated proteins in the nucleus. For example cdc2 kinase phosphorylates the HMGA1 proteins on specific amino acid sequences (Thr^{53} – Thr^{78}) in G_2/M phases of the cell cycle effecting its attraction for binding to DNA [214, 215]. HMGA1 proteins also are involved in signaling pathways that start from the cell surface. Recently it has been reported that CK2 phosphorylates HMGA1 protein at its serine residues (Ser^{102}- Ser^{103}) activating a signaling pathway which involves phsophoinositol-3 kinase and pp70 S6 kinase[215]. Enzyme Ca^{2+}/phospholipid dependent kinase (PKC) modifies HMGA1 proteins by phosphorylating Thr^{24}, Ser^{44} and Ser^{64} residues reducing its DNA binding properties [214, 220].

HMGA1 proteins also undergo acetylation which, like phosphorylation, plays a specific function. After the formation of enhanceosomes, GCN5/PCAF acetylates Lys^{71} in HMGA1 protein inducing chromatin remodeling as well as stabilizing the transcription activating complex. In contrast, CBP/p300 acetylates Lys^{65} in HMGA1 proteins, which leads to destabilization and disruption of the enhanceosome complex and shut down of transcription [220, 221]. In addition to the post-translational modifications mentioned above, HMGA proteins are also subject to methylation. For example, methylation occurs on Arg^{24} within one of the AT-hooks of HMGA1a which has been seen in tumor cells [220].

Two mechanisms have been suggested to explain transcriptional regulation by HMGA1. In the first mechanism HMGA1 plays a role acting as an anti-repressor, competing with histones for binding to the scaffold attachment regions (SARs) [218, 219, 221] of specific DNA sections and causing a more open chromatin that is more easily recognized by transcriptional activators [221]. Alternatively, HMGA1 can attach to certain AT-rich promoter elements, altering DNA conformation at a level that enhances their affinity for

multiple transcription factors. The binding between transcription factors and DNA is further stabilized by protein-protein interactions with HMGA1 [214, 221].

HMGA protein subfamily members are mainly localized in the nuclei of normal cells except during the S and G_2 phases of the cell cycle, when a minor protein fraction reversibly is moved out of the nucleus and into the mitochondria [222]. In cells that overexpress HMGA1 this highly regulated shuttling is disrupted and the proteins are found in the mitochondria at all stages of the cell cycle [223]. When inside the mitochondria, HMGA1 binds to the regulatory D-loop of mitochondrial DNA. Increased levels of mitochondrial HMGA1 interferes with the replication of mitochondrial DNA and results in the reduction of mitochondrial DNA. HMGA1 binds to the regions in the D-loop with a high A/T content referred as conserved sequence blocks (CSBs), which regulate mitochondrial DNA replication. When HMGA1 binds to this region it introduces bends and other structural change, which result in reduction of mitochondrial replication and decreased amounts of mitochondrial DNA [224].

Like mitochondrial DNA levels, mitochondrial mass is also influenced by intracellular levels of HMGA1. Mitochondrial biogenesis is controlled by closely regulated pathways which involve transcription of both nuclear and mitochondrial genomes [224]. The nuclear genome encodes 98% of the protein content of mitochondria. HMGA1 proteins are well characterized components of transcription factors involved in both up and down regulation of genes [225]. Therefore, the reduction in mitochondrial mass in cells overexpressing HMGA1 is due to changes in nuclear genes that encode for mitochondrial proteins whose altered expression contributes to mitochondrial reduction [224]

Microtubule Associated Protein Tau (*MAPT*)

Tau proteins are unfolded microtubule-associated proteins which bind to, and play a role in, the accumulation and stabilization of microtubules [226]. In neurons, tau is seen ubiquitous in axons [227], however several studies indicate a role for tau in dendrites [228]. Six tau isoforms are expressed in the adult brain, and they are generated by alternative splicing of the *MAPT* gene on chromosome 17q21.31 [229]. The tau isoforms vary from one another by the inclusion of exclusion of a 29-amino acid or 58-amino acid insert in the N-terminal region and by the presence or absence of a 31 amino acid repeat in the C-terminal region of the protein. The inclusion of exon 10 is associated with the production of three isoforms with four repeats each, and its absence is seen in the formation of another three isoforms with three repeats each (**Figure 6**). The repeats and the adjacent sequences make up the microtubule-binding domains of tau, with four-repeat tau having a bigger affinity initiate microtubule accumulation than three-repeat tau [230, 231].

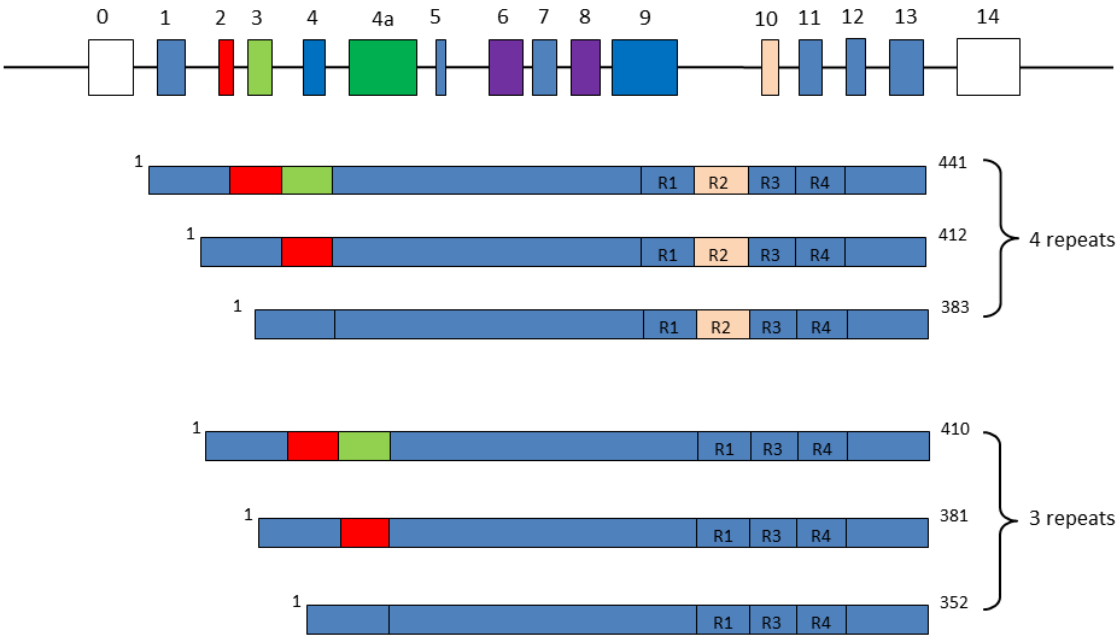

Figure 6: Human brain tau isoforms. *MAPT* and the six tau isoforms expressed in an adult human brain. *MAPT* consists of 16 exons (E). Alternative mRNA splicing of E2 (red), E3 (light green), and E10 (orange) gives rise to the six-tau isoforms (amino acids 352–441).

Equal ratios of three-repeat and four-repeat tau are observed in the brains of healthy adults [230]. Alternative splicing of *MAPT* is similar in different brain regions. Although, localized changes in the alternative splicing of *MAPT* may increase sporadic tauopathies. In the young human brain, only the shortest tau isoform (ie, three-repeat tau with no amino-terminal inserts) has been seen. However tau is expressed in many forms in different animals but the isoform ratios are not conserved. Tau isoforms with three, four, or five repeats have been observed in the brains of adult chickens, whereas in adult rodents predominately tau proteins with four repeats have been seen (207).

Tau aggregation

Tau proteins accumulate into tangles via its tandem repeats, with the N-terminal region and the C- terminal [226, 232, 233]. In AD, after cell death, tau tangles can remain in the extracellular space as so-called ghost tangles made up of largely of the tandem repeat region. The generation of tau tangles has a deleterious effect, and recent studies have demonstrated that tangles made of the tandem repeats region of tau can cause neurotoxicity [234]. Human tauopathies which are result of mutations in *MAPT*, tau aggregate do not aggregate in the extracellular space after cell death. It is still unclear why in tauopathies tangles are not seen in the extracellular space after cell death.

Model organisms for Alzheimer's disease

Although *in vitro* analyses have proven effective, there ability to replicate the complexities of cellular system are limited. In order to thoroughly investigate cellular

biology *in vivo* system must be used. There is, and has been, a need to develop appropriate *in vivo* systems to investigate AD. Importantly *in vivo* animal models provide readily available techniques and experimental approaches that are denied to human studies.

Invertebrate models of Alzheimer's disease

The nematode *Caenorhabditis elegans* has multiple presenilin genes: *sel-12, hop-1* and *spe-4*. *sel-12* has a key role in LIN-12/Notch signalling [235]. Furthermore, it was demonstrated that mutations in *sel-12* and *hop-1* leads to a reduction in the temperature memory. Interestingly, the human PSEN1, but not the familial AD mutant (PSEN1 A246E), has been shown to be able to rescue this phenotypic changes seen in the *C. elegans*, which suggest an evolutionarily conserved control of neural morphology and function by presenilin [236]. Receptors of the LIN-12/Notch have been shown to regulate cell–cell interactions during development, and presenilins homologues are essential for cleavage of Notch intracellular domain (NICD) which modulaes the transcription of key genes involved in the animals differentiation [237].

Expression of human $A\beta_{42}$ peptide using the muscle specific unc-54 promoter/enhancer in *C. elegans*, results in the formation of inclusions comprised of amyloid is seen in the worm which then undergoes paralysis [238]. Mutant worms with neuronal $A\beta_{42}$ expression have deficiencies in odorant preference associative learning behavior and the serotonin controlled behaviors [239]. In addition, mutant *C. elegans* have been generated that express $A\beta_{42}$ peptide with a single amino acid change, Leu17Pro and Met35Cys, which are not able to form amyloid deposits, indicating that these are important residues for plaque formation [240]. Additionally, the transcribed Aβ in *C. elegans* is not the full length 1–42 (amino acids) as anticipated, but a 3–42 truncated product. *In vitro* studies showed that $A\beta_{3-42}$ self-aggregates like $A\beta_{1-42}$, but with higher rate and forms fibrillar structures. The $A\beta_{3-42}$ peptide is also a more potent initiator of $A\beta_{1-40}$ aggregation [241]. These results support *C. elegans* as a good model to investigate the toxic effects of $A\beta_{3-42}$.

Drosophila expresses an APP ortholog [242] and all components of the γ-secretase complex [243]. While a β-secretase-like enzyme was found in flies [244], it exhibits very low β-secretase activity [245]. The *Drosophila* APP ortholog dAPPl region corresponding to the Aβ peptides lacks significant homology with its human homologue [242]. As a result, no endogenous Aβ is generated in the fly. However, overexpression of the β-secretase-like protein leads to cleavage of dAPPl, resulting in the generation of a fragment similar to the human Aβ peptide [244]. Furthermore, this fragment can cluster and cause age dependent behavioral deficiencies and neurodegeneration [244].

Beyond endogenous Aβ formation, flies models have been produced to study human $A\beta_{42}$ induced toxicity and neurodegeneration [246-248]. Greeve and co-workers produced a

triple mutant fly expressing human APP (hAPP), human β-secretase (hBACE) and *Drosophila* presenilin (dPsn) with point mutations linked to familial AD mutations N141I, L235P and E280A [247, 249]. These flies where seen to have age-dependent neurodegenerative difficulties such as photoreceptor cell loss, severe degeneration of their projecting axons and early lethality [247]. Finelli *et al*. generated fly lines expressing Aβ peptides which allowed an in-depth analysis of Aβ aggregation as forced overexpression of $A\beta_{40}$ and $A\beta_{42}$ peptides can be induced in different cell types including neuronal cells. [250]. Both peptides have been seen to accumulated in the brain of the fly and only $A\beta_{42}$ aggregates to form deposits [251]. As a result, only $A\beta_{42}$ expressing flies show age-dependent and dose dependent neurodegeneration. Short-term memory impairment and locomotor deficiencies where observed in aged flies [248].

Mouse model of Alzheimer's disease

Mouse and rat are the most frequently used experimental model organisms for a great variety of human disease. Their short reproductive cycle, their high birth rate and small size make them relatively easy to maintain and manage [252]. These models have provided essential understanding of the overall physiological role of PSENs, as well as their role in neurodegenerative disease. PSEN proteins are present at early stages of development in the embryo, where the play an important role in the regulation of cell division. *PSEN1* and *PSEN2* mRNA is ubiquitous in the neuro-epithelial cells during early development. In adulthood expression of *PSEN* is reduced but can be detected mainly in the neurons of the cortex, hippocampus and cerebellum [253].

To better understand the functions of genes encoding proteins involved in Alzheimer's disease, such as AβPP, β-secretase, or PRESENILINs, knockout mouse models have been generated. For example, Presenilin 1 (Psen1) knockout mice develop neurodegeneration of the cerebral cortex and deterioration of memory and synaptic functions with increasing age [254]. These observed complications can be rescued by crossing these mice to a transgenic line expressing human *PSEN1*. A similar phenotype is also seen in *Notch1* knockout mice, which show skeletal and somite defects [254]. Also, expression in transgenic mice of human *PSEN1* under the mouse prion promoter that drives transgene expression from E8.5 onwards, rescues the developmental phenotype of $Psen1^{-/-}$ mice [252, 253]. While $Psen1^{-/-}$ mice show embryonic lethality, $Psen2^{-/-}$ mice are viable and fertile and only show a mild pulmonary phenotype without CNS abnormalities. Abnormal expression of Notch ligand and down regulation of Notch target genes demonstrates that knockdown of both these genes results in the loss of Notch signaling [255].

To generate AD animal models exhibiting senile plaques and Aβ associated neuropathology different types of transgenic mice that express human APP and other genes involved in AD have been designed [256]. The first AD animal model with Aβ neuropathology was generated using platelet-derived growth factor-β promoter driving a human *APP* mini-gene encoding the APP V717F mutation associated with familial AD (PDAPP mouse) [256, 257]. Theses mice show numerous extracellular Aβ deposits,

neuritic plaques, synaptic loss, astrocytosis, and microgliosis. Aβ deposition in these mice is associated with neutrophil changes, but not with overt neuronal loss. Other transgenic models such as the "APP 23" transgenic mice bearing the Swedish mutation of AβPP (K670N/M671L) develop congophilic plaques with neuritic changes and dystrophic cholinergic fibers, suggesting a causal link between Aβ deposition and cholinergic degeneration[256].

Triple transgenic (3xTg-AD) mice which harbor $PS1_{M146V}$, APP_{Swe}, and tau_{P301L} transgenes have also been developed. 3xTg AD mice progressively develop extracellular Aβ plaques and neurofibrillary tangles (NFT). These mice exhibit deficits in synaptic plasticity, including long-term potentiation (LTP) that occurs prior to extracellular Aβ deposition and tangles [258].

Zebrafish model of Alzheimer's disease

Danio rerio (zebrafish) provide an effective vertebrate animal model for investigating human cellular and developmental biology. Zebrafish are easy to maintain, small, and are sexually reproductive after 3 months, enabling the quick establishment of a transgenic line. Their embryos develop rapidly, with gastrulation complete after 10 hours post fertilization (hpf), while the organs are formed and functional within the first 5 days. This rapid, external development of a predominantly transparent embryo highlights their potential as a cell biology investigative model. Many *in vivo* experiments can be conducted, allowing dynamic cellular processes and early embryo development to be observed. The injection of morpholino antisense oligonucleotides (MOs) into zebrafish embryos has enabled the quick and easy assessment of gene function. These are typically 25 bases in length and designed to bind to a complementary sequence of RNA. Morpholinos hybridized with mRNA can interfere with progression of the ribosomal initiation complex from the 5' cap to the start codon. This prevents translation of the coding region of the targeted transcript [259]. MOs efficiently block translation initiation or mRNA splicing. Embryos injected with an MO at the one-cell stage can effectively show a loss of gene function [260]. Over hundred embryos can be injected per hour, providing an effective method for genetic screens.

High genome conservation between human and zebrafish. The human *PSEN1* and *PSEN2* genes both have orthologues in zebrafish, *psen1* and *psen2* [261]. Also, other components of the γ-secretase complex, *Aph-1*, *Psenen* and Nicastrin (*Ncstn*) have been indentified in zebrafish. Zebrafish contain two homologues of the human *AβPP* gene, *appa* and *appb,* which show 70% homology to human APP_{695} at the amino acid level. The Aβ-encoding region as well as the transmembrane domain of human AβPP is well conserved in zebrafish [262].
Zebrafish Psen1 (similar to its counterpart in humans) is a membrane spanning protein. The catalytic sites in human PSEN1 are conserved in zebrafish [263]. *psen1* transcripts are expressed maternally and ubiquitously throughout embryo development [264]. The full length holoprotein is rapidly cleaved into two stable fragments, the N-terminal

fragment (NTF) and the C-terminal fragment (CTF). *In vitro,* studies showed that zebrafish *psen1* is able to replace human *PSEN1* and is very efficient in producing $A\beta_{42}$ from AβPP containing the Swedish FAD mutation (K670N/M671L) [265].

It was demonstrated that by injecting a *psen1* translation blocking morpholino (MO) into fertilized embryos that a phenotype arises that resembles the mouse *Psen1* knockout phenotype [264]. After the injection, overall levels of Psen1 CTF fragments are reduced whereas full length protein and the high molecular weight complexes containing Psen1 are not affected. Also, reduction in the expression of *hairy-related 1(her1),* the orthologue of human *HES7*, is seen. This is a Notch target gene, indicating a reduction in Notch signaling due to loss of Psen1 [264]. While in mice *psen2* knockdowns produce a very mild phenotype showing subtle pulmonary fibrosis and hemorrhage during aging, in zebrafish, *psen2* knockdownt using MO gives rise to phenotypes resembling loss of *psen1* activity, including enlarged brain ventricles and reduced pigmentation. *neurogenin1* (*neurog1*) expression is mediated by the Notch signaling pathway. Injection of *psen2* MO increased *neurog1* expression in the spinal cord due to decreased Notch signaling [262]. Injection of MOs blocking translation of psen1 and psen2 increased the number of Rohon-Beard dorsal sensory neurons, which results in a decrease in the number of trunk neural crest cells [263].

Other components of γ-secretase, Psenen/Pen-2 (encoded by *psenen*) and Aph-1 (encoded by *aph1b*) have been identified in zebrafish. Like *psen1*, both of the genes encoding these proteins are maternally expressed and are at their highest level of expression at 12hpf of development. Injection of embryos with MOs blocking *aph1b* or *psenen* expression decreases Psen1 CTF levels, indicating that, as in mammals, Aph1b and Psenen are essential for stabilization of Psen1 fragments. Psenen knockdown results in a severe phenotype, including opaque regions throughout the embryo indicating cell death. Being part of the γ-secretase complex, Aph1b and Psenen are involved in Notch signaling. Injection of MOs blocking *aph1b* and *psenen* decreases Notch target gene *her6* expression and increases *neurog1* expression. This indicates decreased Notch signaling and γ-secretase activity [262]. The orthologue of human NCT has been identified in zebrafish and shares 56% identity [266]. The inhibition of γ-secretase complex activity by N-N-(3,5- Difluorophenacetyl-l-analyl)-5-phenylglycine t-butyl ester (DAPT), has been shown to reduce Aβ peptide levels in the brain [267] and impairs somitogenesis and neurogenesis in developing embryos [268]. DAPT impairs Notch processing by disrupting Presenilin activity to prevent Notch signaling driving cell fate decisions [268].

1.2 Summary of chapter II-VI and the links between them

Zebrafish embryos have a unique combination of characteristics that allows genetic manipulation and analysis of molecular pathways implicated in neurodegenerative diseases. The embryos are numerous, macroscopic, external to the mother and transparent making them easy to inject and observe. Changes in different aspects of their rapid

development can be used as bioassays to assess gene activity. In **chapter II** of this thesis, we present evidence from a number of different assays that acute exposure to hypoxia or chemical mimicry of hypoxia increases oxidative stress in zebrafish brain tissue. We demonstrated that intracellular ROS levels are significantly increased in zebrafish brains exposed to actual hypoxia or chemical mimicry of hypoxia using NaN_3. Our results are consistent with the idea that insufficient oxygen supply may cause the oxidative stress and alterations in antioxidant enzymes observed early in the development of Alzheimer's disease. In previous work from our lab we have analysed the response to low oxygen levels of genes encoding proteins required for formation of the Aβ peptide that accumulates in the brains of people with Alzheimer's disease. We showed that, similar to in humans, expression of these genes seems to be increased in zebrafish brains under hypoxia [269, 270].

In **chapter III** of this thesis we examine the evolutionary conservation of PS2V and model the K115Efx10 mutation in zebrafish to investigate its effect on gene expression profiles, γ- secretase activity. In this chapter we show evidence for an important role of PRESENILIN genes in cellular responses to low oxygen (hypoxia). The PS2V splicing isoform of human *PSEN2* transcripts is generated under hypoxic conditions through induction of HMGA1a that binds to exon 5 sequence in transcripts. We show that an orthologue of the PS2V isoform, PS1IV, exists in the zebrafish. We also use our zebrafish assay system to examine the activity of the putative truncated protein product of the K115Efx10 allele of human *PSEN2* and show that it can also boost γ-secretase activity. The novel splice product of zebrafish *psen1*, "PS1IV" codes for a much smaller peptide then PS2V but, nevertheless is capable of boosting γ-secretase activity. In **chapter IV** we show that microarray analysis of PS1IV function demonstrates roles in modulation of a wide variety of gene products. In our results shows that production of this PS1IV is accompanied with activation of stress response genes such as interleukin 1 Beta (*IL1B),* tyrosine hydroxylase *(TH)*, and myelin expression factor (*MYEF)* which leads to triggering apoptosis and autophagy. We also demonstrate that PS1IV is an important contributor in signaling pathways associated with AD.

In **chapter V** we describe that while PS2V appears absent from mice and rats it is present in guinea pigs (and probably most other mammals). In this chapter we investigated the guinea pig, *Cavia porcellus*, as a model for Alzheimer's disease (AD), both in terms of the conservation of genes involved in AD and the regulatory responses of these to a known AD risk factor - high cholesterol intake. Unlike rats and mice, guinea pigs possess an Aβ peptide sequence identical to human Aβ. Consistent with the commonality between cardiovascular and AD risk factors in humans, we saw that a high cholesterol diet leads to up-regulation of BACE1 (β-secretase) transcription and down-regulation of ADAM10 (α-secretase) transcription which should increase release of Aβ from APP. We show that PS2V formation is up-regulated by hypoxia and a high-cholesterol diet while,

consistent with observations in humans, Aβ concentrations are raised in some brain regions but not others.

We have previously identified two paralogues (co-orthologues) of *MAPT* in zebrafish, denoted *mapta* and *maptb* and have shown that both genes are expressed in the developing central nervous system [271]. Similar to human *MAPT*, a complex pattern of alternative splicing of the *mapta* and *maptb* transcripts occurs. Zebrafish *mapta* gives rise to transcripts encoding 4R-6R isoforms, whereas *maptb* is predominantly expressed as a 3R isoform. In **chapter VI** we extend our examination of expression of the zebrafish tau co-orthologues to study their response to actual hypoxia and chemical mimicry of hypoxia in explanted adult fish brains. We observe increases in the overall levels of both *mapta* and *maptb* transcripts due to specific increases in the levels of *mapta* 6R and *maptb* 4R transcript isoforms. This is consistent with dramatically decreased levels of transcripts of the zebrafish orthologue of the human *TRA2B* gene that codes for a splicing factor proposed to regulate alternative splicing of *MAPT* transcripts.

Chapter II

THE COMPARISON OF METHODS FOR MEASURING OXIDATIVE STRESS IN ZEBRAFISH BRAINS.

STATEMENT OF AUTHORSHIP

The comparison of methods for measuring oxidative stress in zebrafish brains

Seyyed Hani Moussavi-Nik (Candidate/First Author)

• Development of PCR primers and Q-PCR analysis on:
  - Hypoxia and chemical mimicry of hypoxia treated adult zebrafish brain
• Performed lipid hydroperoxide assay on hypoxia and chemical mimicry of hypoxia treated adult zebrafish brain.
• Performed H_2DCF assay on hypoxia and chemical mimicry of hypoxia treated adult zebrafish brain.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Signed
…… Date

Kevin Croft (Co-author)

• Editing of paper
• Development of assay for F_2- and F_4-Isoprostanes on hypoxia and chemical mimicry of hypoxia treated adult zebrafish brain.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Signed
…… Date

Trevor A. Mori (Co-author)

• Development of assay for F_2- and F_4-Isoprostanes on hypoxia and chemical mimicry of hypoxia treated adult zebrafish brain.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.
Signed
…….. Date

Michael Lardelli (co-author)

Planned the research and supervised development of work for Moussavi-Nik.
Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.
Signed …… Date

Moussavi Nik, S.H., Croft, K., Mori, T.A. & Lardelli, M. (2014). The comparison of methods for measuring oxidative stress in Zebrafish brains.
Zebrafish, v. 11 (3), pp. 248-254

Chapter III

ALZHEIMER'S DISEASE-RELATED PEPTIDE PS2V PLAYS ANCIENT, CONSERVED ROLES IN SUPPRESSION OF THE UNFOLDED PROTEIN RESPONSE UNDER HYPOXIA AND STIMULATION OF γ-SECRETASE ACTIVITY

STATEMENT OF AUTHORSHIP

Alzheimer's disease-related peptide PS2V plays ancient, conserved roles in suppression of the unfolded protein response under hypoxia and stimulation of γ-secretase activity and

Seyyed Hani Moussavi-Nik (Candidate/First Author)

- Development of PCR primers and RT- and Q-PCR analysis on:
  - Hypoxia and chemical mimicry of hypoxia treated morpholino and mRNA injected zebrafish embryos
  - Hypoxia and chemical mimicry of hypoxia treated guinea pig cells
- Bioinfomatic analysis of PS2V binding site in different species.
- qRT-PCR of UPR related genes.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Signed
……Date

Morgan Newman (Co-author)

- Editing of paper
- Injection of HMGA1 morpholino and mRNA.
- Development and injection of UPR assay
- Zebrafish Notch assay (Except where noted)

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Lachlan Wilson (Co-author)

- Performed all Appa assay experiments, statistical analysis and injections.
- Performed all HMW western blot analysis and corresponding injections.
- Performed all preliminary IP experiments, injections and preparations of IP samples.
- Performed zebrafish Notch assay on K115Efx10.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Esmaeil Ebrahimie (Co-author)

- Microarray data analysis and gene expression network construction

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Signed
……Date

Simon Wells (Co-author)

• Preliminary work on Notch assay analysis.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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……Date

Giuseppe Verdile (co-author)

• Human cell culture experiments on Notch and APP (preliminary and not in the paper).
• Analysis of human PS2V in human cell culture.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Ralph Martins (co-author)

Supervised development of work for Verdile..

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Michael Lardelli (co-author)

Planned the research, wrote the manuscript, acted as corresponding author and supervised development of work for Newman, Wilson, Moussavi-Nik and Wells.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Moussavi Nik, S.H., Newman, M., Wilson, L., Ebrahimie, E., Wells, S., Musgrave, I., Verdile, G., Martins, R.N. & Lardelli, M. (2015). Alzheimer's disease-related peptide PS2V plays ancient, conserved roles in suppression of the unfolded protein response under hypoxia and stimulation of γ-secretase activity.
Human Molecular Genetics, v. 24 (13), pp. 3662-3678

Chapter IV

A ZEBRAFISH HOMOLOGUE OF ALZHEIMER'S DISEASE ASSOCIATED PRESENILIN ISOFORM PS2V REGULATES INFLAMMATORY AND OTHER RESPONSES TO HYPOXIC STRESS

STATEMENT OF AUTHORSHIP

A zebrafish homologue of Alzheimer's disease-associated PRESENILIN isoform PS2V regulates inflammatory and other responses to hypoxic stress.

Esmaeil Ebrahimie (Co-author)

• Microarray data analysis and gene expression network construction and wrote the manuscript.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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Seyyed Hani Moussavi-Nik (Candidate/Co-author)

• Development of PCR primers and Q-PCR analysis on:
  - Hypoxia and chemical mimicry of hypoxia treated morpholino and mRNA injected zebrafish embryos
• RNA extraction from treated embryos.
• Analysis of Microarray data.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Morgan Newman (Co-author)

• Injection of zebrafish embryos with morpholino.
• Editing of manuscript.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Mark Van Der Hoek (Co-author)

• Performed microarray and preliminary analysis

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Michael Lardelli (co-author)

Planned the research and supervised development of work for Ebrahimie, Moussavi-Nik, and Newman.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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……..Date

A zebrafish homologue of Alzheimer's disease-associated PRESENILIN isoform PS2V regulates inflammatory and other responses to hypoxic stress

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Abstract

The changes in gene transcription underlying Alzheimer's disease (AD) and their consequences for cell biology are not yet well understood. Mutations in the *PRESENILIN* genes *PSEN1* and *PSEN2* can cause familial Alzheimer's disease (FAD) that usually shows early onset before 65 years of age. Genetic variation in these genes does not appear to contribute to sporadic Alzheimer's disease (SAD) with late onset. However, an alternatively spliced form of *PSEN2* transcript encoding a truncated peptide named PS2V has been observed to be upregulated in sporadic AD brains and is induced by hypoxia. Mice and rats do not possess PS2V but a cognate isoform, PS1IV, forms in zebrafish. PS1IV is induced under hypoxia (or mimicry of hypoxia using sodium azide exposure) and we can selectively block its formation using a morpholino antisense oligonucleotide. To investigate the function of PS1IV (and suggest possible roles for *PS2V* in humans) we performed microarray analysis of changes in the transcriptome of zebrafish embryos at 48 hours post fertilization (hpf) under mimicry of hypoxia with and without inhibition of PS1IV formation. Various methods of genetic network analysis were subsequently applied to the data to suggest cellular functions altered by the absence of PS1IV. We observed changes in the expression of genes controlling inflammation (particularly SAD-associated *IL1B* and *CCR5*), vascular development, the unfolded protein response, protein synthesis, calcium homestasis, catecholamine biosynthesis, TOR signaling and cell proliferation. The role of *PS2V* in SAD and other diseases, including schizophrenia, deserves closer study, particularly when hypoxia is implicated in the pathology.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder with several pathological characteristics, including severe neuronal loss, glial proliferation, extracellular deposition of senile plaques composed of amyloid-β, and deposition of intracellular neurofibrillary tangles (reviewed in (1, 2)). The majority of AD cases are sporadic, with more than 15 million people affected worldwide. However, the cause of sporadic AD is poorly understood and therapeutic approaches are not yet well developed.

The *PRESENILIN1* (*PSEN1*) and *PRESENILIN2* (*PSEN2*) genes are known to be associated with the pathology of AD (reviewed in (3)). These genes generate transmembrane proteins located primarily in the endoplasmic reticulum (ER), which are involved in the production of amyloid-β through provision of γ-secretase activity. PRESENILIN proteins modulate the unfolded protein response that is a defense mechanism against ER stress (4).

One phenomenon observed in sporadic AD brains is an increase in alternative splicing of *PSEN2* transcripts to produce a truncated isoform named "PS2V" (5). PS2V protein accumulates in intracellular inclusion bodies termed PS2V bodies. These are observed in pyramidal cells of the cerebral cortex and the hippocampus of sporadic AD patients during early stages (6, 7). *In vitro* experiments indicate that PS2V protein impairs the unfolded protein response thereby sensitizing cells to various ER stresses and significantly stimulating the production of the AD-associated peptides, amyloidβ40 and amyloidβ42 (6). PS2V also changes the conformation of the protein tau, which is a major component of neurofibrillary tangles (8). Interestingly, PS2V was seen to be elevated in brains from people with schizophrenia to a greater extent even than in sporadic AD brains (although the sample size was small 40).

Using human neuoblastoma SK-N-SH cells, Sato et al. showed that the exclusion of exon 5 from *PSEN2* transcripts that forms PS2V mRNA is induced by hypoxia but not other forms of cellular stress (6). Under hypoxia, expression of the protein HIGH MOBILITY GROUP AT-HOOK 1 isoform a (HMGA1a) is induced in neurons and this binds to a sequence in exon 5 of *PSEN2* transcripts. This interferes with spliceosome complex function causing exon 4 to be ligated to exon 6. This causes a frameshift and generates a premature termination codon in exon 6 sequence (5, 9). Remarkably, PS2V increases gamma-secretase activity and production of Amyloidβ (Aβ) peptide from APP. Our previous research suggests that PS2V requires the presence of full-length PSEN protein to do this (10). Interestingly, the pathogenic K115Efx10 mutation of PSEN1 (causing of FAD) causes formation of an open reading frame very similar to that coding for PS2V suggesting that PS2V upregulation in sporadic AD brains may play a pathogenic role. We recently documented that PS2V is not a uniquely human isoform but is widely conserved in most mammals but not rats and mice

(11). Indeed, we showed that zebrafish possess a PS2V-cognate isoform, PS1IV, regulated by a conserved mechanism (i.e. involving a zebrafish orthologue of HMGA1a) but generated from the fish's PSEN1 orthologous gene (*psen1*) rather than its PSEN2 orthologue (*psen2*). Both human PS2V and zebrafish PS1V stimulate γ-secretase activity and suppress the unfolded protein response (Moussavi Nik, et al., 2014 submitted).

We recently demonstrated an ability to block specifically the binding of zebrarfish Hmga1a protein to *psen1* transcripts using an antisense morpholino oligonucleotide (Moussavi Nik, et al., 2014 submitted). This blocks formation of PS1IV under hypoxia while still allowing formation of transcripts encoding full-length protein. Since so little is known regarding the function of PS1IV or its cognate human isoform PS2V, we exploited our ability to manipulate PS1IV to investigate changes in the zebrafish transcriptome caused by its absence during hypoxia. We used a comprehensive microarray detecting zebrafish mRNAs to observe transcriptome-level changes in gene expression. This data was then subjected to various forms of genetic network analysis to reveal greater biological meaning from the observed expression changes. The results provide evidence-supporting modulation by PS1IV of numerous processes that, in humans, are implicated in AD pathology and other diseases.

Materials and Methods

Ethics statement

All animal experiments including experiments on genetically modified organisms were conducted under the auspices of the University of Adelaide's Animal Ethics Committee and Institutional Biosafety Committee.

Zebrafish husbandry, morpholino injection and exposure to NaN_3

Danio rerio were bred and maintained at 28 °C on a 14 h light/10 h dark cycle. Embryos were collected from natural mating from Tubigen strain (Tu), grown in embryo medium (E3) and staged (12). MoHmga1aBindBlock morpholino was synthesized by GeneTools LLC (Corvallis, OR, USA) and injected at 0.5mM (diluted to this concentration with MoCont as previously described (13) at the one-cell stage. For exposure of embryos to sodium azide (NaN_3, Sigma-Aldrich CHEMIE Gmbh, Steinheim, Germany), this was performed at 100μM from 6 hpf until embryos reached developmental stages equivalent to those attained under normoxia at 48 hpf at 28.5 °C (30 embryos were used for each concentration).

Oligonucleotides

The sequences microRNA primers, and qPCR primers used in this study are given in Supplemental Data 1.

Microarray experiment, experimental design, and statistical analysis

For microarray analysis, total RNA was extracted from different injection batches using the RNeasy Mini Kit according to the manufacturer's specifications (Qiagen GmbH, Hilden, Germany). For each sample, the RNA concentration was determined with a NanoVue™ UV–vis spectrophotometer (GE Healthcare Life Sciences, Fairfield, USA). RNA integrity and quality were then estimated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and the RNA integrity number (RIN) index was calculated for each sample. Only RNAs with a RIN number >7.0 were processed further.

To evaluate genome-wide changes in gene transcription related to the presence or absence of PS1IV under mimicry of hypoxia, we performed hybridisation analysis using the Zebrafish Gene 1.0 ST Array (Affymetrix Inc. Santa Clara, CA). Briefly, 300ng of total RNA derived from embryos was converted to amplified sense strand cDNA using the Ambion WT Expression Kit (Life Technologies, Carlsbad, CA). The resulting sense cDNAs were fragmented and Biotin end labelled using the Affymetrix Genechip WT Terminal Labeling Kit prior to hybridisation to the arrays at 45 °C for 16 hours. Finally, the signal intensity of the chip was scanned using a GeneChipR Scanner 3000TG and analysed using the Partek Genomics Suite software (www.partek.com). Cel files were imported and intensities adjusted for GC content using Partek's own method prior to RMA background correction and quantile normalization (14-16). Each treatment had four biological replicates.

The final list of genes significantly ($p \leq 0.05$) over-expressed or under-expressed as a result of blocking PS1IV formation under hypoxia was generated using the following formula:
Impact of PS1IV splicing blockage in hypoxia condition = [Comparison of (HMGA1aBindBlock) vs (Control Morpholino) in chemical Hypoxia] – [Comparison of (HMGA1aBindBlock) vs (Control Morpholino) in Normoxia] – [Comparison of (Control Morpholino) vs (Uninjected) in chemical Hypoxia] – [Comparison of (Control Morpholino) vs Uninjected in Normoxia]. A Bayesian t-test using FlexArray package (McGill University, Canada) was employed to find significantly over-expressed/under-expressed genes ($p \leq 0.05$) at each of the dual comparisons in the above formula as previously described (17). Removal of genes with significant alteration in the control comparisons increases the accuracy of uncovering genes influenced specifically by PS1IV expression.
Gene ontology analysis was carried out using the "comparative GO" web application (18, 19).

Statistical-based pathway discovery and regulatory network analysis after loss of PS1IV under mimicry of hypoxia

To understand the consequences of loss of PS1IV under hypoxia, we used an integrative approach of "gene set enrichment" (based on Fisher's exact test) (20) with union selected subnetworks (pathways) (21). This reveals important altered pathways as well as underlying regulatory mechanisms altering gene expression patterns in an integrative network.

Pathway discovery

For determination of statistically altered pathways after loss of PS1IV splicing under mimicry of hypoxia, the following steps were performed:

1- an enriched mammalian database of gene/protein/small RNA interactions, ResNet, was implemented in the Pathway Studio 10 package (Elsevier). ResNet is a database of biological relations, ontologies and pathways, compiled by Ariadne (Elsevier) for mammalian research (20, 21). One of the major advantages of this database is inclusion of information on the subcellular localization of gene prouducts which provides the oppotunity to evaluate the contribution of different cell compartments to biological pathways.

A range of interaction sets are deposited in the ResNet database such as regulation, expression, promoter binding, molecular transport, protein modification, binding, molecular synthesis, chemical reaction, direct regulation, miRNA effect, protein complex, and small molecule function. Furthermore, we updated the network database Pathway Studio 10 using Medscan language programming via text mining (22) with the keywords of names of over-expressed and under-expressed genes after *PS1IV* loss of splicing, Alzheimer's disease, and hypoxia to enrich the deposited networks in our reference database. MedScan is an NLP (Natural Language Processing) application for reading scientific literature and extracting protein-centric biological relations (22).

2- using as input the set of genes showing significantly altered genes expression after loss of PS1IV, different possible pathways to which these genes may contribute were called from the updated database of the Pathway Studio package.

3- the gene set enrichment concept was used to distinguish and select important pathways. Gene set enrichment measures the enrichment of different networks/pathways by an imported list of genes/miRNAs and highlights the statistically enriched pathways at $p \leq 0.05$, based on various statistical tests and particularly Fisher's exact test (20). As an example, if a particular pathway has 15 genes and 14 out of 15 genes of this network are present in the list of those displaying altered expression after loss of PS1IV, this pathway has a high probability of existence according to Fisher's exact test.

Integrative regulatory network construction

For construction of an integrative regulatory network, various algorithms such as "add neighbours (with varying ranges of directly and indirectly interacting neighbours – "expansions" of 1, 2 or 3)", "add direct interaction", "add shortest path", "add common targets", "add common regulators", and "union selected subnetworks" (17, 21, 23-25) were compared.

The add neighbours algorithm adds a new interacting element (direct or indirect interaction) to an imported gene entry if that new element is in within defined interacting range. Here, expansions of 1, 2, and 3 were tested. Add direct interaction only adds direct interactions to the imported gene list and produces the smallest networks. The next algorithm was the shortest path algorithm. Two elements in the network may interact with each other via many different routes. The shortest path algorithm selects the shortest route as that is the most probable to occur. The add common targets algorithm searches for interactions that are affecting a shared target. Add common regulators looks for common regulators of imported genes such as transcription factors. Union selected pathway is a reliable algorithm which attempts to join together statistically significant pathways (from the previous stage of pathway discovery) and finds the underlying relationship between pathways based on the shared elements. Due to the ability of transcription factors (TFs), ligands, and microRNAs, to regulate multiple systems, it can be expected that these play central roles in joining various pathways. In fact, union selected pathway can be seen as a crosstalk discovery tool.

Topological analysis of regulatory networks

Topological analyses of the regulatory networks of over-expressed, under-expressed as well as the combined set of over- and under-expressed genes (constructed using union selected subnetworks) were performed using the cyto-Hubba package (26, 27) in Cytoscape software (28). Centrality indices including Degree, clustering coefficient, Edge Percolated Component (EPC), Maximum Neighborhood Component (MNC), Density of Maximum Neighborhood Component (DMNC), Maximal Clique Centrality (MCC) and centralities based on shortest paths, such as Bottleneck (BN), EcCentricity, Closeness, Radiality, Betweenness, and Stress were used to identify the highest-ranked genes (hubs) in the structure of the regulatory networks using the cyto-Hubba plugin (26, 27, 29). For details regarding the employed indices, please see the cyto-Hubba URL: http://hub.iis.sinica.edu.tw/cytoHubba/supplementary/index.htm. The genes which received the highest scores in the above mentioned indices were selected as the highest-ranked genes (hubs) in the regulatory networks. The 10 highest-ranked genes according to Bottleneck score were visualised in Cytoscape for the regulatory networks of over-expressed andunder-expressed genes, and for the combined set of over- and under-expressed genes.

Validation of network nodes by quantitative real-time PCR (qPCR)

The relative standard curve method for quantification was used to determine the expression of experimental samples compared to a basis sample. For experimental samples, target quantity was determined from the standard curve and then compared to the basis sample to determine fold changes in expression. Gene specific primers were designed for amplification of target cDNA and the cDNA from the ubiquitously expressed control gene *eef1a1l1*. The reaction mixture consisted of 50ng/μl of cDNA, 18 μM of forward and reverse primers and *Power* SYBR green master mix PCR solution (Applied Biosystems Foster City, CA, USA).

To generate the standard curve, cDNA was serially diluted (100 ng, 50 ng, 25 ng, 12.5 ng). Each sample and standard curve reaction was performed in triplicate for the control gene and experimental genes. Amplification conditions were 2 min at 50 °C followed by 10 min at 95 °C and then 40–45 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification was performed on an ABI 7000 Sequence Detection System (Applied Biosystems) using 96 well plates. Cycle thresholds obtained from each triplicate were averaged and normalized against the expression of *eef1a1l1*, which has previously been demonstrated to be suitable for normalisation for zebrafish quantitative PCR (qPCR) (30). Each experimental sample was then compared to the basis sample to determine fold changes of expression.

Validation of predicted miRNAs by quantitative real-time PCR (qPCR)

MicroRNA PCRs were performed on replicated biological samples using TaqMan microRNA assays (Life Technologies). Briefly, a multiplex cDNA reaction was performed on 10ng of RNA using a TaqMan MicroRNA Reverse Transcription Kit together with specific TaqMan microRNA assay primers (Life Technologies). Real-time PCR was performed in triplicate with a 1:3 dilution of cDNA using specific TaqMan MicroRNA assays and TaqMan Universal Mastermix (Life Technologies) on a Rotorgene 6000 series PCR machine (Corbett Research). The relative expression of microRNA was calculated using the comparative Ct method with U6 as the endogenous reference. A list of primers used for miRNA qPCR assays is given in Supplemental Data 1.

Results and Discussion

The full names of genes/proteins are given in the section Abbreviations (see later).

Transcriptome alteration after PS1IV splicing blockage and genetic network analysis overview

To understand the functional genomics changes modulated by PS1IV, we blocked the induction of PS1IV splicing formation that commonly occurs under hypoxic conditions. We then used microarray analysis to identify genes with significantly ($p \leq 0.05$) increased or decreased transcript levels. We describe these genes as being "over-expressed" or "under-expressed" respectively (Supplemental Data 2 and Supplemental Data 3).

Within cells, the transcripts and proteins encoded by genes can have many functionally interacting partners. "Pathways" are sets of genes with biologically important interactive relationships. The Pathway Studio 10 software package (based on the Fisher Exact Test) accepts a set of genes with significantly altered expression and evaluates the genes with respect to their contribution to all the possible pathways in its database. It then ranks these pathways in terms of statistical significance to create a list of "statistically significant pathways". This approach offers a more comprehensive level of understanding of biological mechanisms compared to analysis of individual (separate) gene expression changes. We used the Pathway Studio 10 software package to examine the sets of genes showing significantly increased or decreased transcript levels when PS1V is blocked during mimicry of hypoxia (incubation in 100 μM sodium azide - hereafter referred to simply as "hypoxia"). Pathways with a statistical significance of $p \leq 0.05$ were then ranked.

Once ranked lists of statistically significant pathways had been determined, we then used an algorithm "union selected pathway" to attempt to join different pathways into larger aggregate networks (integrative networks). Networks were produced based on those genes with increased transcript levels, those with decreased transcript levels, and genes with either increased or decreased transcript levels (see later). The union selected pathway algorithm looks for elements shared between pathways that could act as links between them. This procedure is valuable as a test of the veracity of the existence of the identified pathways within our experimental system since pathways that show greater numbers of shared elements are less likely to be spurious.

A number of other methods for network analysis were also applied to our sets of genes that are "over"- and "under"-expressed in the absence of PS1IV. These other methods are summarised in Materials and Methods. In general, these analyses confirmed the major features of the networks produced by union selected pathway analysis. "Union selected

pathway" was the most efficient network prediction algorithm for combining the statistically significant pathways and unravelling the regulatory components (transcription factors, microRNAs, and ligands). The veracity of the networks predicted by union selected subnetworks was confirmed by qPCR analysis of predicted changes of transcript levels for a number of genes identified as nodal within the networks. In the discussion that follows we have focused on the union selected pathway analysis since it is the method producing the most statistically rigorous output. The "union selected pathway" algorithm has been recently used in the discovery of transcriptomics-based regulatory networks of biological phenomena such as apoptosis and stem cell differentiation (24, 31).

In the work described below, microarray analysis was performed to assess expression of genes expressed in zebrafish. However, since numerous human genes have "co-orthologues" in zebrafish (due to a whole genome duplication that occurred early in the teleost evolutionary lineage (32), and since the GO and Pathway Studio databases contain predominantly mammalian genetic information, we refer to genes using their human orthologue names. Raw microarray expression data gene designations are given in Supplemental Data 2 and 3.

Genes with altered expression after loss of PS1IV

PS2V is a truncated isoform of PSEN2 that shows elevated expression in the brains of people with sporadic AD. However, the cellular role of PS2V cannot be investigated using mouse or rat models since the rapid evolution of some of their AD-related genes has resulted in loss of their ability to induce this isoform (11). In contrast, zebrafish produces a PS2V-cognate isoform named PS1IV (32). We can block formation of PS1IV in embryos subjected to hypoxia by injection of the morpholino oligonucleotide Hmga1aBindBlock (Moussavi Nik et al. submitted). We exploited this phenomenon to investigate the effects on cellular function of loss of PS1IV under hypoxia using microarray analysis and subsequent gene network analysis.

Zebrafish embryos were injected at the 1-cell stage with MoHmga1aBindBlock or the negative control morpholino MoCont. They were then exposed to chemical hypoxia from 36 hpf to 48 hpf before extraction of mRNA and analysis of gene expression on the Affymetrix Zebrafish Gene 1.0 ST Array. A number of control microarray comparisons were also made to identify genes for which expression is affected by the act of injection or by the non-specific effects of the morpholino oligonucleotides (Figure 1). Bayesian t-tests produced lists of genes with significantly increased or decreased transcript levels ("expression") in the comparisons. We describe these below as genes "over-" or "under-expressed" in response to decreased PS1IV formation under hypoxia. The final list of genes with significant ($p \leq 0.05$) over- or under-expression was generated after removal of any genes showing significantly altered expression in the controls (see Figure1 and Table 1).

Among the genes showing over-expression under hypoxia without PS1IV (i.e. normally repressed by the formation of PS1IV) were numerous genes involved in modulation of immune responses (*HRH2*, *IL17RA*, *CD22*, *ACY1*, *IRF4*, *XCL2* etc.), in regulation of TOR signalling (*RICTOR*, *PKD1*) in regulation of blood pressure and formation of vascular structures (e.g. *AGTR1* and *ANGPTL2*), and in cell proliferation (*PIM3*, *ACY1*, *OLFM4*) (Supplemental Data 2). This set of genes also included two genes known to be involved in neurological conditions, *GAK* (associated with Parkinsons Disease) and *CAMTA1* (associated with cerebellar ataxia, nonprogressive, with mental retardation (Supplemental Data 2). Two non-coding RNAs were also seen in the list of genes over-expressed in the absence of PS1IV, si:dkey-167i21.2 and si:ch73-110p20.1.

A greater number of genes were identified as showing significant under-expression under hypoxia without PS1IV (i.e. normally dependent on PS1IV for stable or increased expression under hypoxia) These included genes encoding transcription factors or proteins involved in chromatin remodelling (e.g. *NKX3-2*, *SRCAP*, *GBX2*, *TCF25*, *SMARCA4*, *POU4F2*), genes involved in ER function (*CALR*), intracellular signalling (*OCRL*, *AIDA*, *PLEKHA4*, *PLCB1*, *MAPKAPK5*) and a considerable number of ribosomal proteins (see Table 1).

Significant pathways formed by genes over-expressed after loss of PS1IV under hypoxia

Genes over-expressed after loss of PS1IV were mined for their ability to construct statistically significant pathways ($p \leq 0.05$) based on the "gene set enrichment" approach (20) using the Pathway Studio 10 software package. The possible significant pathways and their corresponding *p*-values are presented in Table 2. The underlying relationships and their relevant references are presented in Supplemental Data 4. The pathways are: neighbours of *GABARAP* (Gamma-Aminobutyric Acid Receptor-Associated Protein), *PDLIM5* (PDZ And LIM Domain 5), MIR20A (microRNA 20a), LPS (lipopolysaccharides), BTK (Bruton A gamma globulinemia), and *NOTCH2* (notch2). These pathways and their interactions (relationships) are visualised in Figure 2. *GABARAP* encodes a ligand-gated chloride channel that mediates inhibitory neurotransmission and interacts with the cytoskeleton. GABARAP protein has a direct regulatory effect on the angiotensin receptor AGTR1 that regulates blood pressure and vascular development. GABARAP also mediates membrane expression of AGTR1 (Figure 2A) (33). Interestingly, GABARAP is also involved in apoptosis and autophagy (34-39). As presented in Figure 2B, *PDLIM5* may be involved in the observed over-expression of *AGTR1* and another gene, *PKD1*, after loss of PS1IV (40, 41). *PDLIM5* has been implicated in bipolar disorder, depression, and schizophrenia via regulation of dendritic spine morphogenesis in neurons (42, 43). This is especially interesting when one considers that the human homologue of PS1IV, PS2V, is greatly increased in the brains of some cases of schizophrenia (44). It has recently been suggested that *PDLIM5* plays an

important role in heart development and cardiomyocyte expansion (45). Since mutations in both human *PSEN1* and *PSEN2* can cause Dilated Cardiomyopathy (DC) this suggests that the *PRESENILIN* genes with *PDLIM5*, *PKD* and *AGTR1* may form an interesting genetic network involved in cardiovascular disease.

One pathway is formed by *NOTCH2* as a neighbour of *FOXE3*. *NOTCH* genes encode transmembrane receptors that signal directly to the nucleus. When NOTCH receptors are cleaved by PSEN proteins within γ-secretase complexes, their intracellular domains move into the nucleus to act as transcription factors (46). NOTCH receptors have important roles in maintenance of undifferentiated stem cell states, particularly under hypoxic conditions (47, 48). During zebrafish embryo development, the gene *foxe3* is expressed mainly in the developing lens of eyes that are relatively much larger than in humans. The *foxe3* gene is also shows restricted expression in the brain and in the hatching gland (46), a structure not found in mammals. This reminds us that our microarray analysis involves all the cells of a developing zebrafish at 48 hpf and not only those with relevance to the brain. This means that the networks we subsequently identify should not be interpreted as representing single overall regulatory networks within a single cell type. Some of the pathways will exist only within particular cell types and some apparent linkages between putative pathways may be artefactual.

A pathway illustrating the ability of microRNAs to coordinate regulation of multiple gene transcripts is formed by the apparent inhibitory effect of the microRNA *MIR20A* on *PKD1*, *AGTR1*, and *CAMTA1* (Figure 2C). Since these 3 genes are over-expressed in the absence of PS1IV, we would predict *MIR20A* to be downregulated in this circumstance. *MIR20A* expression is not measured by the Affymetrix Zebrafish Gene 1.0 ST Array but subsequent qPCR analysis of its expression under normoxia and mimicry of hypoxia and in the presence or absence of PS1IV supports that PS1IV may be important for maintaining levels of *MIR20A* expression under hypoxia (Figure 3). *MIR20A* belongs to the miR-17 microRNA precursor family that includes *miR-20a/b*, *miR-93*, and *miR-106a/b*. These microRNAs negatively regulate gene expression through complementarity to the 3' UTR of specific target messenger RNAs (miRBase, http://www.mirbase.org/). High expression levels of the *miR-17* family cause defects in lung and lymphoid cell development and are involved in many diseases (49).

From our analysis, another microRNA, *MIR1-1*, is implicated in a putative pathway regulating *NOTCH2* and *GAK* (Figure 2 F). Decreased NOTCH signaling has been suggested to underlie the development of AD pathology (50) so the downregulation of *NOTCH2* by PS1IV under hypoxia may be significant. Decreased expression of GAK, CYCLIN G-ASSOCIATED KINASE, has been shown to inhibit NOTCH signaling and to increase neural

cell death in zebrafish (51). GAK has also been associated with Parkinsons Disease (PD) in GWAS analyses (52), consistent with its interaction with the PD-associated protein α–synuclein (53). Previously, we observed increased expression of *cyclin G2* gene expression when zebrafish *psen1* or *psen2* activity was suppressed in embryos in a morpolino-based study (54). We have also argued that insufficient delivery of oxygen to the brain may play an important role in AD pathogenesis (55) and, in this light, it is interesting that both the MIR1-1- and MIR20A-coordinated pathways described here involve genes important for vascular function and development (e.g. see (56) and (57)). The analysis summarised in Figure 4 also illustrates that both these pathways are potentially linked through the transcription factor ESTROGEN RECEPTOR 2, ESR2. The *ESR2* gene shows homology to the *Hairy/enhancer of split* (*HES*)-related genes that are typically directly activated by NOTCH signaling. The estrogen estradiol has been shown to up-regulate *Agtr1* in rat heart (58) .

Finally, lipopolysaccharides are known to have positive regulatory effects on all the receptors seen to be over-expressed after loss of PS1IV (*AGTR1, HRH2, IL17RA, CD22,* and *NOTCH2*) (59-63). Together these form a statistically significant pathway (Figure 2D). Lipopolysaccharides commonly induce inflammatory responses (64) while hypoxia (that induces PS2V and PS1IV formation) is also pro-inflammatory (e.g. (65)). The fact that PS1IV upregulates expression of the pro-inflammatory cytokine IL1B and the chemokine receptor CCR5 (see later) but also uniformly suppresses expression of the above receptors that are downstream of pro-inflammatory factors suggests that the truncated *PRESENILIN* transcript isoforms induced by hypoxia act as important modulators of inflammatory responses.

Prediction of a regulatory network underlying genes over-expressed after loss of PS1IV

Transcription factors and microRNAs commonly establish the crosstalk and coordination between different pathways since any one transcription factor or microRNA usually has the ability to regulate multiple gene transcripts.

To detect potentially coordinated pathways, genes significantly over-expressed after loss of PS1IV under hypoxia were subjected to various network construction algorithms including "add interacting neighbours (interacting range of 1, 2, and 3, as mentioned in Material and Methods)", "add direct interaction", "add shortest path", "add common targets", "add common regulators", and "union selected subnetworks".

The results for the union selected subnetworks algorithm are presented below. The algorithm for union selected subnetworks seeks to join different significant pathways based on shared elements. For construction of union selected subnetworks, this algorithm was applied to our

list of statistically significant pathways shown in Table 2. The network generated by this procedure is presented in Figure 4. Significant features of this network are discussed below. Also, Supplemental Data 5 demonstrates the underlying relationships of the network.

The transcription factor encoded by *ATF2* (*ACTIVATING TRANSCRIPTION FACTOR 2*, also known as *CREBP1*) regulates *PKD1* and *ANGPTL2* within this network. Loss of *ATF2* has been shown to affect mouse Psen1 expression and to cause cranial motoneuron degeneration during brain development (66). The ATF2 protein is involved in regulation of cellular responses to multiple stresses (67). The role of *MIR20A* as a key pathway coordinator controlling expression of *CAMTA1, AGTR1* and *PKD1* is evident (Figure 4). The *CAMTA1* and *AGTR1* genes are described above but *PKD1* encodes a protein that has been observed in astrocytes (68) and has been shown to activate the JAK-STAT signaling pathway (69) Another microRNA suggested to coordinate a pathway, *MIR1-1* (see above) regulates NOTCH2 and MRC2 receptor expression and expression of GAK in the Golgi apparatus. In the network analysis, the number of interactions for any gene product can be used as an index of that gene's regulatory importance. The structure of our network of genes over-expressed after PS1IV loss highlights the products of *PKD1, AGTR1, ANGPTL2*, and *NOTCH2* as central regulatory elements since these each show large numbers of interactions.

The results of an alternative strategy for network assembly – the shortest path method – are presented in Supplemental Data 6 and emphasise the importance of the genes *CAMTA1* and *AGTR1* as coordinating regulatory nodes. Calmodulin has a considerable number of interactions within that network. Noticeably, *CAMTA1* expression is under the control of many microRNAs, such as mir20A and mir34A emphasing both its function importance and the selective pressure for its tight regulatory control. This is not unexpected when one considers the important role that Ca^{2+} signaling and the PRESENILIN proteins play in interactions between the ER and mitochondria. *CAMTA1* encodes a Ca^{2+}-regulated transcription factor and has been observed to be expressed in brain where changes in *CAMTA1* expression have been associated with early-onset nonprogressive cerebellar ataxia and mild mental retardation (70-72). The relationships of this network are presented in Supplemental Data 7.

Significant pathways formed by genes under-expressed after loss of PS1IV under hypoxia

When formation of PS1IV is suppressed during hypoxia, a number of genes show decreased expression. These genes require PS1IV for normal or increased expression during chemical mimicry of hypoxia and are listed in Table 1. We used these genes to construct pathways using the Pathway Studio 10 software (as described above for those genes with increased expression in the absence of PS1IV).

Significant pathways generated using the under-expressed genes are shown in Table 3 and Figure 5. Many of the pathways are related to apoptosis and immune response, including genes such as *CASP5*, *NLRP12), IL1B, TRIM22* and *CCR2*. The underlying relationships of these pathways are presented in Supplemental Data 8. It is apparent that *IL1B* is shared in many pathways. The regulatory pathway with the greatest significance (smallest p-value) is controlled by the transcription factor *MEF2D* (myocyte enhancer factor 2D) (Figure 4C). *MEF2D* is a stress-induced gene, which is involved in cardiac muscle development, as well as neuronal differentiation and survival. *MEF2D* plays diverse roles in cell growth, survival and apoptosis via the p38 MAPK pathway. It has been suggested that *MEF2D* is a critical protein in the regulation of neuronal apoptosis and autophagy (73-76). Another important pathway coodinates around Ca^{2+} ion concentration (Figure 5D) again emphasizing the relationship between *PRESENILIN* gene function and this ion.

Prediction of a regulatory network underlying under-expressed genes after loss of PS1IV

The regulatory network for under-expressed genes was constructed based on the union selected pathway algorithm as discussed above. This network is presented in Figure 6 and its underlying relationships are given in Supplemental Data 9. A variety of cellular organelles and structures respond to modulation by PS1IV including the mitochondria, endoplasmic reticulum, Golgi apparatus, nucleous, and plasma membrane emphasing the functional significance of this PRESENLIN isoform (Figure 6).

The following nodes are central in the generation of the regulatory network of under-expressed genes: (1) *IL1B*, (2) *CCR5*), (3) *TH*, (4) *CALR*, (5) *SMARCA4* and (6) *MEF2*. According to its Gene Ontology classification of *CCR5*, based on ComparativeGO database (18, 19), CCR5 is involved in aging and positive regulation of apoptosis. It has previously been suggested to have a modulatory effect on the activated microglia seen in AD brains (77) and is elevated in the peripheral blood mononuclear cells of AD patients (78). As can be seen in Figure 6, *TH* and *IL1B* represent intensive nodes in the network. *TH* encodes the enzyme *TYROSINE HYDROXYLASE* that is rate limiting in the catecholamine biosynthetic pathway required for production of dopamine. Interestingly, hypoxia is known to regulate TH activity in selected areas of the brain (79). It has recently been documented that disruption of *TH* activity in humans causes Segawa syndrome, an inherited form of infantile Parkinsonism (80, 81). TH has also been seen to modulate the release of inflammatory cytokines so the observed coupling of the TH and IL1B nodes (below) in our network is not surprising. Also intriguing is the existence of another gene associated with Parkinson disease as a node in our network, *LEUCINE-RICH REPEAT KINASE 2*, *LRRK2*. Mutations in *LRRK2* are one of the most common causes of familial Parkinson disease. The protein product of *LRRK2* binds to the product of the *TUBULIN, BETA* gene, *TUBB*, that is the most under-expressed gene when

PS1IV is absent under hypoxia. LRRK has also been seen to stimulate protein expression by acting on Argonaut proteins to repress microRNA action (82).

Cytokines are signaling compounds and major mediators of immune responses which are involved in stress response, cell differentiation, and apoptosis. The IL1B protein is a member of the interleukin 1 cytokine family which is proteolytically processed to its active form by CASPASE 1 (CASP1/ICE). The importance of IL1B in sporadic AD is demonstrated by identification of polymorphisms at this gene locus associated with increased AD risk (83). *IL1B* mRNA is highly significantly under-expressed in the absence of PS1IV and this gene forms a major node in our genetic network. Thus PS1IV contributes significantly to induction of *IL1B* expression under hypoxia. In our network, *IL1B* communicates with *CCR5* the product of which has important roles in mediation of neuroinflammatory responses to excitotoxicity (84). In rats, *CCR5* is expressed by microglia in the developing brain and predominantly in the endothelial cells of the adult brain (85). It is highly up-regulated in the brain by hypoxia-ischaemia. Interestingly, expression of *CCR5* has recently been observed to be increased in the blood of people with Alzheimer's disease (86) which may support that hypoxia plays a role in the pathogenesis of this disease. However, some researchers have reported that hypoxia can inhibit upregulation of *CCR5* in mouse macrophages (87). In our predicted network, the transcription factor encoded by *STAT4* increases the expression of *CCR5* and *IL1B*. STAT4 protein is well recognized as playing a stimulatory role in inflammation including in the brain (88).

The family of *MEF* transcription factors figures prominently in our network of under-expressed genes. These transcription factors control neuronal survival, apoptosis, and autophagy (75, 76). We also see involvement of the gene encoding CREATINE KINASE, MITOCHONDRIAL 1B, (*CKMT1B*), a protein of the inner membrane of mitochondria involved in lipid transfer between membranes. The gene *CALR*, that encodes a stress response protein binding Ca^{2+} ions in the ER and that is also found in the nucleus possibly acting as a transcription factor (89) is an important node in this network. Interestingly, in the ER, CALR binds to PROGRANULIN, (a protein implicated in frontotemporal dementia), where it forms a complex together with protein disulfide isomerases (90). The mitochondrial associated membranes (MAM) are foci for oxidative protein folding (disulphide bond formation) and are the major subcellular location for PRESENILIN protein expression (91). Changes in PRESENILIN expression affect the lipid constitution of the MAM that is a site of oxygen use and that produces high levels of reactive oxygen species (ROS). ROS induces expression of PS2V via upregulation of HMGA1a to alter *PSEN2* transcript splicing (92)). Numerous mutations in *PSEN1* are linked to frontotemporal dementia (usually in association with AD). Thus, our network analysis supports involvement of the PSEN proteins in frontotemporal dementia via regulatory interactions with PROGRANULIN in the MAM under hypoxic conditions.

SMARCA4 is another node in the network (Figure 6). *SMARCA4* encodes a calcium signaling related protein with helicase and ATPase activities that can regulate the expression of genes by altering their chromatin structure (93). Interestingly, significant down regulation of *SMARCA4* has been reported in severe AD (94).

MAPKAPK5, encoding a protein of the serine/threonine kinase family, governs another node in the network. This kinase is expressed in response to cellular stress and proinflammatory cytokines (95, 96). As a tumor suppressor protein, the major functions of *MAPKAPK5* are activation of apoptosis and mediation of senescence (97). Interestingly, *MAPKAPK5* is involved in amyloidosis and yellow fever diseases (98). This supports the idea that PS2V might be important for the accumulation of amyloid in AD.

In our network of under-expressed genes, the microRNAs *MIR137* and *MIR26A1* potentially negatively regulate the nodal gene, *PHOSPHOLIPASE C, BETA-1*, (*PLCB1*), that encodes a key protein regulating signal transduction pathways involving IP3 and diacylglycerol (DAG). Deletions of *PLCB1* have been observed in the brains of people with schizophrenia (99) which may be significant since PS2V has also been observed to be greatly elevated in schizophrenia brains (44). This supports that neural hypoxia may be an important environmental influence in this disease (100) via PS2V.

Finally, a considerable number of genes encoding ribosomal proteins are under-expressed in the absence of PS1IV, for example RPL9, RPS15A, RPL28, RPL26 and RPS19. The stimulation of expression of these proteins by PS1IV under hypoxia appears consistent with the action of this truncated PRESENILIN protein to repress the UPR (101). The UPR usually acts to suppress protein synthesis when cellular stresses (e.g. lack of oxygen for oxidative protein folding) lead to increased levels of unfolded proteins (101). As mentioned above, the stimulation of LRRK2 expression by PS1IV is also consistent with its opposition to the UPR since this should increase protein synthesis. Notably, among the genes normally suppressed by PS1IV expression, the gene with most significantly altered expression is *HSPA8* encoding the chaperone HEAT-SHOCK 70-KD PROTEIN 8. Upregulation of chaperone protein expression is an important element of the UPR. However, the action of PS1IV on other UPR actors is less easy to explain. PS1V stimulates *CALR* expression (see above), the product of which is involved in the UPR and is normally upregulated by ER stress (102). The CALRETICULIN protein can form complexes with the Ca^{2+} pump ATPase, Ca(2+)-TRANSPORTING, FAST-TWITCH 1 (ATP2A1, also known as SERCA) and disulphide isomerases required for oxidative protein folding (103). PRESENILIN1 holoprotein binds ATP2A1 and is, itself, upregulated by unfolded protein stress (104). Indeed, the PRESENILIN1 holoprotein has been observed to act a chaperone (105, 106).

Contribution of over and under-expressed genes after loss of PS1IV in pathway discovery

The genes observed to be over- or under-expressed in our study were detected after a single experimental manipulation, i.e. blockage of PS1IV expression under hypoxia. Therefore, we can expect both the over- and under-expressed genes to show regulatory interactions within a global network. To examine this, the combined set of over- and under-expressed genes was subjected to pathway discovery using the "gene set enrichment" approach followed by integrative network construction using the "union selected subnetwork" algorithm. Supplemental Data 10 shows pathways enriched by this global gene set. The regulatory network underlying these genes regulated by PS1IV is presented in Supplemental Data 11 and its relationships in Supplemental Data 12.

The combined network does not reveal very significant new nodes but does highlight the intensity of the nodal roles played by *IL1B*, *PLCB1, NOTCH2, AGTR1, SMARCA4*, *CALR*, *PKD1* and *HSPA8*. The regulation by PS1IV of processes in the ER, mitochondria, nucleus and on the plasma membrane is evident. The contribution of the ER and mitochondrial dysfunction to induction and progression of AD has been discussed recently (105, 107, 108). It seems that PS2V may play a significant role in this context.

Literature mining based network analysis of under-expressed genes after loss of PS1IV highlights PS1IV involvement in cancer and cardiomyopathy

During our construction of various networks, one based on the shortest path algorithm analysing genes under-expressed after loss of PS1IV and incorporating disease terms (to extract relationships between these genes and different diseases from the scientific literature, see Material and Methods) was of particular interest. The identified network and its underlying interactions (Supplemental Data 13 and Supplemental Data 14) emphasized the pathological importance of changes in expression of IL1B, HSPA8, TH and CALR. It suggests that PS1IV's human homologue, PS2V, might play roles in the progression of cancer and in preeclampsia. Changes in the expression of the under-expressed genes *TH*, *IL1B*, *PLCB1* and *AMPD1* are associated with heart failure. This is interesting since mutations in both human *PSEN1* and *PSEN2* exist that are thought to cause dilated cardiomyopathy (109).

Validation of some of network nodes (hubs) by RT-qPCR in validating network nodes

To confirm the validity of the constructed networks, RT-qPCR was performed for a subset of those genes located at network nodes (Figure 7). The results showed high correspondence between the microarray and RT-qPCR results. *CAMTA1* showed more than a 2-fold increase in mRNA level in both microarray and qPCR analyses. On the other hand, *IL1B* was down

regulated by more than 2-fold in both analyses. Both methods of analysis showed *PKD1* to be significantly over-expressed after inhibition of PS1IV formation under hypoxia while *MAPKAPK5* and *SMARCA4* were significantly under-expressed.

Decreased expression of MIR20A after loss of PS1IV under hypoxia

In network analysis, a "seed" is defined as the central gene (node) in a pathway/pathway if it has the highest number of interactions with other pathway members. As seen in Figures 2 and 4, *MIR20A* is the seed of an interesting pathway which negatively modulates expression of important genes that are over-expressed after loss of PS1IV. These genes include *PKD1*, *AGTR1*, and *CAMTA1*. We have analysed the expression of *MIR20A* in the presence and absence of PS1IV under hypoxia in zebrafish (Figure 3). Interestingly, the expression of *MIR20A* appears to be decreased after loss of PS1IV (by 1.6 fold on average although p is only 0.1) . As microRNAs bind to the mRNA of target genes to decrease their expression it is expected that down–regulation of *MIR20A* will result in up-regulation of its targets in the network, i.e. *PKD1*, *AGTR1*, and *CAMTA1*.

It should be noted that changes in the expression levels of some microRNAs can cause disproportionately greater changes in the expression of their target mRNAs. This is because microRNAs can function at much lower concentrations than mRNAs and need not be translated before binding to mRNAs to rapidly affect gene expression. Therefore, typically a fold change of 1.2 – 1.5 is used as a lower threshold when identifying microRNA s with altered expression (110, 111).

The analyses above show that extending examination of the consequences of gene/isoform dysregulation from simplistic observation of statistically significant changes in gene expression to construction of statistically significant theoretical gene interaction pathways (networks) enhances our understanding of the normal functions of the dysregulated genes/isoforms Thus, gene network analysis will be necessary to understand the pathological mechanisms underlying complex disorders such as AD. The fact that, despite publication of over 60,000 scientific papers on AD, there is no firm consensus either on either its underpinning molecular mechanisms or its treatment reinforces the need to implement more advanced analytical strategies such as machine learning (data mining), network analysis, promoter architecture analysis, promoter-binding based network, and gene ontology illustration to reveal the cellular changers underlying AD pathology (19, 112-117) .

Topological analysis of regulatory networks

To identify those genes central to the structure of the regulatory networks, Bottleneck, DMNC, MNC and MCC centrality indices were calculated. The results are presented in

Supplemental Data 15 and the genes are ranked based on overall scores of importance in various indices. Also, the 10 highest-ranked genes according to Bottleneck are presented in Figure 8.
AGTR1, *NOTCH2*, LPS, are *PKD1* are central hubs in the network structure of genes over-expressed after loss of PS1IV under mimicry of hypoxia (Supplemental Data 15). MIR20A received a high rank in network topological indices (Supplemental Data 15, Figure 8)

The Inflammatory response genes *IL1B* and *CCR5* are central in the regulatory network of genes under-expressed after loss of PS1IV under mimicry of hypoxia (Supplemental Data 15). Also, STAT4, a transcription factor involved in stimulation of brain inflammation (88), forms a hub in this network (Supplemental Data 15, Figure 8).

In the regulatory network constructed from the combined set of over- and under-expressed genes after loss of PS1IV, the highest ranked genes (hubs) were: *IL1B*, *TH*, *LPS*, *CCR5*, *AGTR1*, *HSPA8*, *PKD1*, *CALR*, and *MAPKAPK5*.$Ca^{2+}$was also identified as a hub. Overall, the topological analyses support our previous discussion of the importance of these genes/factors in the function exercised by PS1IV.

Conclusion

Zebrafish PS1IV is homologous with the human PS2V isoform of PSEN2 that is upregulated in AD brain. Mice and rats lack a PS2V homologue but we have recently demonstrated that the function of human PS2V in suppressing the UPR and increasing γ-secretase activity has been conserved in PS1IV despite nearly half a billion years of divergent evolution(118). We have also previously observed that the pathological function of the *PRESENILIN* genes cannot be correctly understood in physiologically abnormal systems (Moussavi Nik, et al., 2014 submitted). Therefore, the ability to manipulate endogenous gene expression in zebrafish embryos in subtle and physiologically relevant ways opens up the opportunity to analyse the function of PS1IV in order to increase our understanding of the possible roles of PS2V in Alzheimer's disease.

When analyzing the effects of loss of PS1IV on gene expression, network analysis can provide greater insight into function than simply identifying those genes with greatest and/or most significantly changed expression. This is because small changes in the expression of genes at nodal positions in networks can have greater systemic effects on cell biology than large changes in the expression of non-nodal genes. One example of this is the *TUBB* gene that shows the most statistically significant and greatest fold change in expression of any gene in our analysis but that is not very enlightening in terms of understanding the functional consequences of PS1IV loss. (Interestingly, however, problems with tubulin polymerization into microtubules have been observed in AD brains (119) and, of course, AD pathology is associated with abnormal aggregation into neurofibrillary tangles, NFTs, of

MICROTUBULE-ASSOCIATED PROTEIN TAU, MAPT (119). In contrast, it was very significant to see that PS1IV is required to maintain or increase the expression of the nodal gene *CALR* under hypoxia since the CALR protein is known to exist in complexes with the PGRN protein implicated in FTD. CALR protein (120) has also been shown to bind the ATP2A1 protein that binds PSEN1. This supports a putative functional link between the major genetic locus mutated in familial AD (PSEN1) and the second most commonly mutated gene in familial FTD (*PGRN*). Interestingly, there are mutations in *PSEN1* that are thought to cause FTD rather than FAD and these have been observed to differ in their effects on subcellular calcium transport (121, 122). This emphasizes the importance of Ca^{2+} in PRESENILIN function. Since the PRESENILINs and ATP2A1 are all MAM-resident proteins, the data above imply that PGRN will also show localization in the MAM. Of course, since PGRN possesses a very high number of disulphide bonds (123), the oxidative protein folding that occurs in the MAM must be central to PGRN function. Reassuringly, the involvement of CALR, a protein important for quality control of protein folding and for signal transduction (123), showed that our microarray and network analyses are consistent with the known roles of PS1IV and PS2V in modulation of the UPR.

Unexpectely, we saw that PS1IV is important for the upregulation of *1L1B* and *CCR5* that, in humans, have been strongly implicated in the inflammation that characterises AD brains. A large number of other cellular processes are also controlled by PS1IV that, in humans, are implicated in AD. This suggests that PS2V plays an important role in the development of sporadic AD brain pathology. Since hypoxia is important for induction of PS2V formation this supports that insufficient brain oxygenation may be fundamental driver of such pathology. This is consistent with evidence that serum markers of hypoxia can distinguish people who progress from mild cognitive impairment (MCI) to AD versus from those who do not (116). Also, hypoxia has been seen to upregulate production of the AD-associated peptide Aβ in cultured human neuroblastoma cells and to greatly raise Aβ in serum (124). Thus, PS2V may form a facilitating link between hypoxia and a number of pathological processes in AD brains. It is possible that blockage of PS2V action in AD brains might inhibit some of these pathological processes such as the inflammation provoked by IL1B expression or might relieve suppression of the UPR that normally restricts generation of inappropriately folded, aggregation-prone proteins.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

Figures and Figure Legends

Figure 1

Microarray comparisons to detect statistically significant changes in gene expression including the control comparisons used to refine the set of genes for which expression is altered by loss of PS1IV (under mimicry of hypoxia).

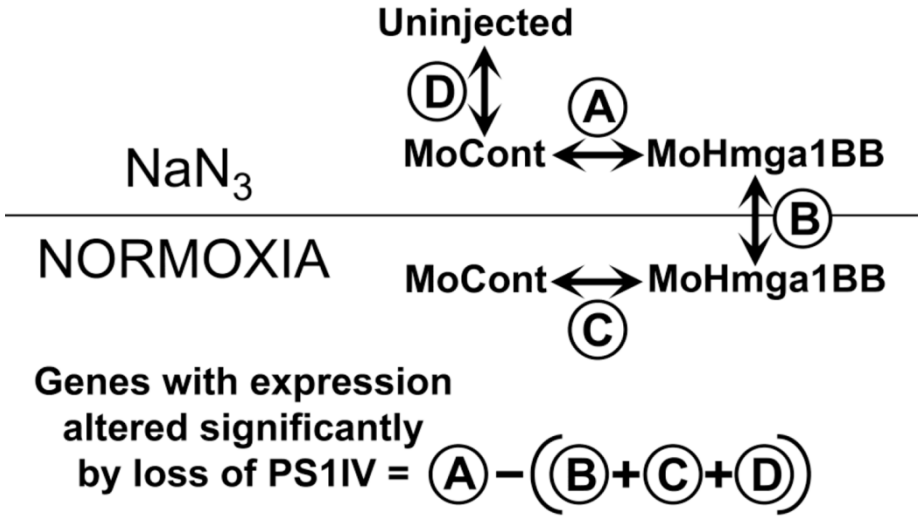


Figure 2

Schematic representation of some of the pathways for over-expressed genes after loss of PS1IV under mimicry of hypoxia. (A) Neighbors of *GABARAP* (Gamma-Aminobutyric Acid Receptor-Associated Protein), (B) Neighbors of *PDLIM5* (PDZ And LIM Domain 5), (C) Neighbors of MIR20A (microRNA 20a), (D) Neighbors of *LPS* (lipopolysaccharides).

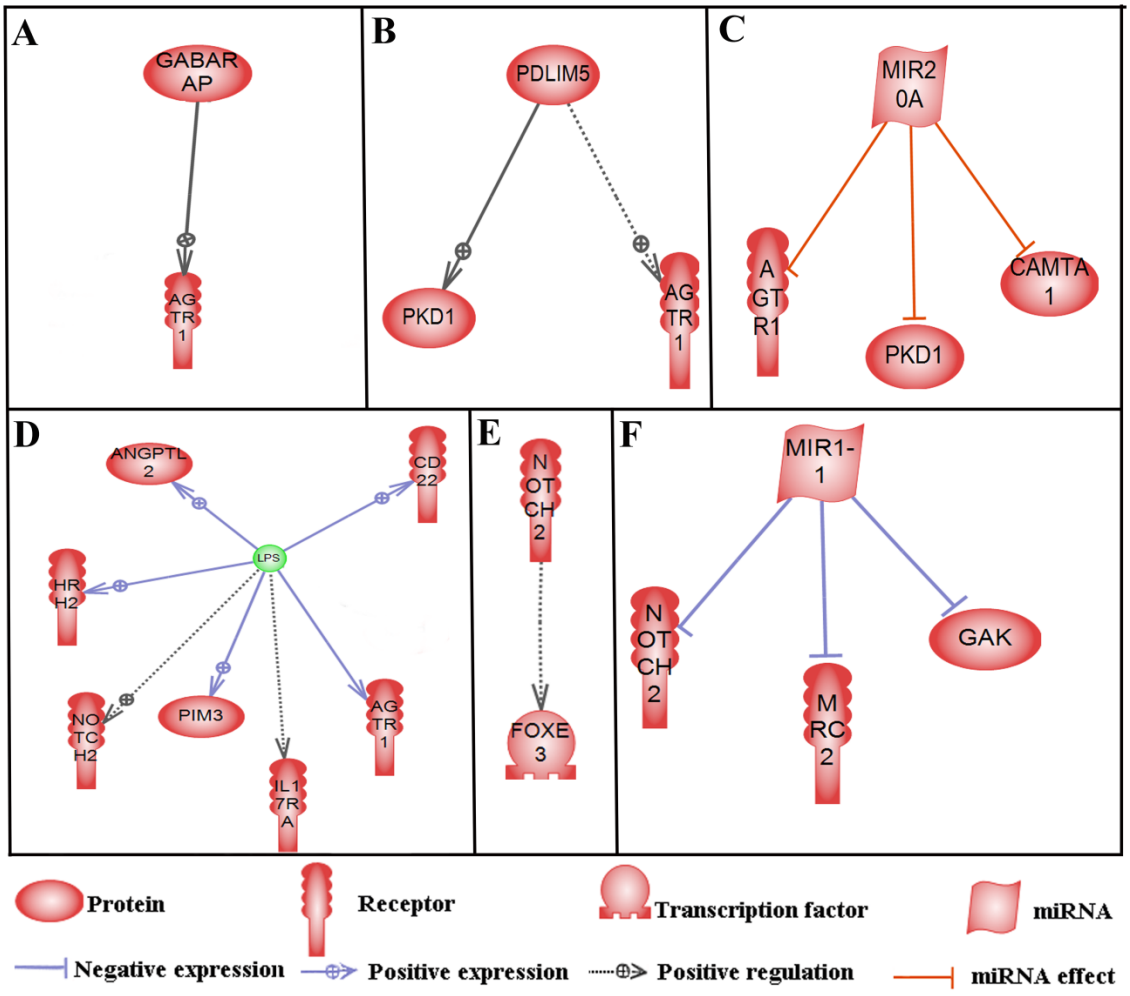


Figure 3

qPCR analysis supported regulation of miR-20A by the presence of PS1IV. This is indicated by decreased expression of miR-20A in embryos injected with the Hmga1aBindBlock morpholino. Values are normalised against those for Hmga1aBindBlock in normoxia. The letters represent the significant difference at P<0.1 calculated using a Bayesian t-test.

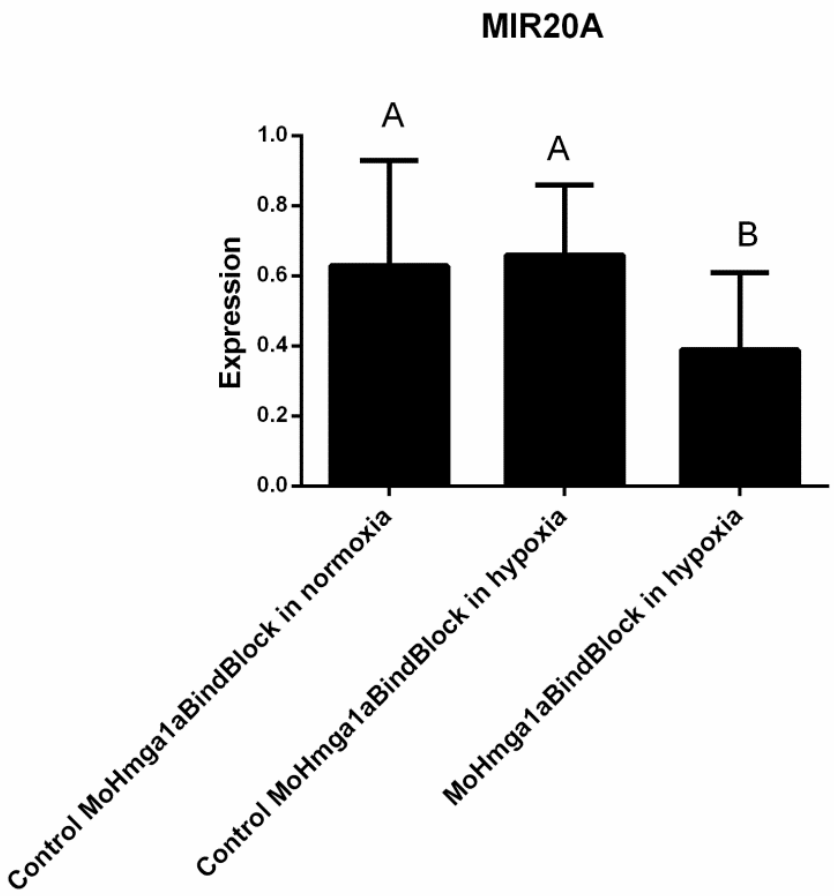


Figure 4

Regulatory network for genes over-expressed after loss of PS1IV under mimicry of hypoxia. This network was predicted using the union selected pathway algorithm.

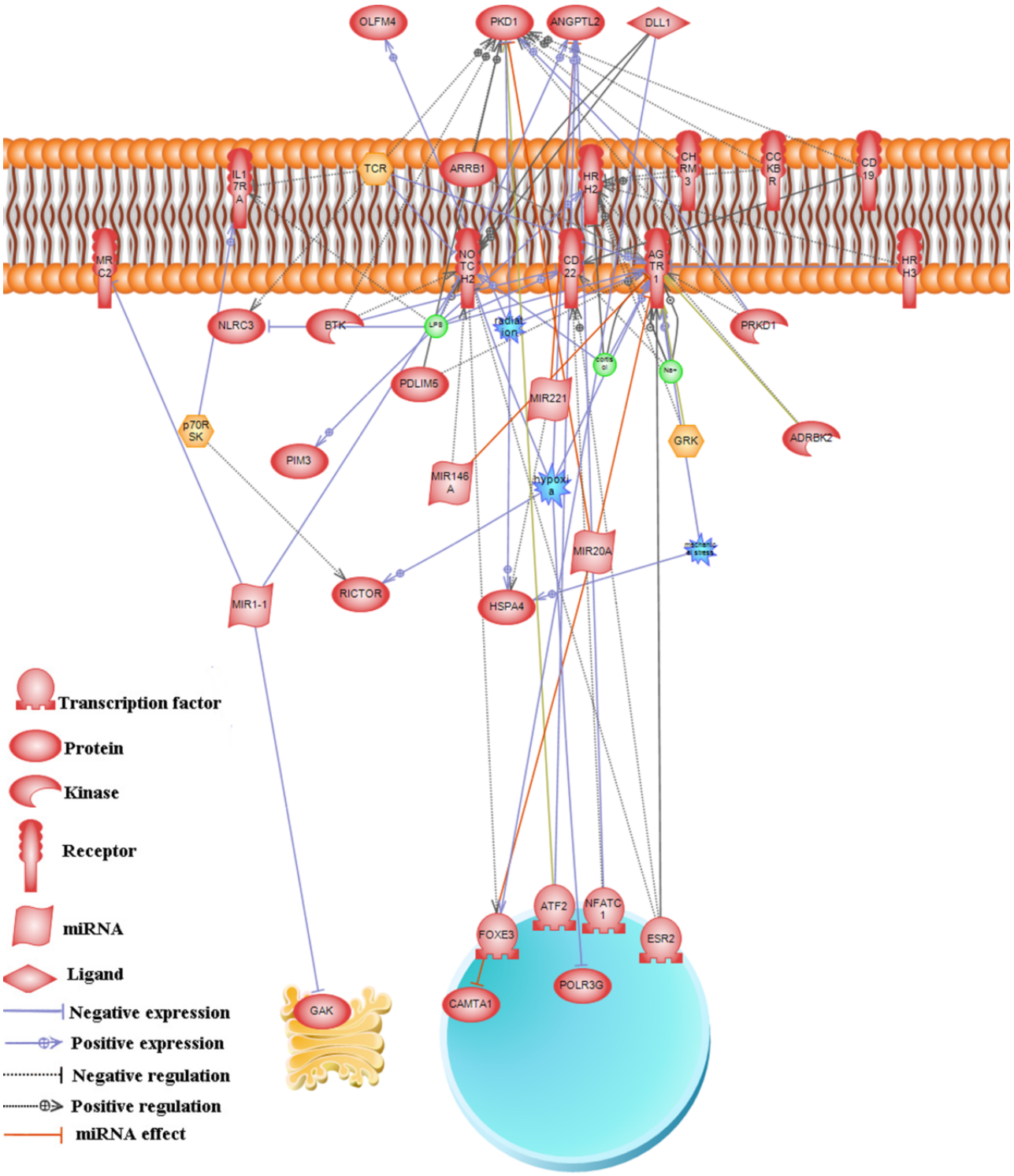

OLFM4
PKD1
ANGPTL2
DLL1
TCR
ARRB1
IL17RA
CHRM3
CCKBR
CD19
MRC2
NOTCH2
CD22
AGTR1
HRH3
NLRC3
BTK
LPS
radiation
PRKD1
PDLIM5
MIR221
p70RSK
PIM3
GRK
ADRBK2
MIR146A
hypoxia
MIR20A
MIR1-1
RICTOR
HSPA4
ATF2
NFATC1
FOXE3
ESR2
POLR3G
CAMTA1
GAK
Transcription factor
Protein
Kinase
Receptor
miRNA
Ligand
Negative expression
Positive expression
Negative regulation
Positive regulation
miRNA effect

Figure 5

Significant pathways generated by under-expressed genes after loss of PS1IV under mimicry of hypoxia. (A) Neighbors of *CASP5*. (B) Neighbors of *TRIM22*. (C) Neighbors of *MEF2D*. (D) Neighbors of Ca^{2+}.

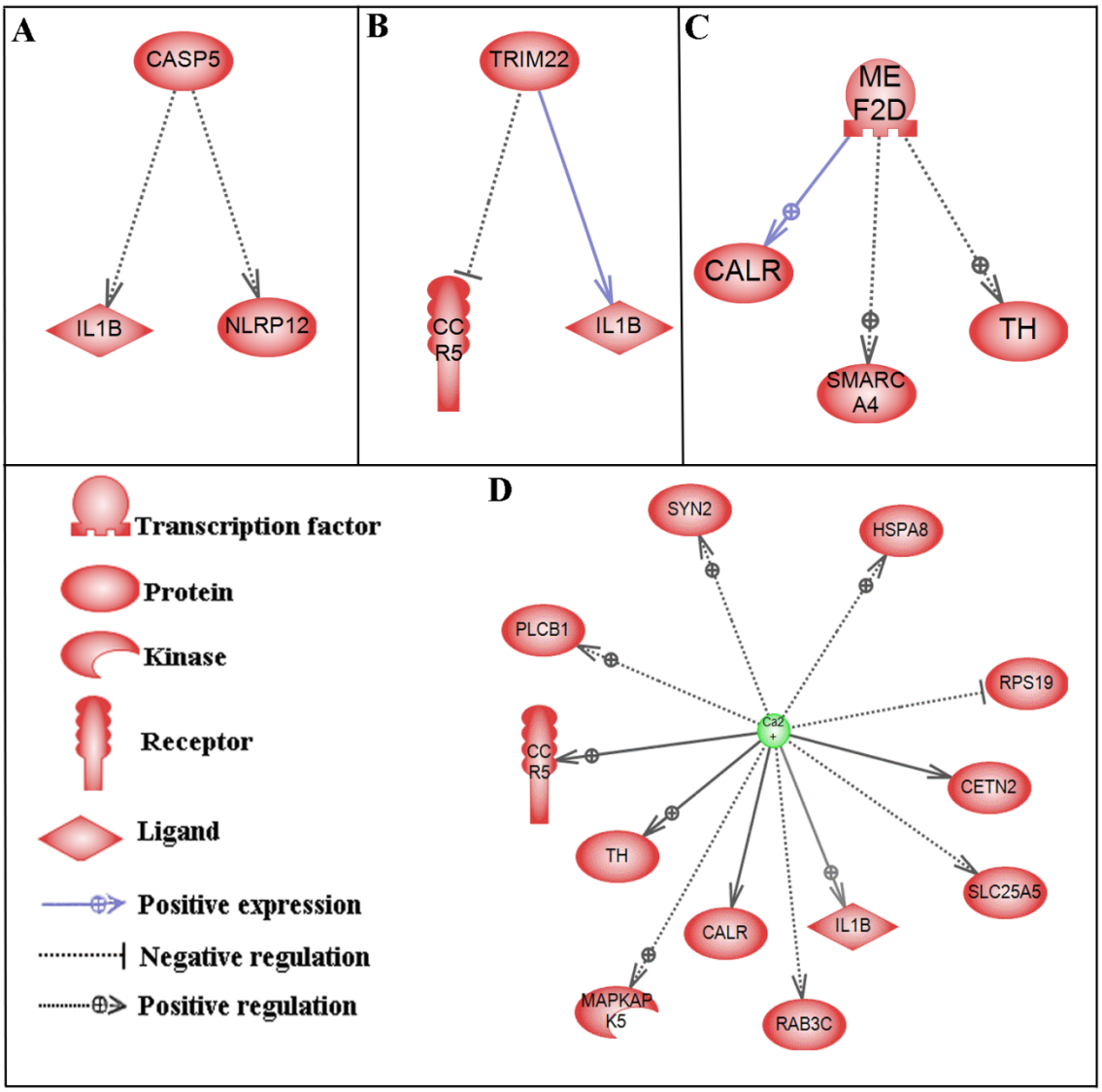


Figure 6

Regulatory network for genes under-expressed genes after loss of PS1IV under mimicry of hypoxia. The network was constructed using the union selected pathway algorithm.

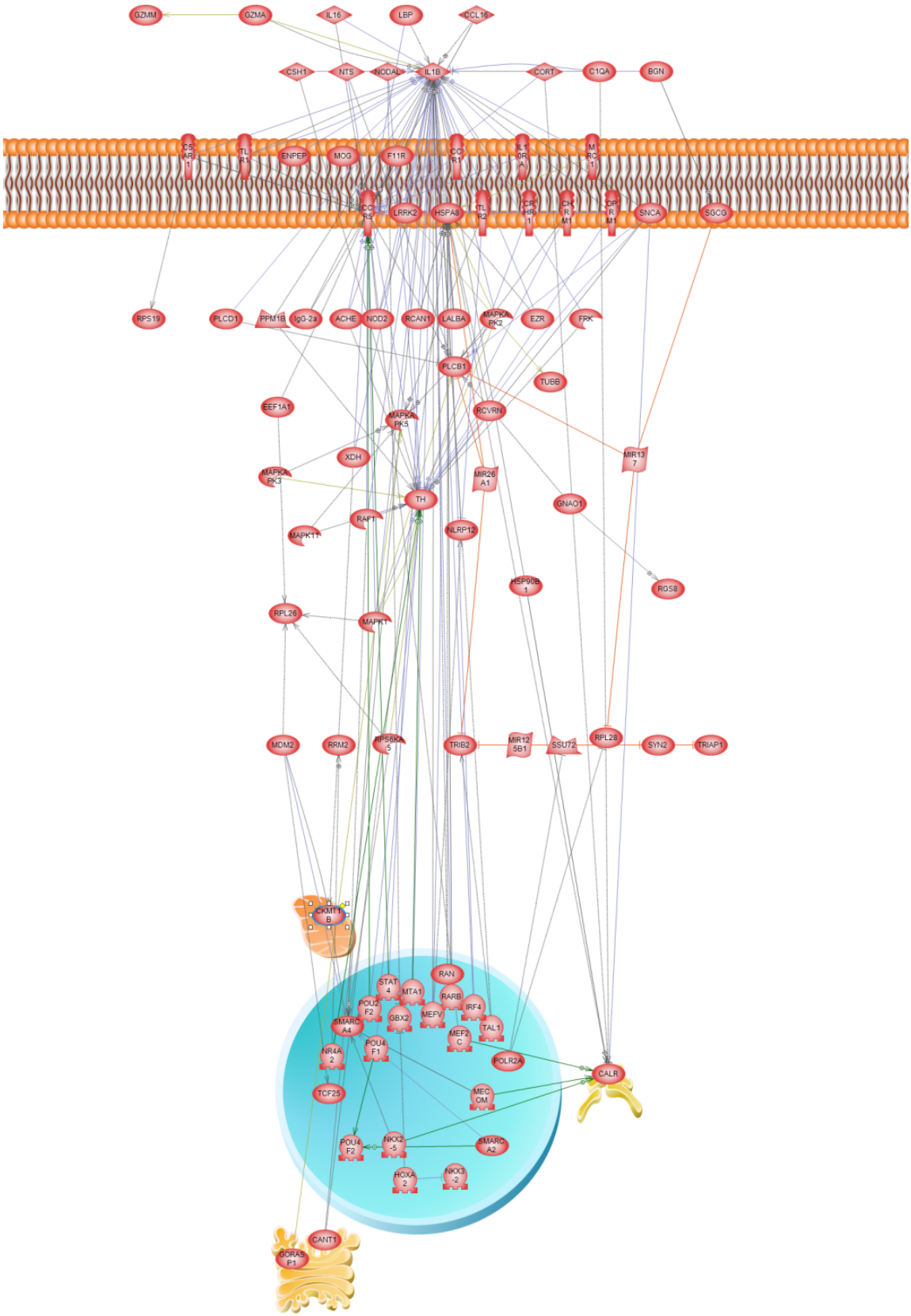

GZMM
GZMA
IL16
LBP
CCL16
CSH1
NTS
NODAL
IL1B
CORT
C1QA
BGN
ENPEP
MOG
F11R
LRRK2
HSPA8
SNCA
SGCG
RPS19
PLCD1
PPM1E
IgG-2a
ACHE
NOD2
RCAN1
LALBA
EZR
FRK
PLCB1
TUBB
EEF1A1
RCVRN
XDH
TH
RAP1
MAPK1
NLRP12
GNAO1
RGS8
HSP90B1
RPL26
MDM2
RRM2
TRIB2
RPL28
SYN2
TRIAP1
CKMT1B
STAT4
MTA1
RAN
RARB
POU2F2
IRF4
GBX2
MEFV
SMARCA4
TAL1
MEF2C
NR4A2
POU4F1
POLR2A
CALR
TCF25
NKX2-5
SMARCA2
POU4F2
HOXA2
NKX3-2
CANT1
GORASP1

Figure 7

Comparison of the results of microarray and qPCR analyses for validation of nodal genes in the networks after loss PS1IV under mimicr of hypoxia. *CAMTA1* and *PKD1* are seen to be over-expressed by both methods. *IL1B*, *MAPKAPK5* and *SMARCA4* are seen to be under-expressed by both methods.

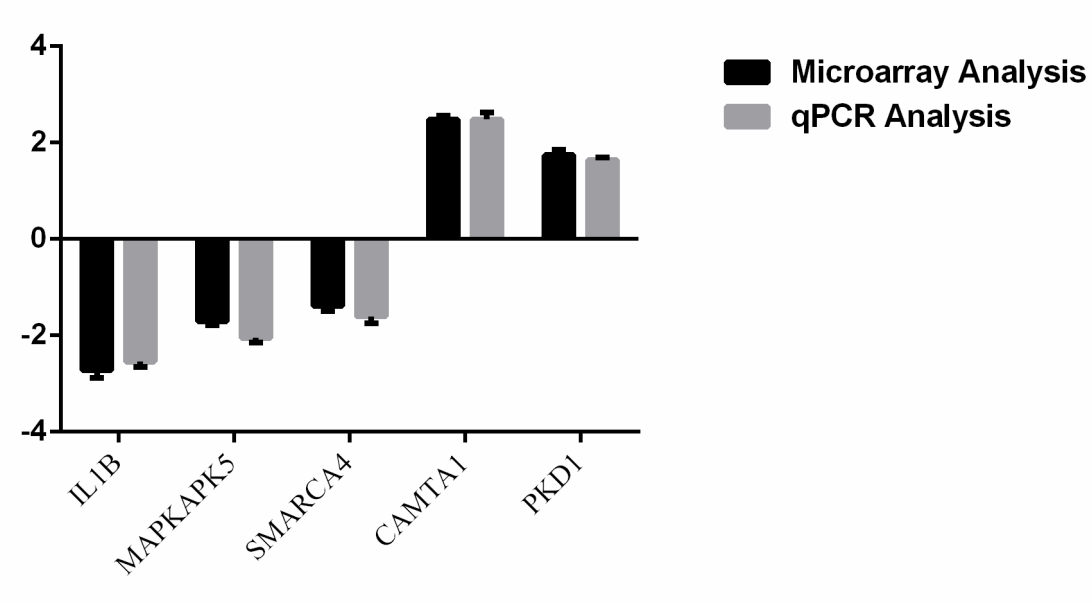
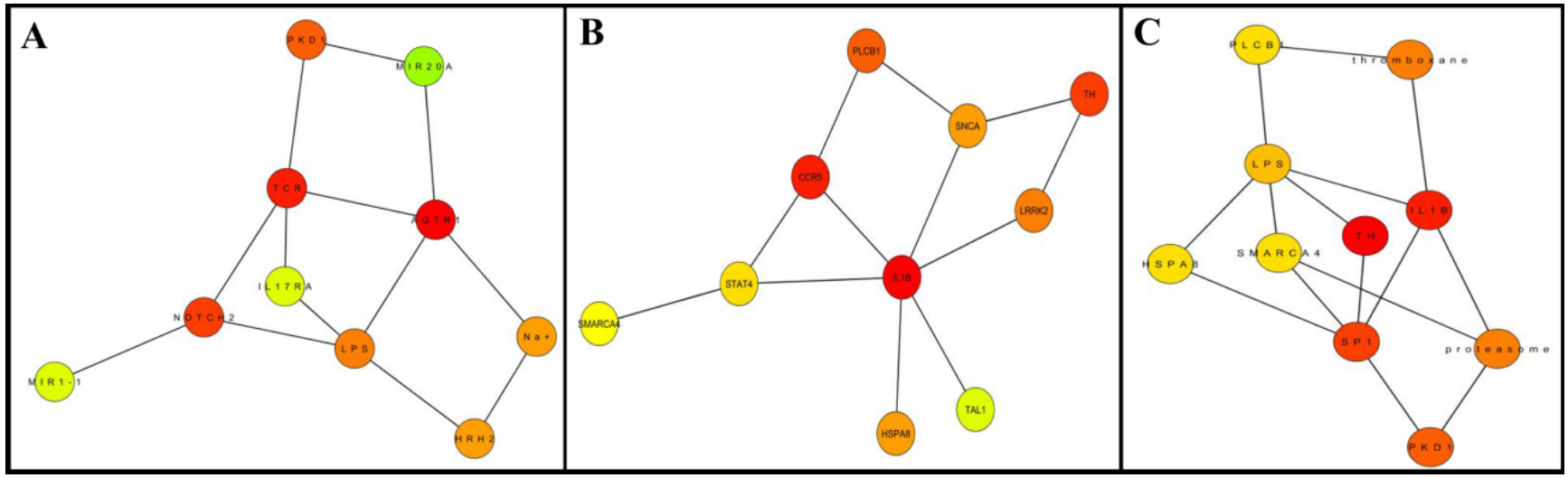


Figure 8

The 10 highest ranked genes (hubs) in the regulatory networks of genes (A) over-expressed, (B) under-expressed, and (C) the combined set of these genes after loss of PS1IV under mimicry of hypoxia according to the Bottleneck (BN) centrality index.



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Abbreviations

ACY1	AMINOACYLASE 1
AGTR1	ANGIOTENSIN RECEPTOR 1
AIDA	AXIN INTERACTOR, DORSALIZATION ASSOCIATED
ANGPTL2	ANGIOPOIETIN-LIKE 2
AMPD1	ADENOSINE MONOPHOSPHATE DEAMINASE 1
ATF2	ACTIVATING TRANSCRIPTION FACTOR 2
BTK	Bruton A gamma globulinemia
CALR	CALRETICULIN
CAMTA1	CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 1
CASP5	CASPASE 5, APOPTOSIS-RELATED CYSTEINE PEPTIDASE
CCR2	CHEMOKINE (C-C MOTIF) RECEPTOR 2
CD22	CD22 MOLECULE
CKMT1B	CREATINE KINASE, MITOCHONDRIAL 1B
CREBP1	CAMP RESPONSIVE ELEMENT BINDING PROTEIN 5
ESR2	ESTROGEN RECEPTOR 2
FOXE3	FORKHEAD BOX E3
GABARAP	GAMMA-AMINOBUTYRIC ACID RECEPTOR-ASSOCIATED PROTEIN
GAK	CYCLIN G ASSOCIATED KINASE
GBX2	GASTRULATION BRAIN HOMEOBOX 2
HSPA8	HEAT SHOCK 70KDA PROTEIN 8
HRH2	HISTAMINE RECEPTOR H2
IL17RA	INTERLEUKIN 17 RECEPTOR A
IL1B	INTERLEUKIN 1, BETA
IRF4	INTERFERON REGULATORY FACTOR 4
LRRK2	LEUCINE-RICH REPEAT KINASE 2
LPS	LIPOPOLYSACCHARIDES
MAPKAPK5	MITOGEN-ACTIVATED PROTEIN KINASE-ACTIVATED PROTEIN KINASE 5
MEF2D	MYOCYTE ENHANCER FACTOR 2D
MEF2	MYOCYTE ENHANCER FACTOR
NKX3-2	NK3 HOMEOBOX 2
NLRP12	NLR FAMILY, PYRIN DOMAIN CONTAINING 12
NOTCH2	NOTCH2
OCRL	OCULOCEREBRORENAL SYNDROME OF LOWE
OLFM4	OLFACTOMEDIN 4
PDLIM5	PDZ AND LIM DOMAIN 5
PKD1	POLYCYSTIC KIDNEY DISEASE 1
PIM3	PIM-3 ONCOGENE
PLCB1	PHOSPHOLIPASE C, BETA 1
PLEKHA4	PLECKSTRIN HOMOLOGY DOMAIN CONTAINING, FAMILY A MEMBER 4
POU4F2	POU CLASS 4 HOMEOBOX 2
PSEN1	PRESENILIN1
PSEN2	PRESENILIN2
SRCAP	SNF2-RELATED CREBBP ACTIVATOR PROTEIN
SMARCA4	SWI/SNF RELATED, MATRIX ASSOCIATED, ACTIN DEPENDENT REGULATOR OF CHROMATIN, SUBFAMILY A, MEMBER 4
STAT4	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 4
TCF25	TRANSCRIPTION FACTOR 25 (BASIC HELIX-LOOP-HELIX)
TH	TYROSINE HYDROXYLASE
TRIM22	TRIPARTITE MOTIF CONTAINING 22
TUBB	TUBULIN, BETA

Chapter V

The Guinea Pig as a Model for Sporadic Alzheimer's Disease (AD): The Impact of Cholesterol Intake on Expression of AD-Related Genes.

STATEMENT OF AUTHORSHIP

The Guinea Pig as a Model for Sporadic Alzheimer's Disease (AD): The Impact of Cholesterol Intake on Expression of AD-Related Genes.

Matthew J. Sharman (Co-Author)

• Editing of manuscript
• Conceived and designed the experiment
• Wrote the manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

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The Guinea Pig as a Model for Sporadic Alzheimer's Disease (AD): The Impact of Cholesterol Intake on Expression of AD-Related Genes

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Abstract

We investigated the guinea pig, *Cavia porcellus*, as a model for Alzheimer's disease (AD), both in terms of the conservation of genes involved in AD and the regulatory responses of these to a known AD risk factor - high cholesterol intake. Unlike rats and mice, guinea pigs possess an Aβ peptide sequence identical to human Aβ. Consistent with the commonality between cardiovascular and AD risk factors in humans, we saw that a high cholesterol diet leads to up-regulation of BACE1 (β-secretase) transcription and down-regulation of ADAM10 (α-secretase) transcription which should increase release of Aβ from APP. Significantly, guinea pigs possess isoforms of AD-related genes found in humans but not present in mice or rats. For example, we discovered that the truncated PS2V isoform of human PSEN2, that is found at raised levels in AD brains and that increases γ-secretase activity and Aβ synthesis, is not uniquely human or aberrant as previously believed. We show that PS2V formation is up-regulated by hypoxia and a high-cholesterol diet while, consistent with observations in humans, Aβ concentrations are raised in some brain regions but not others. Also like humans, but unlike mice, the guinea pig gene encoding tau, *MAPT*, encodes isoforms with both three and four microtubule binding domains, and cholesterol alters the ratio of these isoforms. We conclude that AD-related genes are highly conserved and more similar to human than the rat or mouse. Guinea pigs represent a superior rodent model for analysis of the impact of dietary factors such as cholesterol on the regulation of AD-related genes.

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Introduction

Murine models of Alzheimer's disease (AD) have played an important role in providing significant insight into mechanisms underlying disease pathogenesis and are still currently the most commonly used for pre-clinical drug screening. Rodent models themselves are poor natural models of AD and do not exhibit pathological hallmarks of the disease [deposition of beta amyloid (Aβ) and aggregation of tau as neurofibrillary tangles], partly due to differences in Aβ and tau species and aggregation states of these proteins. Thus, transgenic models, expressing familial AD (FAD) associated mutations in key components of Aβ metabolism [i.e. amyloid precursor protein- APP and/or presenilins (PS) genes) and tau *(MAPT)]* have been developed [reviewed in [1]]. The relevance of these models to the more common late onset AD (LOAD) which is associated with a complex aetiology, maybe questioned. Further, limitations of the murine models associated with transgene expression [2], differences in genetic background [3] and confounding issues with the presence of both human and endogenous murine Aβ and tau [4], has prompted growing interest in further investigating non-transgenic animal models, such as the guinea pig (*Cavia porcellus*).

To date, the presence of neurofibrillary tangles or compact senile plaques has not been reported in the guinea pig brain. A recent report has showed that the closely related *Octodon degus* exhibits an age dependent accumulation of these neuropathological markers of AD [5], suggesting that guinea pigs may show similar age related changes, however comprehensive ageing studies in guinea pig are lacking. Nevertheless, studies revealing that APP in guinea pig is highly conserved with that of humans and that the Aβ sequence is identical [6], [7] prompted the use of

this rodent model in assessing amyloid lowering therapeutics [8], [9], [10], [11] and hormonal regulation of Aβ metabolism [12], [13], [14].

Guinea pigs are also an excellent non-transgenic animal model in which to study the mechanism underlying the effects of cardiovascular risk factors, nutrition and drug interventions on AD-like pathology as they are the only small animal model that closely mimics human lipoprotein and cholesterol metabolism [15]. In contrast to other rodents and most species used for studying lipid metabolism, guinea pigs carry the majority of their plasma cholesterol in LDL, the atherogenic lipoprotein, similar to humans making them a unique animal model with which to study cholesterol and lipoprotein metabolism [15]. They are also excellent models to evaluate dietary interventions as they show aortic plaque accumulation when challenged with a hypercholesterolemic diet [15], [16], [17].

The guinea pig has not been widely used to assess the impact of dietary interventions on AD related pathology, such as Aβ accumulation. One possible reason for the guinea pig not being widely used in such studies is that apart from APP and Aβ, the conservation of AD-related genes and their regulatory responses to major risk factors involved in AD, has not been thoroughly explored. Considering this we investigated whether certain AD genes, particularly those involved in APP and Aβ metabolism, are conserved in guinea pigs and we assessed the regulation of these genes under conditions of the major risk factor - high dietary cholesterol intake.

Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes of the National Health and medical research Council (NHMR&C). The protocol was approved by the Committee on the Ethics of Animal Experiments of Edith Cowan University (Approval number 05-A17). The guinea pigs were anaesthetised with isoflurane prior to euthanasia. All efforts were made to minimise suffering.

Sequence Alignments of AD-related Genes

To analyse the sequential similarity of AD-related genes in human and rodent models, blastp analysis was performed, using the NCBI blast engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi?). Default parameters were used, with the exception that a gap existence penalty of 10 and a gap extension penalty of 1 were applied. The Sequence Similarity Score was calculated as shown at http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html and was the main parameter used to judge conservation between the human AD-related genes and their orthologues in rodent models.

Animals

Sixteen male Hartley guinea pigs weighing 500 to 600 *g* were obtained from the Biological Sciences Animal Unit at the University of Western Australia (Perth, WA, Australia). At the beginning of the study, animals were randomly assigned to one of two experimental groups, a control diet, and a high-cholesterol diet for 12 weeks.

Guinea pigs were housed in a controlled environment at 22°C on a 12 hour day/night cycle (light from 0700 to 1900 h). Diet and water were consumed *ad libitum*. The guinea pigs were weighed before and during each week of the study to monitor their health. Compared to animals fed the control diet, no significant changes were observed in food consumption or body weight of animals fed the cholesterol diet (Figure S1).

For analysis of the response of PS2V to a hypoxia mimetic in guinea pig brains, brains from three culled adult guinea pigs were collected from the colony maintained by the Veterinary Services Division of IMVS Pathology in Adelaide. Brains were divided into left and right halves before cutting into small (≤1 mm diameter) pieces and incubation for 6 hours in either DMEM medium plus FCS or this medium containing 100 μM NaN_3 followed by mRNA extraction and qPCR (see below). The same procedure was used to test for PS2V formation in an adult mouse brain collected from another research project at The University of Adelaide.

Diets

The research diets were prepared and pelleted by Specialty Feeds (Glen Forrest, WA, Australia). The control diet consisted of 0.25% cholesterol, 34% fat, 25% protein and 41% carbohydrate (Table S1). This cholesterol diet has commonly been used in guinea pigs to cause hypercholesterolemia and induce atherosclerotic plaque accumulation [16], [17], [18], [19]. This amount of dietary cholesterol corresponds to an absorbed amount equal to 1.5 times the daily cholesterol synthesis rate in guinea pigs [18] and is the equivalent to 1,875 mg cholesterol per day in the human situation. The control diet used consisted of 0.01% cholesterol, 34% fat, 25% protein and 41% carbohydrate. Both the cholesterol and control diets contained the same macronutrient composition and differed only in the cholesterol content. The experimental diets were weighed daily to monitor food intake.

Tissue Collection and Sample Preparation

Guinea pigs were euthanized under isoflurane vapours and blood was obtained via cardiac puncture. Serum and cerebrospinal fluid (CSF) samples were collected and stored at −80°C for subsequent analysis of CSF and serum cholesterol. Animals were transcardially perfused with phosphate-buffered saline (PBS) with heparin (10 IU/mL) and the brains collected and snap frozen in liquid nitrogen. The dissected brain sections for protein analysis were homogenized in 1:3 in PBS, pH 7.4 containing protease inhibitor cocktail tablets (Roche Diagnostics, Castle Hill, NSW, Australia) as described previously [20]. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

Measurement of Aβ by ELISA

A sensitive double-antibody sandwich ELISA was used for the detection and measurement of brain and CSF Aβ. Brain homogenates were diluted 1:10 with tissue homogenisation buffer, pH 7.4 (250 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA) and Aβ extracted from brain homogenates with 0.4% diethylamine (DEA), 100 mM NaCl [21]. CSF samples were also diluted 1:10 with PBS, prior to analysis. The Aβ ELISA assay was performed as previously described by Mehta et al. [22]. Briefly, brain and CSF Aβ levels were measured in prepared samples (100 μL) using monoclonal antibody WO2 as the capture antibody, with rabbit antiserum R208 (specific for Aβ40), kindly provided by Dr. Pankaj Mehta (NYS Institute for Basic Research, Staten Island, NY, USA) used as the detection antibody.

Cholesterol Analysis

Serum cholesterol concentrations were determined using the Amplex Red Cholesterol kit (Molecular Probes, Leiden, Netherlands). Serum samples were assayed in duplicate using black 96-

well plates. Plates were incubated in the dark for 30 min at 37°C and read using a FLUOstar OPTIMA multi-detection microplate reader (BMG Labtech Inc, Offenburg, Germany) at an absorption/emission spectrum of 560 nm/615 nm. The cholesterol concentrations of the samples were calculated from a cholesterol standard curve.

Quantitative RT-PCR Analysis of ADAM10, BACE1 and PS2V Transcripts

Total RNA was extracted from frozen Guinea pig brain tissues, using Qiagen RNeasy Lipid Tissue Mini Kit (Cat No. 74804). The quality of extracted total RNA was assessed on 1% agarose gel and the quantity was determined spectrophotometrically using the Nano-spectrum instrument (Thermo Fisher Scientific). First strand cDNA was synthesised following the production manual, using Bioline cDNA synthesize Kit (Cat No. Bio-65025), and was stored at −20°C for future PCR.

The relative standard curve method for quantification was used to determine the expression of experimental samples compared to a basis sample. For experimental samples, target quantity was determined from the standard curve and then compared to the basis sample to determine fold changes in expression. Gene specific primers were designed for amplification of target cDNA (Table 1) and the cDNA from the ubiquitously expressed control gene *RPS-16*. The reaction mixture consisted of 50 ng/μl of cDNA, 18 μM of forward and reverse primers and Power SYBR green master mix PCR solution (Applied Biosystems, Foster City, USA).

To generate the standard curve cDNA was serially diluted (100 ng, 50 ng, 25 ng, 12.5 ng). Each sample and standard curve reaction was performed in triplicate for the *RPS-16* gene and experimental genes. Amplification conditions were 2 min at 50 °C followed by 10 min at 95 °C and then 40–45 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification was performed on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster city, USA) using 96 well plates. Cycle thresholds obtained from each triplicate were averaged and normalized against the expression of *RPS-16*. Each experimental sample was then compared to the basis sample to determine fold changes of expression. Each experiment was conducted three times and triplicate PCRs were performed for each sample.

Sequence Prediction of Guinea pig Mapt and Subsequent RT-PCR Analysis

To predict the full sequence of guinea pig Mapt, tblastn analysis against guinea pig ESTs and genomes was performed, using human Mapt protein sequence as a query. The resulting fragments were then assembled based on overlapping sequence and their genomic location guided by information in the guinea pig Ensembl genome browser (http://www.ensembl.org/). RT-PCR was performed to identify 3R and 4R or 0N, 1N and 2N transcripts of *Mapt* using primer pairs outlined in table 1. The PCR products were then examined on 1% agarose gel and sequenced.

Statistical Analyses

Means and standard deviations were calculated for all variables using conventional methods. A students t-test was used to evaluate significant differences among the two groups of animals for the Aβ levels in the CSF and brain, levels of BACE1, ADAM10 and, PSEN2 and PS2V transcripts and serum cholesterol levels. Raw p values, degrees of freedom and t values are shown within the figure and figure legend. All values represent mean ± SEM of 8 animals per group. A criterion alpha level of $P<0.05$ was used for all statistical comparisons. All data were analysed using SPSS version 15.0 (SPSS, Chicago,IL).

Results

Sequence Similarities of AD-related Genes between Human, Mice Rats and Guinea Pig

Although, the sequence identity of guinea pig Aβ to that of human has been well established [(Figure 1, [7], [6])], genetic similarities with other AD related proteins have not been well documented. Therefore, we investigated the similarities of AD-related proteins between common rodent models (i.e. guinea pigs, mice and rats) and humans. These included, the Aβ parent molecule, APP and its processing enzymes, β-APP CLEAVING ENZYME 1 (BACE1) and A DISINTERGRIN AND METALLOPROTEINASE 10 (ADAM10); two critical components of the mutli-subunit γ-secretase enzyme, PRESENILIN1 (PSEN1), and PRESENILIN2 (PSEN2), Aβ clearance proteins APOLIPOPROTEN E (APOE) and INSULIN DEGRADING ENZYME (IDE) and the component of neurofibrillary tangles, MICROTUBULE

Table 1. Forward and reverse primers used for the qRT-PCR of *ADAM10*, *BACE1*, *PSEN2*, *PS2V* and *MAPT* transcripts.

Gene	Forward Primer	Reverse Primer
RPS16	5′-AGACAGCTACAGCCGTGGCACAT-3′	5′-CAGAAGCAGAACAGGCTCCAGTAACTT-3′
ADAM10	5′-GTGATCGCCCAGATATCCAGT-3′	5′-GAACCCCATCATCAAAGTCTCG-3′
BACE1	5′-GAGATCGCCAGGCTCTGTG-3′	5′-CCACGATGCTCTTGTCATAGTTG-3′
PS2V (RT-PCR)	5′-ACGGTCAGCTTCATCCAG-3′ (in *Psen2* exon 3)	5′-TCAGGAAGAGCGTGGGGTAA-3′ (in *Psen2* exon 7)
PSEN2 (qPCR)	5′-CCGCTGCTACAAGTTCATCCA-3′	5′-CCACGTTGTAGGTCTTGAGCACT-3′
PS2V (qPCR)	5′-GCTTTCATCCACGGCTG-3′ (spans *Psen2* exon 4/6 junction)	5′-CCGAGGTAGATGTAGGTGAAC-3′ (in *Psen2* exon 6)
MAPT (RT-PCR for 3R and 4R)	(5′-ACTCCACCCAAATCACCCTCCTC-3′)	(5′-TTGATGCTGCCAGTGGAAGAGAC-3′)
MAPT (RT-PCR for 0N, 1N and 2N)	(5′- TTCTCCTCCACTGTCCTCTTCTG-3′)	(5′- GTGTCTCCAATGCCTGCTTCTTC-3′)
MAPT (qPCR for full length)	5′-TCCACCGAGAACCTGAAGCA-3′	5′-GATGTTGCCTAGCGAGCGG-3′
MAPT (qPCR for 3R)	5′-GGAAGGTGCAAATAGTCTACAAACC-3′	5′-CGCTCGCTAGGCAACATCTC-3′
MAPT (qPCR for 4R)	5′-TAGCAACGTCCAGTCCAAGTGT-3′	5′-CGCTCGCTAGGCAACATCTC-3′

Note that guinea pig exon designations are according to the cognate exons in human since annotation of the guinea pig genome sequence is currently rudimentary.
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ASSOCIATED PROTEIN TAU (MAPT). For each protein, a "pblast" test was performed to compare sequence similarities between guinea pigs, mice or rats and humans. The "Sequence Similarity Score" was used as the main parameter to determine the level of sequence similarity. Results from the analysis are shown in Table 2. As expected, all three rodents mostly show very similar levels of sequence similarity of AD-related genes to their human orthologues (See Table 2). However, this is not the case for Psen1 where guinea pig Psen1 shows 96% identity to human PSEN1 but the mouse and rat proteins show 92% and 93%, respectively. PSEN1 is the major FAD locus in humans and over 200 mutations are known to affect different amino acid residues (aa). For this reason up to a 4% difference in sequence identity for this 467 aa protein may well be significant in terms of function.

Alignment of the PSEN1 protein sequence of rat, mouse and guinea pig to that of human PSEN1 (Figure 2) reveals a number of residues throughout the protein that are conserved in guinea pigs but not rats or mice. These residues appear to be concentrated within the N-terminus and large hydrophilic loop. Of the 100 residues in the PSEN1 protein in which FAD associated missense mutations occur, only one residue (serine) is conserved in guinea pigs but not conserved in, mice or rats. The mutation affecting this residue, S212Y, occurs in transmembrane 4 and has recently been identified in a family with FAD and shown to be associated with increased brain amyloid load, brain hypometabolism and increased Aβ42 production [23].

The PS2V Marker of AD Pathogenesis is not Unique to Humans

The analysis of overall sequence similarity shown in Table 2 can conceal important differences in isoform formation generated by alternative splicing. An example of this is the splice donor sites present in exon 3 of human PSEN1 which result in variants that differ in a four amino acid (VRSQ) motif [24]. The presence or absence of this motif at the 3′ end of exon 3 affects the binding of the GDP dissociation inhibitor that recycle rab GTPases important for vesicle trafficking [25]. The donor splice site is not conserved in mice, resulting in the inability of the motif to be alternatively spliced leading to only the longer isoform of PS1. The imbalance of longer to shorter PSEN1 isoforms has been speculated to lead to differences in Aβ production [25].

This prompted us to investigate whether there are species differences in isoforms of PSEN2, resulting from alternative splicing. A normal truncated PSEN2 isoform "PS2V" was identified by Sato and colleagues [26] and has implications in AD, since it shows increased expression in AD brains and up-regulates Aβ production [27], [28]. Human neuronal cells under oxidative stress induce expression of the HIGH MOBILITY GROUP AT-HOOK 1 (HMGA1) protein [29], [30]. This binds to specific sites within exon 5 of human *PSEN2* transcripts leading to exclusion of exon 5 and ligation of exon 4 and exon 6 sequences. The ligation of exon 4 to exon 6 sequences results in a frameshift that terminates the open reading frame in exon 6 and results in translation of a truncated PSEN2 protein isoform named PS2V (Figure 3 A and B).

Sequence alignment analysis of the HMGA1 binding site on PSEN2 in human, mouse, rat and guinea pig and other mammals revealed that this sequence is completely conserved in guinea pigs but not conserved in mice and rodents (Figure 3). Consistent with this, we were unable to detect PS2V transcript formation in PC12 (rat pheochromocytoma) cells and mouse brain following treatment with NaN_3 to mimic hypoxia (see Materials and Methods, data not shown), supporting that HMGA1a could not bind to the *PSEN2* transcripts of these rodents to cause alternative splicing. To test for PS2V formation in guinea pigs we extracted mRNA from guinea brains exposed to NaN_3 and then RT-PCR was conducted using primers amplifying cDNA spanning exons 3 and 7 of *Psen2*. This revealed the presence of a smaller cDNA fragment predicted from exclusion of exon 5 sequence (Figure 3D). qPCR using a primer binding over the exon 4/6 junction (and so amplifying only PS2V cDNA) showed that hypoxia mimicry significantly increases PS2V transcript levels (Figure 3E).

PS2V is Up-regulated by the AD Risk Factor Cholesterol Intake

Unlike rats and mice, guinea pigs metabolise cholesterol in very similar manner to humans. Since high cholesterol intake is a risk factor for AD and guinea pigs possess the AD marker PS2V, we examined whether PS2V levels are affected in the presence of this

	1 10 20 30 40 43
Consensus Sequence	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
***1. Homo sapiens* (Human)**	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
2. Bos taurus (Cattle)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
3. Macaca mulatta (Rhesus monkey)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
4. Pteropus vampyrus (Large flying fox)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
5. Felis catus (Domestic Cat)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
6. Canis familiaris (Domestic dog)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
7. Loxodonta africana (African Bush Elephant)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
***8. Cavia porcellus* (Guinea Pig)**	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
9. Tupaia belangeri (Nothern Treeshrew)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
10.Oryctolagus cuniculus (European Rabbit)	DAE-FRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
11.Dipodomys ordii (Kangaroo Rat)	DAE-FRHDSG**F**EV**R**HQKLVFFAEDVGSNKGAIIGLMVGGVVIA
12.Mus musculus (House mouse)	DAE-F**G**HDSG**F**EV**R**HQKLVFFAEDVGSNKGAIIGLMVGGVVIA
13.Rattus norvegicus (Rat)	DAE-F**G**HDSG**F**EV**R**HQKLVFFAEDVGSNKGAIIGLMVGGVVIA
14.Spermophillus tridecemlineatus (Leopard ground squirrel)	DAE-FRHDSG**F**EV**R**HQKL**? ?????**VGSNKGAIIGLMV**A**GVVIA
15.Daino rerio (Zebrafish)	DAE**E**-RH**S - - -** EV**Y**HQKLVFFAEDV**S**SNKGAIIGLMVGGVVIA

Figure 1. Amino acid residue sequence alignment of Aβ in humans and that predicted for guinea pig, rat and mouse. Black shading indicates identical residues. Red box represents residues from mouse and rat Aβ that differ from those in human and guinea pig Aβ sequences.
doi:10.1371/journal.pone.0066235.g001

Table 2. Sequence Similarity Comparison of AD-related proteins, between Humans and species of rodent: Sequence Similarity Scores and Sequence Identity (%) shown for each gene.

	APP	PSEN1	PSEN2	BACE1	ADAM10	APOE	IDE	MAPT*
Guinea Pig	1269 (97%)	759 (96%)	761 (96%)	1001 (97%)	1316 (96%)	416 (70%)	1880 (97%)	655 (90%)
Mouse	1261 (97%)	740 (93%)	764 (96%)	1009 (96%)	1308 (96%)	442 (71%)	1848 (96%)	652 (89%)
Rat	1269 (97%)	743 (92%)	745 (95%)	1010 (96%)	1313 (96%)	410 (70%)	1849 (96%)	660 (90%)

*The MAPT sequence of guinea pig was predicted.
doi:10.1371/journal.pone.0066235.t002

risk factor. Guinea pigs were fed a normal diet or a cholesterol diet for 12 weeks. Serum cholesterol concentrations were significantly increased in the cholesterol group compared to the control group (7.1 ± 4.9 vs. 3.6 ± 1.4 mMol/L respectively, $p = 0.0017$) at the completion of the 12 week intervention, confirming the effect of the cholesterol diet. To examine relative full length *PSEN2* and PS2V levels we then extracted mRNA from the frontal cortex and cerebellum for synthesis of cDNA followed by qPCR (Figure 4 A and B). Compared to control, full length *PSEN2* transcript levels increased ~2 fold in both regions. The increase in PSEN2 expression is consistent with previous findings where human neuroblastoma cells were exposed to LDL-cholesterol [31]. However, we show dramatic increases in PS2V levels were observed where levels increased 4 fold and 6 fold in the frontal cortex (A) and cerebellum (B), respectively compared to control fed animals. The impact of cholesterol on PS2V levels was above that seen for *PSEN2* levels ($p<0.01$, and $p<0.001$ compared to *PSEN2* levels in cholesterol fed animals).

Aβ and Genes Involved in Aβ Synthesis are Up-regulated by High Cholesterol Intake

Forced expression of PS2V in neuroblastoma cells increases γ-secretase activity and cleavage of Aβ from APP [27]. Also, increases in dietary cholesterol are known to correlate with higher Aβ cerebral load and changes in the APP processing enzymes BACE1 and ADAM10 [32], [33], [34], [35], [36]. Therefore, we tested whether increased cholesterol intake also affects the levels of Aβ, ADAM10 and BACE1 in guinea pig brains. Aβ was assessed by measuring levels in the cerebrospinal fluid (CSF), frontal cortex and cerebellum. Analysis of CSF Aβ40 levels showed a significant increase in Aβ40 in the cholesterol group compared to the control group (Figure 5A). Analysis of cerebral Aβ40 levels showed a significant increase in the frontal cortex for the HC group compared to the control group. There were no differences observed in the cerebellum between groups (Figure 5B). Quantitative RT-PCR (qRT-PCR) was used to assess BACE1 or ADAM10 expression levels. Results show that BACE1 transcript levels were significantly increased (Figure 6 A, B) and ADAM10 levels significantly reduced (Figure 6 C, D) in frontal cortex and cerebellum from guinea pigs fed the HC diet compared to those fed the control diet.

Overall, our findings indicate that cholesterol supplementation to guinea pigs up-regulates, PS2V, BACE1 and down-regulates ADAM10 expression, consistent with promoting Aβ production.

Analysis of Mapt Transcripts in Guinea Pig Brain

Although NFTs are also present in other dementias, they are still an important correlate of AD pathology and tau *(MAPT)* is a component of a toxic triad thought to mediate Aβ neurotoxicity [37], [38]. The strict regulation of *Mapt* transcriptional splicing, especially the maintenance of a 1:1 ratio of the 3R and 4R isoforms (derived from the alternative splicing of Exon10 of the human *Mapt*) has been considered to play an important role in normal MAPT function. Disturbance of the 3R/4R ratio of MAPT has been evident in neurodegenerative diseases such as Frontotemporal dementia (FTD), Corticobasal degeneration (CBD), Progressive supranuclear palsy (PSP) and AD. In human brain, six MAPT isoforms are generated through alternative splicing of Exon 2, 3 and 10 (Figure 7A). The alternative splicing of exon 10 yields two groups of MAPT isoforms with either 3 or 4 microtubule-associate repeats on the C-termini of the protein. Alternative splicing of exon 2 and 3 yields Mapt isoforms with 0 (0N), 29(1N) or 58 (2N) amino acids. Mapt expression in mouse is notable for its lack of an isoform with 4 tubulin-binding repeats (4R) indicating that simple protein aa identity may be a poor indicator of conservation of protein function. Therefore, we sought to analyse the isoforms that could be produced by the guinea pig *Mapt* gene.

The number of tau *(MAPT)* isoforms present in guinea pig brain has not been widely investigated, most likely due to the full sequence of the Guinea Pig Mapt yet to be determined. For sequence analysis we used the predicted sequence of Guinea pig Mapt based on protein sequence alignments, using Guinea pig ESTs (http://blast.ncbi.nlm.nih.gov) and the Ensembl database (http://www.ensembl.org/) and Genome sequence database. As this is a predicted sequence, the true similarity scores may not be accurately reflected. The predicted guinea pig *MAPT* sequence shows a similar degree of identity to human *MAPT* as do those of the other rodents (Table 1). We investigated the presence of *Mapt* transcripts in guinea pig brain by RT-PCR. Two primer pairs were, Gtau0F/4R and Gtau10F/14R were designed, targeting the corresponding region of human exon 2 and 3 and the tubulin-binding repeats domains in Guinea Pig *Mapt*, respectively (see Figure 7A). Using these primers in RT-PCR of mRNA isolated from guinea pig brain, we observed the presence ~600 bp and 500 bp transcripts corresponding to 3R and 4R repeats (Figure 7B) and a single transcript at ~300 bp, corresponding to the 1N isoform.

Having identified the presence of *MAPT* transcripts in guinea pig brain, the impact of cholesterol on total, 3R or 4R *MAPT* transcripts was assessed. Quantitative PCR analysis of frontal cortex, revealed ~5 fold increase in total MAPT levels in cholesterol fed guinea pigs, compared to animals fed a normal diet (Figure 8A). Transcript levels of MAPT3R significantly increased (Figure 8B), whilst no change was observed for MAPT4R transcripts (Figure 8C), resulting in an increase in the 3R/4R ratio (Figure 8D). Overall, the results show that although guinea pigs do not contain all isoforms of MAPT, unlike mice, [39], they contain 3R MAPT transcript which is up-regulated under cholesterol fed conditions.

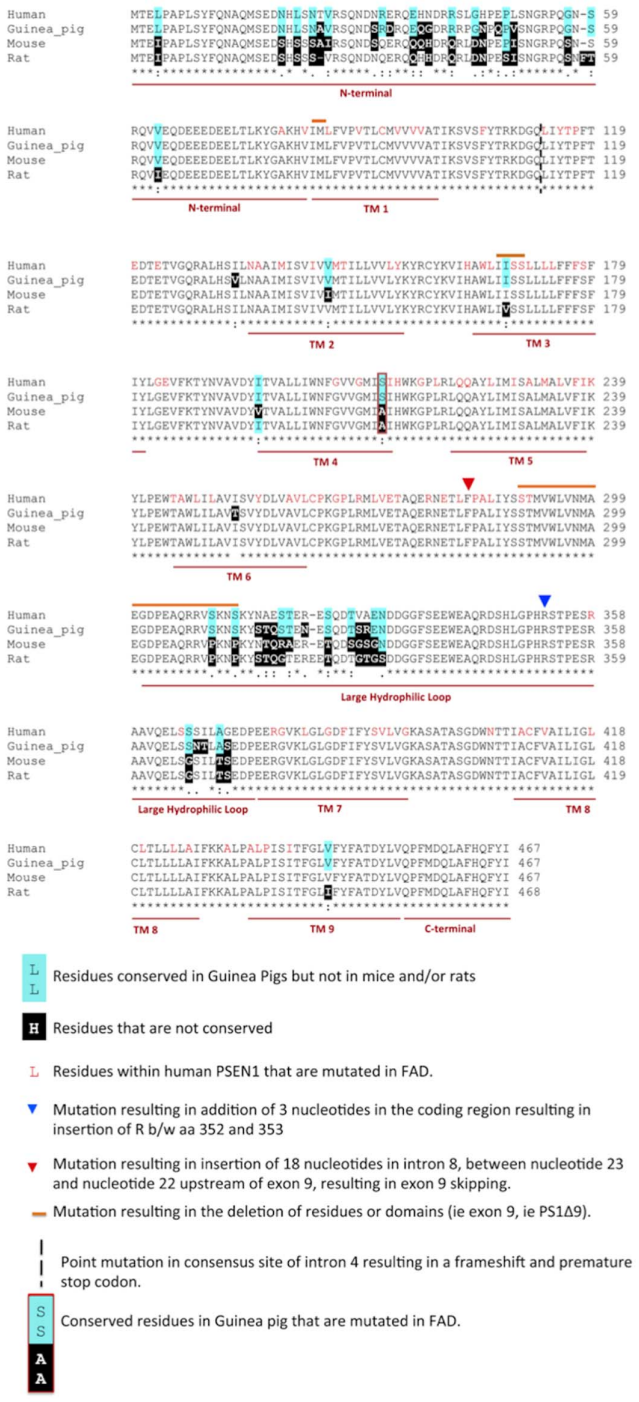


Figure 2. Amino acid residue sequence alignment of human PSEN1 and that predicted for guinea pig, rat and mouse. Residues that are conserved in human and guinea pig but not in the rat, mouse or both are shaded in blue. Rodent residues not conserved in humans are shaded in black. Residues known to be mutated in FAD in human PSEN1 are shown in red text. Only one residue is conserved in guniea pigs (but not mice and/or rats) that is mutated in FAD (S212Y). doi:10.1371/journal.pone.0066235.g002

Discussion

Guinea Pigs Show Closer Sequence and Isoform Similarity of AD Genes to Humans, than do mice and Rats

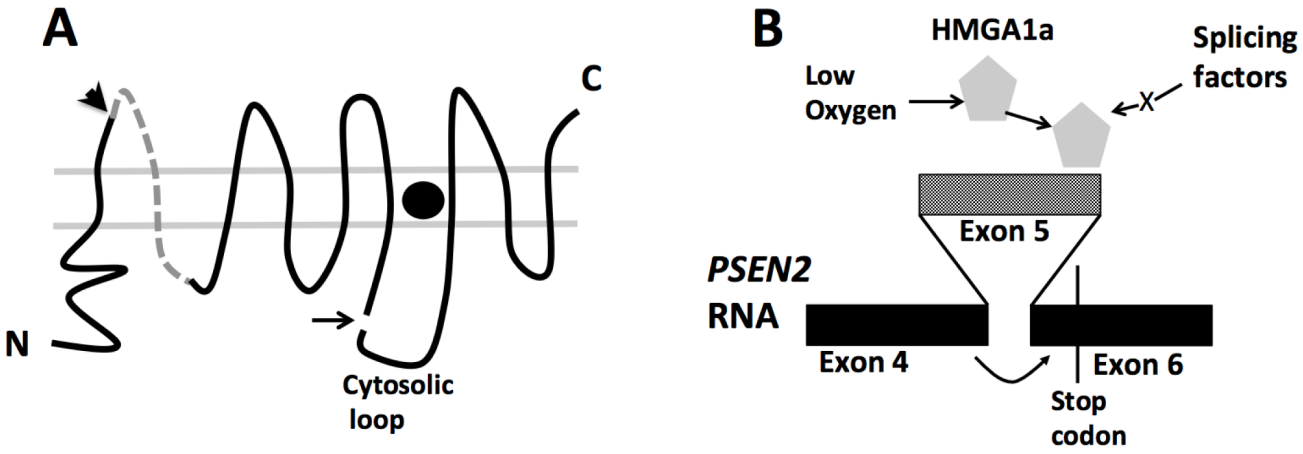
In the current study, in addition to comparing the sequence homology of APP and Aβ in human and the rodent species, mice, rats and guinea pigs, analysis was extended to other AD genes or genes that have been implicated in Aβ metabolism and clearance. All three rodents showed very similar levels of sequence similarity of APP to their human orthologue (97%). Analysis of IDE, ADAM10 and BACE1 also showed similar levels of sequence identity (96/97%), whereas APOE showed only 70% identity. Analysis of PSEN1 revealed that guinea pig PSEN1 showed 96% identity to human PSEN1 but the mouse and rat proteins showed 92% or 93%, respectively. The up to 4% difference in sequence identity between human and rodent may have significant implications in presenilins function and AD related neurodegeneration. This is highlighted by a recent study that analysed the human and mouse brain transcriptome and identified significant differences in transcriptional patterns in AD related genes between human and rodents [40]. Of particular note in that study was that PSEN1 was highly correlated with oligodendrocyte markers only in human brain tissue [40]. Oligodendrocytes are important in axon myelination, where a dysfunction of these cells leads to disruptions in neuronal communication network and neuronal degeneration. This close association of human PSEN1 with oligodendrocyte function may help to explain, in part, significant differences in neurodegeneration observed in human AD brain compared to those observed in mouse models.

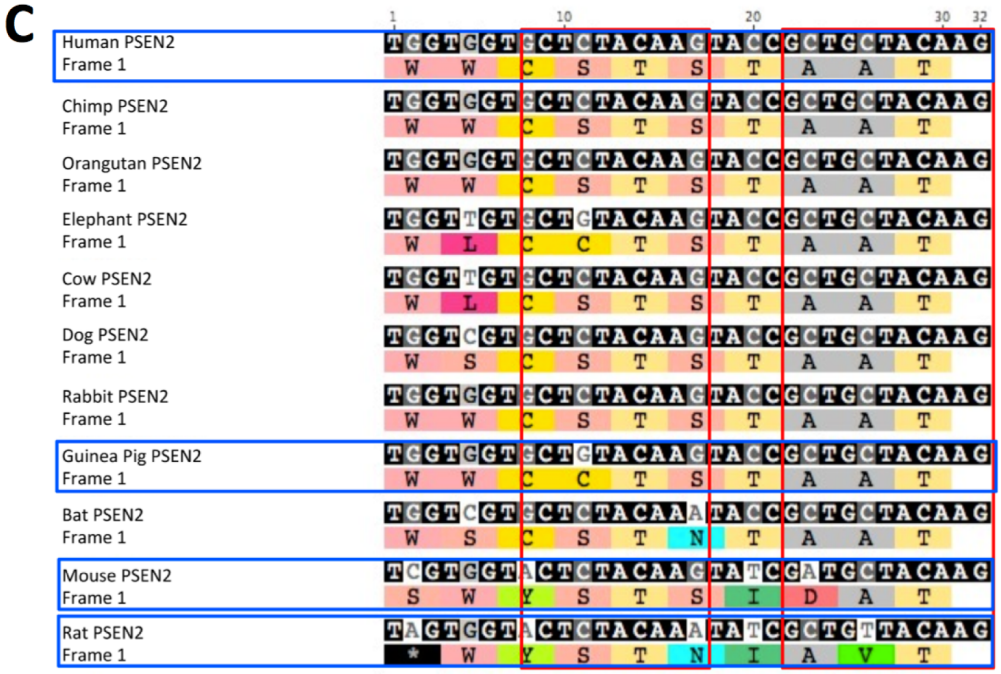
A comparison of human PSEN1 sequence with that of rat, mice and guinea pig showed that this sequence divergence was mainly within the N-terminus and the hydrophilic loop. The residues within the transmembrane domains remain relatively conserved amongst guinea pigs, rats and mice. This is not surprising as these domains have been shown to be important in γ-secretase activity [41], [42], [43], [44], [45]. However, the N-terminal domain and the hydrophilic loop also exhibit important functions. The large hydrophilic loop has been shown to differentially regulate γ-secretase activity on APP and Notch [46] and is also important for γ-secretase-independent functions of the presenilins by interacting with proteins involved in intracellular trafficking [Rab11, [47]], cell-cell adhesion [48], anchoring of membrane proteins to the cytoskeleton [actin-binding protein 280, [49]] and synaptic activity [syntaxin 1A, [50]]. The N-terminal domain has been shown to be important in the formation of PS1 isoforms as a result of alternative splicing, which can impact on activity [25]. The interactions and activities of these domains and the formation of alternative protein isoforms are most likely to be conserved in those species showing greater sequence identity.

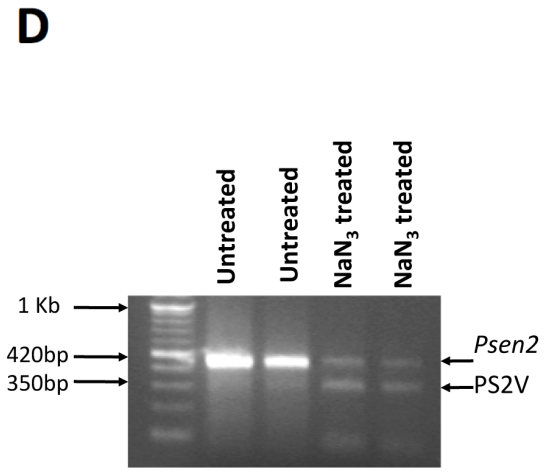
In contrast to PSEN1, analysis of PSEN2 revealed similar levels of sequence identity between human PSEN2 and the PSEN2 genes of mice, rats and guinea pigs. For the first time we demonstrate the presence of transcripts of the PS2V isoform in the guinea pig brain. As discussed below, this has important implications in AD as evidence is mounting that PS2V may play an important role in modulating Aβ metabolism under conditions of hypoxia/oxidative stress.

Guinea Pigs, a more Suitable Small Animal Modelling the Impact of Cholesterol Loading on AD Related Proteins

Studies utilising animal models of AD, including rabbits [32], [33] and transgenic mice, [34], [35], [51] have all shown a strong correlation between serum cholesterol levels and cerebral Aβ production. Our results demonstrate a similar correlation in


A
C
N
Cytosolic loop
B
HMGA1a
Low Oxygen
Splicing factors
X
Exon 5
PSEN2 RNA
Exon 4
Exon 6
Stop codon


C
Human PSEN2 Frame 1
Chimp PSEN2 Frame 1
Orangutan PSEN2 Frame 1
Elephant PSEN2 Frame 1
Cow PSEN2 Frame 1
Dog PSEN2 Frame 1
Rabbit PSEN2 Frame 1
Guinea Pig PSEN2 Frame 1
Bat PSEN2 Frame 1
Mouse PSEN2 Frame 1
Rat PSEN2 Frame 1


D
Untreated
Untreated
NaN3 treated
NaN3 treated
1 Kb
420bp
350bp
Psen2
PS2V

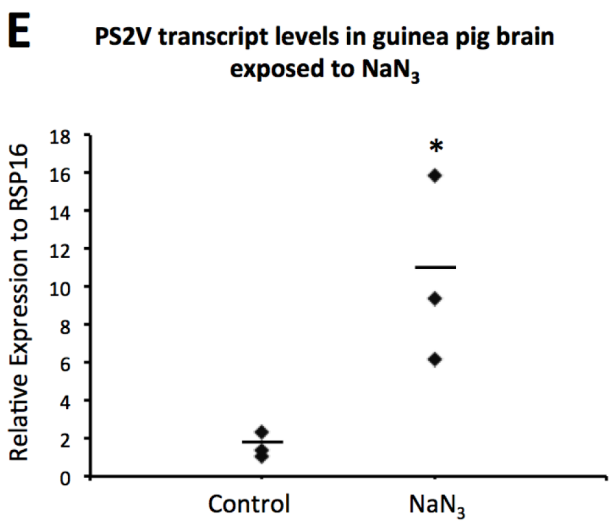

E
PS2V transcript levels in guinea pig brain exposed to NaN3
Relative Expression to RSP16
Control
NaN3

Figure 3. Formation of the PS2V Transcript. A) Presenilin structure in lipid bilayers: Arrowhead indicates boundary between protein sequences derived from exon 4 and 5. Dashed line indicates sequence from exon 5. Arrow indicates endoproteolysis site. Filled circle indicates γ-secretase catalytic site. **B)** PS2V forms when HMGA1a is expressed and binds to exon 5 (lighter shading) of *PSEN2* RNA causing ligation of exon 4 to exon 6 and ORF termination. **C)** Nucleotide sequence alignment of the 3′ end of exon 5 in human *PSEN2* RNA (with corresponding encoded residues) and the cognate exon of other species. Red boxes enclose sequences aligned with the HMGA1a-binding sites in human *PSEN2* RNA. **D)** mRNA from guinea brains exposed to control media or to media containing NaN_3 followed by RT-PCR analysis using primers amplifying cDNA spanning exons 3 to 7 of *Psen2*. In untreated samples a prominent ~420 bp band is observed. In NaN_3 treated samples an additional ~350 bp band is evident representing the cDNA fragment predicted from exclusion of the exon 5 sequence (PS2V). **E)** qPCR using a primer spanning the exon 4/6 junction PS2V cDNA showed up-regulation of PS2V mRNA in samples treated with NaN_3.
doi:10.1371/journal.pone.0066235.g003

guinea pig brain. We showed that, in guinea pigs, cholesterol up-regulates BACE1 and down-regulates ADAM10 expression, which would contribute to the promotion of amyloidogenic processing of APP to generate Aβ. This mirrors previous findings where modulating cholesterol (either through supplementation or depletion) alters the expression BACE1 and ADAM10 *in vitro* [52] and *in vivo* in rat, transgenic mice, dog and rabbit models [32], [33], [34], [35], [53], [36]. This further establishes the suitability of guinea pig as an alternative model to undertake such dietary intervention studies.

Interestingly, the expression profile of PS2V, BACE1 and ADAM10 did not correlate with Aβ40 levels observed in the cerebellum of cholesterol fed animals. Increases in dietary cholesterol have been shown previously in rabbits to increase Aβ levels in the frontal cortex but not in cerebellum [54]. Although APP processing enzymes are expressed in the cortical and limbic areas that develop significant Aβ deposition, high expression is also seen in the cerebellum [55], which does not exhibit significant Aβ pathology. A number of studies have shown that expression of these enzymes is not related to age or regional neuritic plaque burden [55], [56], [57], [58] and suggest that other factors such as Aβ catabolism/clearance may influence the accumulation of Aβ in certain brain regions.

Guinea pigs are the only small animal model in which generation of PS2V has been identified. The PS2V transcript was previously observed in human neuroblastoma cells under conditions of hypoxia-generated oxidative stress and in the brains of individuals with sporadic, late onset AD [27], [28], [59]. Over-expression of PS2V up-regulates Aβ production in neuroblastoma cells [27]. Our results show, for the first time, that an additional stimulus, hypercholesterolemia, simulates PS2V production in addition to up-regulating Aβ synthesis.

The up-regulation of PS2V could be a contributing factor modulating Aβ in hypercholesterolemia. Hypercholesterolemia can lead to vessel wall changes in the brain, leading to hypoperfusion, ischemia and hypoxia [reviewed in ([60])] and evidence indicates that this can contribute to AD pathogenesis. Hypoxia induced by cerebrovascular hypoperfusion in rats lead to accumulation of cerebral Aβ and cognitive deficits [61] and cardiac arrest can rapidly and massively upregulate plasma Aβ levels [62]. Hypoxia has also shown to up-regulate the genes required for Aβ production [63] [64] and here we have shown it to up-regulate PS2V in guinea pig brain. Whether cholesterol up-regulates PS2V, Aβ and Aβ generating genes via impacting on cerebrovascualture, promoting ischemia or hypoxia could not be determined from our data, but could be addressed in *in vitro* or *in vivo* follow up studies by assessing vasculature/hypoxic markers under cholesterol loading conditions. Our data indicate that guinea pigs represent the best *in vivo* model for dissecting the contribution of cholesterol to up-regulation of PS2V and Aβ.

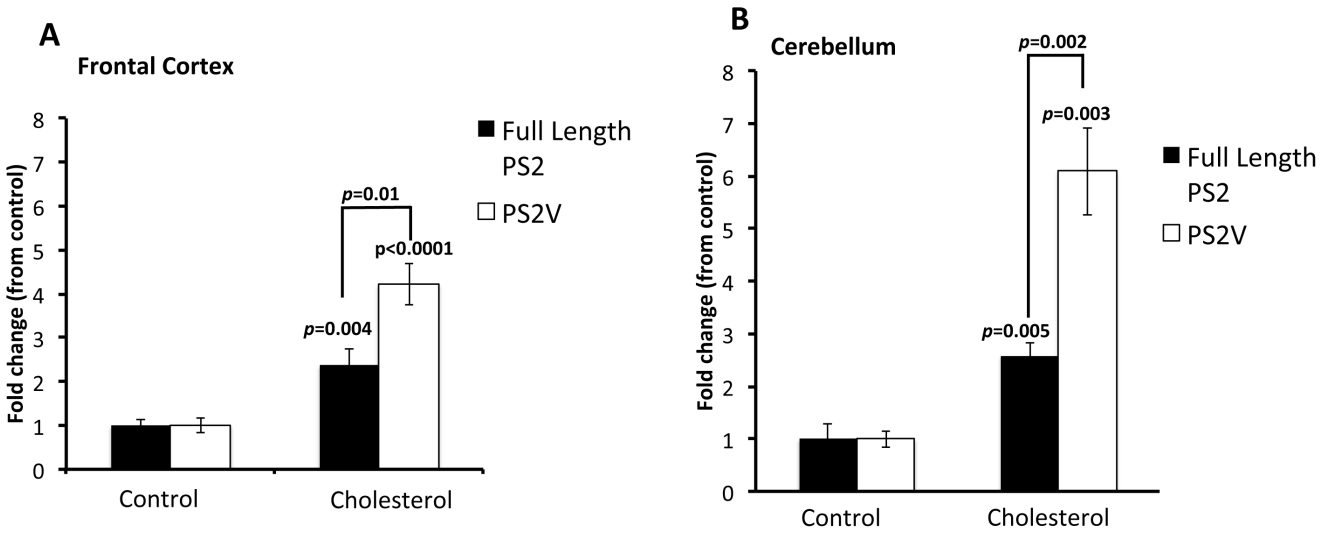


Figure 4. PS2V is up-regulated under cholesterol-fed conditions. Quantitative PCR analysis shows that, in comparison to animals fed a control diet, guinea pigs fed a cholesterol rich diet showed a significant increase in *PSEN2* and *PS2V* transcripts in (**A**) frontal cortex ($p = 0.004$; $t = 3.429$, d.f. = 14 and $p < 0.0001$, $t = 6.841$, d.f. = 14, respectively) and (**B**) cerebellum ($p < 0.005$; $t = 4.484$ and $d.f. = 14$ and $p < 0.003$, $t = 4.763$, d.f. = 14, respectively). The fold increase of PS2V levels in these regions was greater than the increase in full length *PSEN2* levels [4 fold vs 2 fold in frontal cortex ($p = 0.01$, $t = 2.994$, $d.f. = 14$], and 6 fold vs 2 fold in the cerebellum ($p = 0.002$, $t = 3.733$, $d.f. = 14$)]. Data is represented as fold change from control fed animals. Transcript levels were normalised against RPS16. Data represents ± SEM.
doi:10.1371/journal.pone.0066235.g004

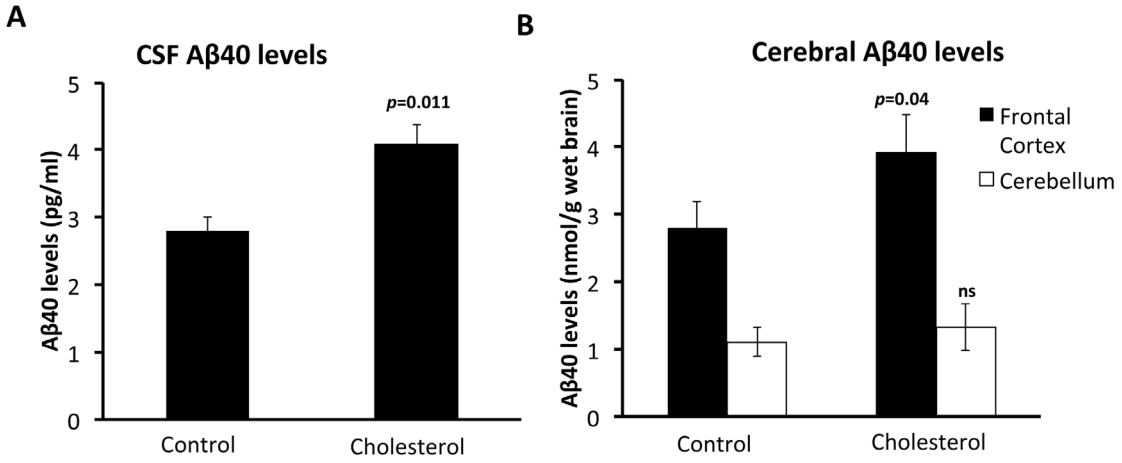


Figure 5. Increased Aβ1-40 levels in the CNS of cholesterol fed guinea pigs. (A) CSF Aβ1-40 levels (pg/mL) in the cholesterol and control fed diet groups following 12 weeks of feeding. Value is significantly increased over those animals fed the control diet ($p = 0.011$, $t = 2.896$, *d.f.* = 14). **(B)** Cerebral Aβ1-40 levels (nmol/g wet tissue) in frontal cortex and cerebellum homogenates from animals fed for 12 weeks on a high cholesterol or control diet. Increases are observed in animals fed cholesterol diet in the frontal cortex ($p = 0.04$, $t = 2.204$, *d.f.* = 14) but not in the cerebellum ($p = 0.501$, $t = 0.684$, *d.f.* = 14, ns). Values mean ± SEM.
doi:10.1371/journal.pone.0066235.g005

Limitations of the Guinea Pig as a Model of AD

Despite the overall advantages over other rodent models, there are limitations to the guinea pig in modelling all aspects of AD pathology including neurofibrillary tangles. There is a distinct lack of knowledge of the tau (*MAPT*) isoforms that exist and whether they are hyperphosphorylated. In our attempts to identify *MAPT* transcripts in guinea pig brain, a predicted sequence was obtained through sequence alignments using Guinea pig ESTs and the Ensembl database. This predicted sequence showed a similar degree of identity to human *MAPT* and thus RT-PCR using human primers was used to identify 3R and 4R repeats of *MAPT*. However, isoforms possessing only one amino terminal insert (1N) were identified while up to three isoforms at this site have been found in human MAPT transcripts (0N, 1N and 2N). Only one other study has investigated tau isoforms in guinea pig brain tissue. That study used an antibody against human tau and only detected the 1N isoform [65]. This supports the result of our RT-PCR analysis in which we could only identify 1N transcripts. The Takuma et al [65] study also identified differences in amino-terminal inserts between mice and rats where 1N and 2N insert types are dominant in rats, whilst 0N and 1N is dominant in mice. The reasons for these species differences in amino-terminal isoforms (and indeed their function) remain unclear. However the dominance of the 1N isoform in human, mice, rats and guinea pigs suggest a conserved role for tau containing this particular N-terminal insert.

Despite the lack of all MAPT isoforms, we show that both 3R and 4R MAPT transcript is present in guinea pig brain and that the 3R/4R ratio was altered due to increases in the 3R transcript. Disturbance of the ratio of 4R to 3R is a feature of AD and neurodegenerative tauopathies. The altered ratio is thought to be due to increases in 4R or reductions in 3R tau levels [66], [67]. However, increased levels of 3R tau have been reported to play a role in the progression of tau pathology particularly at mild-to

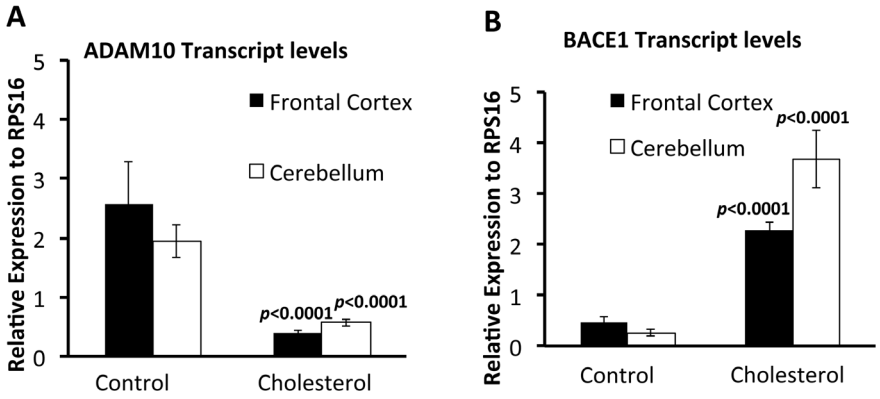


Figure 6. Increased *BACE1* RNA and reduced *ADAM10* RNA expression levels in brain tissue from guinea pigs fed a high cholesterol diet. Quantitative PCR analysis analysis for (A) ADAM10 and (B) BACE1 expression on total RNA extracted from the frontal cortex and cerebellum of guinea pigs fed the control or cholesterol diets. Data is represented as relative expression to RPS16. Compared to animals fed the control diet, ADAM10 expression is significantly decreased in the frontal cortex ($p < 0.0001$, $t = 7.735$, *d.f.* = 14) and cerebellum ($p < 0.0001$, $t = 6.30$, *d.f.* = 14) from animals fed cholesterol. In contrast BACE1 levels are significantly increased in the frontal cortex ($p < 0.0001$, $t = 8.196$, *d.f.* = 14) and cerebellum ($p < 0.0001$, $t = 8.196$, *d.f.* = 14). Values represent ± SEM.
doi:10.1371/journal.pone.0066235.g006

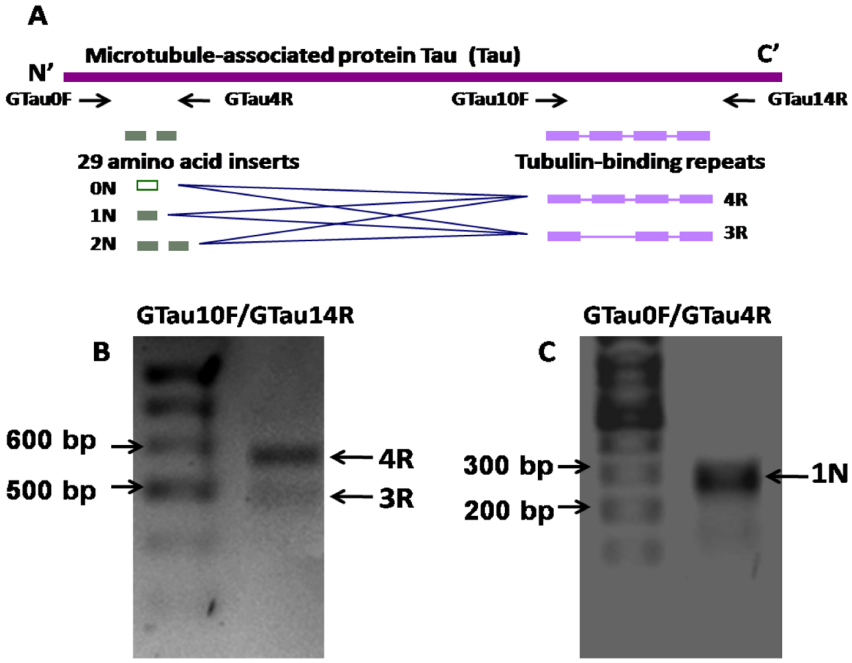


Figure 7. Mapt **isoforms in guinea pig brain. A)** Schematic diagram of the alternative splicing pattern of human *Mapt*. Six Mapt isoforms (0N3R, 1N3R, 2N3R, 0N4R, 1N4R, 2N4R) are generated from alternative splicing of exon2, 3 and 10 of the solo Mapt gene. The alternative splicing of exon2 or/and 3 (green boxes) yields *Mapt* isoforms with 0, 1 or 2 inserts of 29 amino acid residues in the N-termini; whereas, alternative splicing of exon 10 (purple) generates isoforms with either 3 or 4 tubulin-binding repeats in the C-termini. To analyse whether this splicing pattern is conserved in guinea pig, two primer pairs, GTau0F/4R and GTau10F/14R were designed, targeting the corresponding region of the human exon2/3 and tubulin-binding repeats domains respectively in guinea pig Mapt. **B)** RT-PCR *Mapt*, using primer pairs GTau10F/14R.c DNA was isolated from a brain sample from guinea pig fed normal chow diet Two bands representing 3R and 4R Mapt were detected. **C)** RT-PCR of Guinea pig *Mapt*, using primer pairs GTau0F/4R. A single band representing 1N *Mapt* was detected.
doi:10.1371/journal.pone.0066235.g007

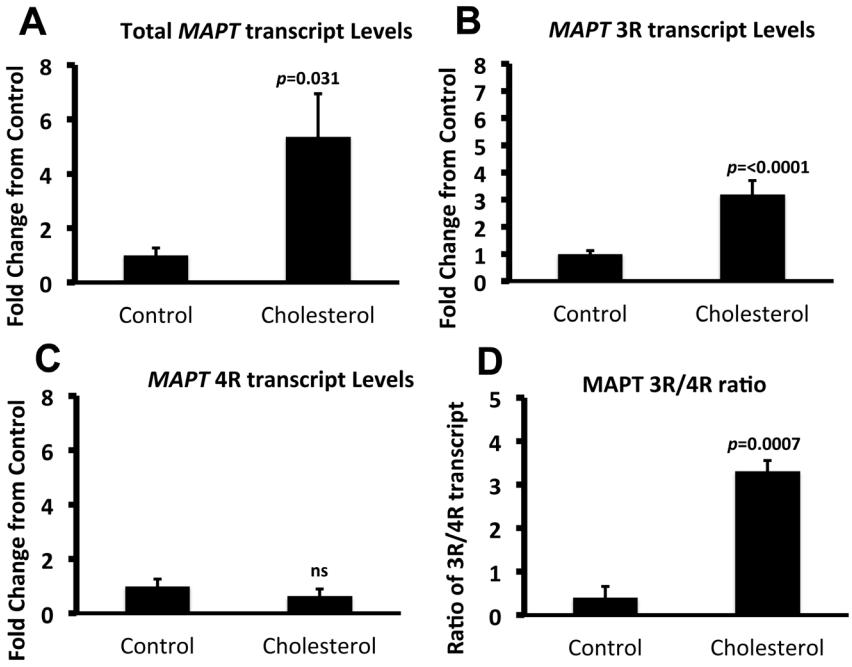


Figure 8. Total *MAPT* and *MAPT*3R transcripts are up-regulated under cholesterol fed conditions. Quantitative PCR analysis shows that, in comparison to animals fed a control diet, guinea pigs fed a cholesterol rich diet showed a significant increase in (A) total *MAPT* ($p = 0.031$, $t = 3.560$, $d.f. = 14$) and (B) *MAPT*3R ($p < 0.0001$, $t = 6.468$, $d.f. = 14$) transcripts but (C) no change was observed in *MAPT4R* transcripts ($p = 0.1320$, $t = 1.60$, $d.f. = 14$, ns). An increased 3R/4R ratio was observed ($p = 0.0007$, $t = 4.326$, $d.f. = 14$). Data is represented as fold change from control fed animals. Transcript levels were normalised against RPS16. Data represents ± SEM.
doi:10.1371/journal.pone.0066235.g008

moderate stages of disease severity [68]; [69]. Further, increases in 3R tau, but not 4R tau were reported in brains of aged obese rats that model the AD risk factor, type-2 diabetes, resulting in increased intracytoplasmic aggregates (that were reactive with antibodies against 3R) and synaptic degeneration [70]. We have shown that another AD risk factor, cholesterol intake, increases 3R transcripts, and although to be directly assessed, would most likely result in increased protein levels.

Taken together with the guinea pig's established role as a model of human lipoprotein and cholesterol metabolism, our findings provide further evidence that they are an alternative *in vivo* model to mice and rats for studying the effects of AD risk factors such as cholesterol on Aβ metabolism and PS2V generation and for evaluating dietary interventions that may have beneficial outcomes in AD.

Supporting Information

Figure S1 Average animal weights (grams) (A) and Average food consumption (grams/day) (B) between the cholesterol and control diet groups over the 12 week experimental diet. Values mean ± SEM.
(TIF)

Table S1 Dietary composition for the control and the cholesterol diet groups. [a]The oil mix contained 49% Copha (solidified coconut oil), 27% safflower oil, and 24% olive oil, and was high in lauric and myristic acids known to cause endogenous hypercholesterolemia in guinea pigs. [b]Mineral and vitamin mixes (AIN_93_G) were formulated to meet the daily requirements for guinea pigs.
(DOCX)

Author Contributions

Conceived and designed the experiments: MJS ML RNM GV. Performed the experiments: MJS SHMN MC DO LW KT MN. Analyzed the data: MJS GV SL MC. Contributed reagents/materials/analysis tools: SL MN. Wrote the paper: MJS GV ML.

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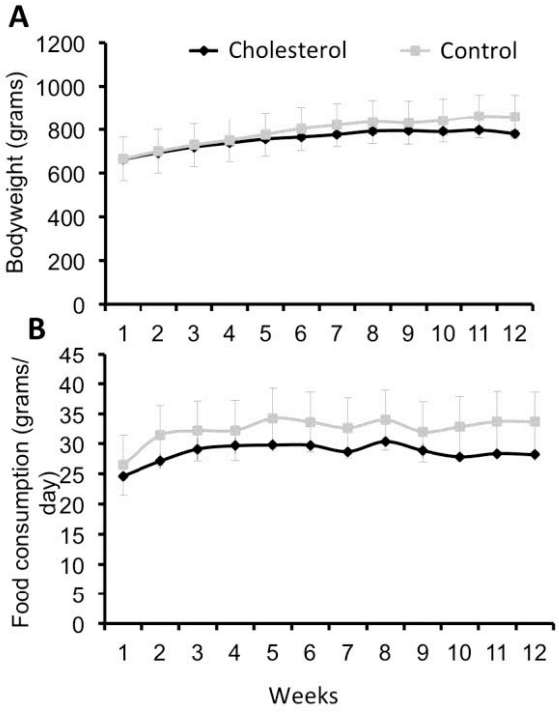
Figure S1.
Average animal weights (grams) (A) and Average food consumption (grams/day) (B) between the cholesterol and control diet groups over the 12-week experimental diet. Values mean ± SEM.



Supplementary Table 1. Dietary composition for the control and the cholesterol diet gro mix contained 49% Copha (solidified coconut oil), 27% safflower oil, and 24% olive oil, a was high in lauric and myristic acids known to cause endogenous hypercholesterolemia guinea pigs. bMineral and vitamin mixes (AIN_93_G) were formulated to meet the daily requirements for guinea pigs.

	Control	**High-Cholesterol**
Ingredient (g/100g diet)		
Sucrose	13.3	13.3
Casein	13	13
Soy Protein	8.69	8.69
Oil Mix [a]	15.092	15.092
Cellulose	10.9	10.9
Guar Gum	2.71	2.71
Wheat Starch	22.562	22.562
Dextrinised Starch	4.6	4.6
DL-Methionine	0.54	0.54
Lime (Fine Calcium Carbonate)	2.4	2.4
Salt (Fine Sodium Chloride)	0.26	0.26
Potassium Dihydrogen Phosphate	1.3	1.3
Potassium Sulphate	0.16	0.16
Potassium Citrate	0.25	0.25
Magnesium Oxide	0.15	0.15
Blue Food Colouring	0.04	-
Red Food Colouring	-	0.04
AIN_93_G_Trace Minerals [a]	0.14	0.14
AIN_93_G_Vitamins [a]	1	1
Choline Chloride 60% w/w	0.5	0.5
STAY Vit C (35% Vit C)	1.15	1.15
Guinea Pig supplement	0.999	0.999
Cholesterol (USP)	0.04	0.25
Protein (% energy)	25	25
Carbohydrate (% energy)	41	41
Fat (% energy)	34	34
Digestible Energy	16.1 MJ/Kg	16.1 MJ/Kg

Chapter VI

HYPOXIA ALTERS EXPRESSION OF ZEBRAFISH MICROTUBULE-ASSOCIATED PROTEIN TAU (MAPTA, MAPTB) GENE TRANSCRIPTS.

STATEMENT OF AUTHORSHIP

Hypoxia alters Expression of Zebrafish Microtubule-associated protein Tau (*mapta, maptb*) gene transcripts.

Seyyed Hani Moussavi-Nik (Candidate/First Author)

- Development of PCR primers and RT- and Q-PCR analysis on:
  - Hypoxia and chemical mimicry of hypoxia treated adult zebrafish Brain
- Bioinfomatic analysis of Tra2b binding site.
- Contributed in research design and wrote the manuscript.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Signed
……Date

Morgan Newman (Co-author)

- Editing of manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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……Date

Menqgi Chen (Co-author)

- Performed Western blots

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Ralph Martins (Co-author)

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Giuseppe Verdile (co-author)

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Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.
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Michael Lardelli (co-author)

• Planned the research and supervised development of work for Moussavi-Nik.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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RESEARCH ARTICLE Open Access

Hypoxia alters expression of Zebrafish Microtubule-associated protein Tau (*mapta, maptb*) gene transcripts

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Abstract

Background: Microtubule-associated protein tau (*MAPT*) is abundant in neurons and functions in assembly and stabilization of microtubules to maintain cytoskeletal structure. Human *MAPT* transcripts undergo alternative splicing to produce 3R and 4R isoforms normally present at approximately equal levels in the adult brain. Imbalance of the 3R-4R isoform ratio can affect microtubule binding and assembly and may promote tau hyperphosphorylation and neurofibrillary tangle formation as seen in neurodegenerative diseases such as frontotemporal dementia (FTD) and Alzheimer's disease (AD). Conditions involving hypoxia such as cerebral ischemia and stroke can promote similar tau pathology but whether hypoxic conditions cause changes in MAPT isoform formation has not been widely explored. We previously identified two paralogues (co-orthologues) of *MAPT* in zebrafish, *mapta* and *maptb*.

Results: In this study we assess the splicing of transcripts of these genes in adult zebrafish brain under hypoxic conditions. We find hypoxia causes increases in particular *mapta* and *maptb* transcript isoforms, particularly the 6R and 4R isoforms of *mapta* and *maptb* respectively. Expression of the zebrafish orthologue of human *TRA2B*, *tra2b*, that encodes a protein binding to MAPT transcripts and regulating splicing, was reduced under hypoxic conditions, similar to observations in AD brain.

Conclusion: Overall, our findings indicate that hypoxia can alter splicing of zebrafish *MAPT* co-orthologues promoting formation of longer transcripts and possibly generating Mapt proteins more prone to hyperphosphorylation. This supports the use of zebrafish to provide insight into the mechanisms regulating *MAPT* transcript splicing under conditions that promote neuronal dysfunction and degeneration.

Keywords: Microtubule-associated protein tau *(MAPT)*, Alternative splicing, Alzheimer's disease, Hypoxia, Zebrafish

Background

The *MICROTUBULE-ASSOCIATED PROTEIN TAU* (*MAPT*) gene encodes the soluble tau protein that is abundant in neurons and functions to assemble and stabilize microtubules to maintain cytoskeletal structure [1]. As a result of alternative splicing of *MAPT* transcripts, six tau protein isoforms ranging from 352 to 441 amino acid residues in length are generated and expressed in the human brain. The isoforms differ by the regulated inclusion or exclusion of two regions of sequence near the N-terminus and the possession of either three (3R) or four (4R) repeat regions, (corresponding to the microtubule-binding domains), towards the C-terminus of tau [2]. The 3R isoform is generated from mRNAs lacking exon 10, while mRNAs containing exon 10 encode 4R tau. These isoforms are normally present at approximately equal levels in the adult human brain [3]. Changes in this isoform ratio and post-translational modifications of the 3R and 4R isoforms affect microtubule binding and assembly [4,5].

Dysregulation of tau splicing is often observed in neurodegenerative diseases with aberrant tau deposition, including frontotemporal dementia (FTD), Pick disease

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(PiD), progressive supranuclear palsy (PSP) [6] and Alzheimer's disease (AD) [7]. Mutations reported in FTD cause aberrant exon 10 splicing, resulting in altered 4R/3R tau ratios [8,9]. In PSP, aggregates of 4R tau predominate, whereas 3R isoforms are found in excess in Pick bodies in the majority of cases of PiD [10,11]. In AD brains, increases in 4R tau isoforms have been reported resulting in altered 4R/3R tau ratios [12]. Neurofibrillary tangles (NFTs), a major pathological hallmark of the AD brain, can result from the phosphorylation of 3R tau, 4R tau or both [13,14]. Thus, any alternations in the levels of these isoforms could promote tangle formation and disease progression. It should be noted that changes in tau protein isoform ratios could result both from changes in the alternative splicing of transcripts and differential changes in the stability of their protein products.

Conditions such as cerebral ischemia and stroke that result in hypoxic conditions in affected brain areas can promote tau hyperphosphorylation and formation of NFTs. Acute hypoxic conditions have been shown to activate kinases that phosphorylate tau resulting in accumulation of phosphorylated tau in neurons [15]. In a rodent stroke model, hyperphosphorylated tau accumulated in neurons of the cerebral cortex in areas where ischemic damage was prominent. This was associated with the up-regulation of the tau phosphorylating enzyme CdK5, and the consequent promotion of the formation of filaments similar to those present in human neurodegenerative tauopathies [16]. It stands to reason that increases in tau isoforms may also contribute to this process by increasing the availability of the tau substrate to phosphorylating enzymes.

The zebrafish, *Danio rerio*, is an emerging model organism for the study of neurodegenerative disease [17]. Zebrafish embryos represent normal collections of cells in which complex and subtle manipulations of gene activity can be performed to facilitate analyses of genes involved in human disease. The zebrafish genome is extensively annotated and regions of conservation of chromosomal synteny between humans and zebrafish have been defined [18]. In many cases zebrafish genes are identifiable that are clear orthologues of human genes. For example, the AD-relevant *PRESENILIN* genes (*PSEN1* and *PSEN2)* have zebrafish orthologues of *psen1* [19] and *psen2* [20] respectively. Tau phosphorylation and subsequent toxicity has been reported in zebrafish over-expressing the FTD associated human tau mutation, P301L [21,22]. However this model does not reflect the pathology of other dementias such as AD where factors that regulate levels of wild-type tau isoforms promote hyper-phosphorylation and neurodegeneration.

We have previously identified two paralogues (co-orthologues) of *MAPT* in zebrafish, denoted *mapta* and *maptb* and have shown that both genes are expressed in the developing central nervous system [23]. (Teleosts appear to have undergone an additional round of genome duplication since their separation from the tetrapod lineage followed by loss of many of the duplicated genes [18]). Similar to human *MAPT*, a complex pattern of alternative splicing of the *mapta* and *maptb* transcripts occurs. Zebrafish *mapta* gives rise to transcripts encoding 4R-6R isoforms, whereas *maptb* is predominantly expressed as a 3R isoform [23] (Figure 1) and is also alternatively spliced to form a "big tau" isoform. In mammals "big tau" is expressed in the peripheral nervous system and other tissues [24-26] while in zebrafish we observed "big tau" expression (at 24 hours post fertilization, hpf) in the trigeminal ganglion and dorsal spinal cord neurons (possibly dorsal sensory neurons) [23]. However, whether hypoxic conditions lead to changes in tau isoform expression has not been widely explored in zebrafish. In the work described in this paper we extend our examination of expression of the zebrafish tau co-orthologues to study their response to actual hypoxia in adult fish brains and to chemical mimicry of hypoxia in explanted adult fish brains. We observe increases in the overall levels of both *mapta* and *maptb* transcripts due to specific increases in the levels of *mapta* 6R and *maptb* 4R transcript isoforms. This is consistent with dramatically decreased levels of transcripts of the zebrafish orthologue of the human *TRA2B* gene that codes for a splicing factor regulating alternative splicing of *MAPT* transcripts in human cells [12]. We also observe an apparent increase under hypoxia in the levels of shorter transcripts of *maptb* relative to "big tau" transcripts of this gene. Overall, our findings indicate that hypoxia can alter splicing of zebrafish MAPT co-orthologues promoting formation of longer transcripts and possibly generating Mapt proteins more prone to hyperphosphorylation. This supports the use of zebrafish to provide insight into the mechanisms regulating *MAPT* transcript splicing under conditions that promote neuronal dysfunction and degeneration.

Results

To determine whether hypoxic conditions regulate alternative splicing in *MAPT* co-orthologues in zebrafish, levels of *mapta* and *maptb* transcripts were assessed in adult zebrafish brains under conditions of actual hypoxia or in explanted adult brains subjected to chemical mimicry of hypoxia caused by NaN_3.

In studies of hypoxia it is common to use chemical agents that can mimic (partially) hypoxic conditions (also known as "chemical hypoxia"). Agents commonly used are cobalt chloride ($CoCl_2$), nickel chloride ($NiCl_2$) and NaN_3. Azides, including NaN_3, have an action on the respiratory chain very similar to that of cyanide. We have previously shown that exposure to aqueous solutions of NaN_3 can induce hypoxia-like responses in zebrafish [27].

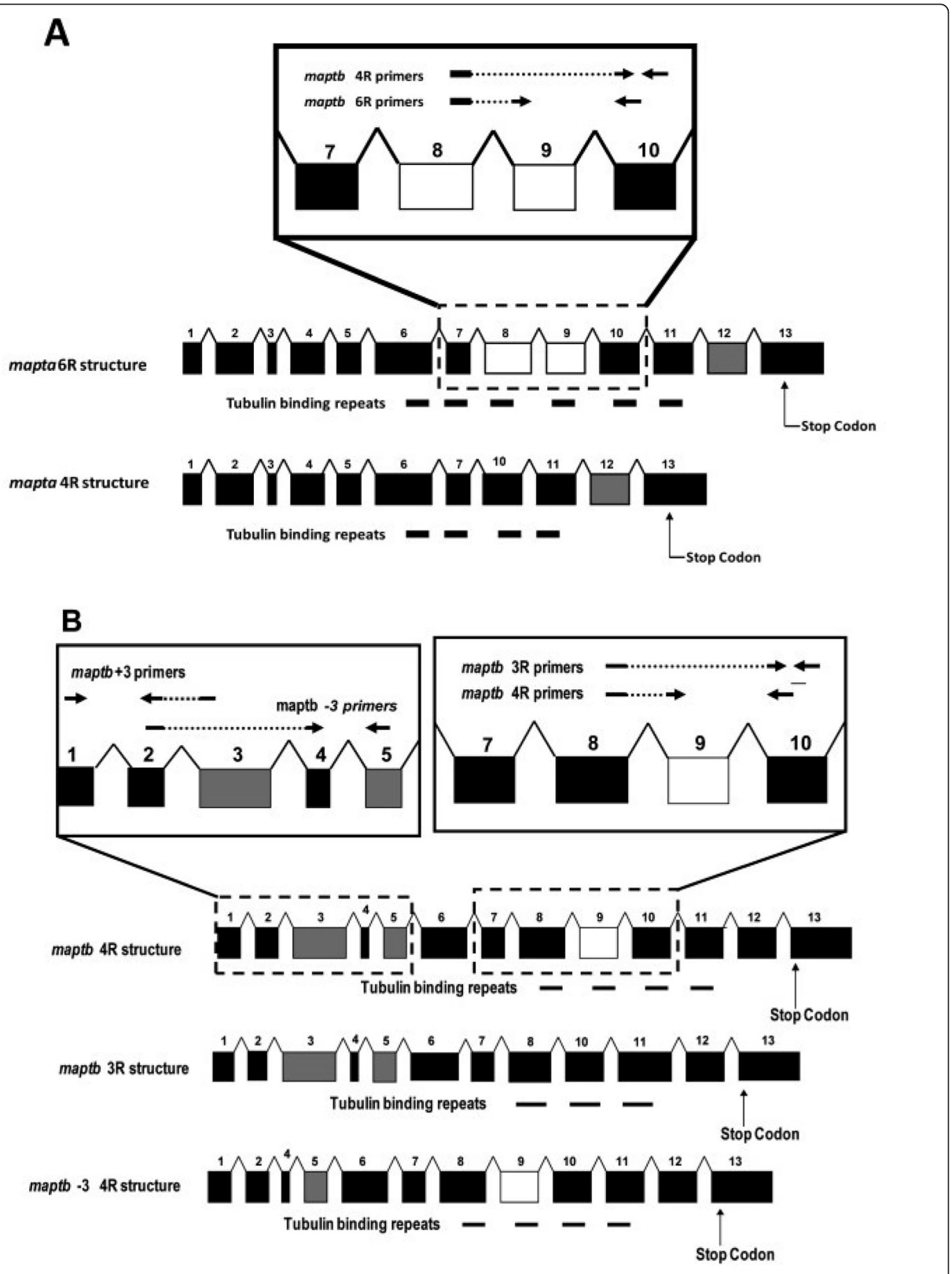

Figure 1 Splicing isoforms of *mapta* and *maptb* mRNA transcripts. Grey and white boxes indicate exons subject to alternative splicing. The black lines below exons indicate those encoding tubulin-binding motifs. Arrows indicate the approximate binding sites of primers used in qPCR analyses of splicing isoforms. **(A)** Exon structure of *mapta*.isoforms **(B)** Exon structure of *maptb* isoforms.

Exposure of adult fish to hypoxia or exposure of explanted adult brains to chemical mimicry of hypoxia increases the overall expression of tau transcripts in zebrafish brains. This was shown by qPCR measurement involving amplification of exonic sequence included in all transcripts of *mapta* or *maptb* (i.e. exon 6 of both genes – see Figure 2A and 2B). We also observed that the pattern of tau transcript splicing differs between hypoxia-exposed brains and controls. In terms of contributing isoforms, expression of the *mapta 6R* isoform was significantly increased, while expression of the *mapta 4R* isoform showed a significant decrease under hypoxia (Figure 2A). We also observed a significantly increased level of expression of *maptb 4R* transcripts, while expression of *maptb 3R* transcripts also showed a significant decrease under hypoxia (Figure 2B). An increase in expression of *maptb 4R* but not *3R* corresponds to an overall increase in the 4R/3R ratio of tau transcripts (Figure 2B).

In rats (and humans) *Mapt* exon 4a contains a large open reading frame. Inclusion of this exonic sequence in *MAPT* mRNAs allows translation of "big tau" protein. Exon 3 of zebrafish *maptb* appears to be equivalent to rat exon 4a in size although no sequence homology is observed. Like rat *MAPT* exon 4a, zebrafish *maptb* exon 3 is subject to alternative splicing [23]. Therefore, we performed qPCR to test whether this alternative splicing event is also influenced by hypoxic conditions. We observed that exclusion of exon 3 (here denoted as *maptb* −3) from zebrafish *maptb* transcripts is significantly increased under hypoxia and chemical mimicry of hypoxia when compared with inclusion of exon 3 (here denoted as *maptb +3*) (Figure 2C).

In humans, differential splicing of *MAPT* transcripts in response to hypoxia can occur due to decreased binding of TRA2 protein to RNA [28]. The *TRA2* gene is duplicated in vertebrates, resulting in two TRA2 proteins with aprpoximately 63% amino acid residue identity in humans [29]. These proteins are denoted TRA2A encoded by the *TRA2A* gene and TRA2B protein encoded by the gene *TRA2B* (also known as *SFRS10*). Nuclear magnetic resonance (NMR) analyses have recently shown that the optimal core RNA target sequence for binding TRA2B protein is AGAA. Conrad *et al.* [12] observed AD-specific changes in TRA2B expression, suggesting a potential mechanism for altered tau in AD. Suh *et al.* [28] also observed a decrease in mouse Tra2b expression leading to a decrease in exon 10 exclusion and 3R-tau expression in cortical neurons after transient occlusion of the middle cerebral artery in mice. To examine whether this behavior is conserved for the zebrafish *mapta* and *maptb* genes we first observed whether hypoxia alters expression of the *TRA2B* orthologous gene, *tra2b*, in zebrafish brains. As shown in Figure 2D both actual hypoxia and chemical mimicry of hypoxia lead to decreased *tra2b* transcript levels presumably indicating reduction in the splice-regulating activity of Tra2b protein. We then examined whether the zebrafish *mapta* and *maptb* genes possess potential Tra2b binding sites within exons encoding tubulin-binding repeats and subject to alternative splicing. Using the online software, ESE finder (http://genes.mit.edu/burgelab/rescue-ese/) [30], zebrafish sequences for *mapta* exon 8 and *maptb* exon 9 were examined for putative tra2b binding sites. We found multiple exonic splicing enhancers (ESEs) but, for each gene, only one appeared significantly similar to the human TRA2B-binding site (Figure 3).

Discussion

The human *MAPT* gene is located on chromosome 17 and contains 16 exons. Alternative splicing of the primary transcript leads to a family of mRNAs, encoding different protein isoforms. In adult human brain, six isoforms are expressed, produced by alternative splicing of exons 2, 3, and 10. Tau isoforms in the CNS contain either three or four copies of a tandem repeat containing tubulin-binding sequences (encoded by exon 10), referred to as 3R and 4R-tau [24]. Optional inclusion of exon 2, or exons 2 and 3, gives rise to N-terminal inclusions of 29 or 58 amino acid residues respectively [24].

In this study we provide evidence that exposure to actual hypoxia and to chemical mimicry of hypoxia leads to overall increases in tau transcript levels and, simultaneously, marked relative changes in the alternative splicing of tau transcripts in adult zebrafish brains. Our results revealed that exposure to acute levels of actual hypoxia or chemical mimicry of hypoxia shifts the production of the predominantly expressed 3R transcript isoform of *maptb* towards formation of the 4R isoform, thus altering the 3R to 4R ratio. The precise regulation of the ratio of expression of 3R relative to 4R MAPT isoforms in human brain has been proposed to be critical for maintaining normal brain function [31]. The disruption of this balance has been found to be correlated with tauopathies [8,32]. We also observed a significant increase in expression of the 6R transcript isoform of zebrafish *mapta* relative to the *mapta* 4R transcript. As far as the behavior in alternative splicing of exons coding for tubulin-binding domain sequences is concerned, our data are in agreement with those of Conrad *et al.* [12] and Ichihara *et al.* showing that, in AD brains, the expression level of exon 10 is altered [33].

Imbalance of the 4R-3R tau isoform ratio has been observed in tauopathies such as FTDP-17 [8], PSP [10], and PiD [34]. An altered 4R-3R tau isoform ratio has also been reported in the spinal cord after sciatic nerve axotomy [35]. Suh *et al.* [28] reported that cerebral ischemia changes the ratio of 4R-3R tau mRNAs and protein levels as well as causing tau hyperphosphorylation. Changes in

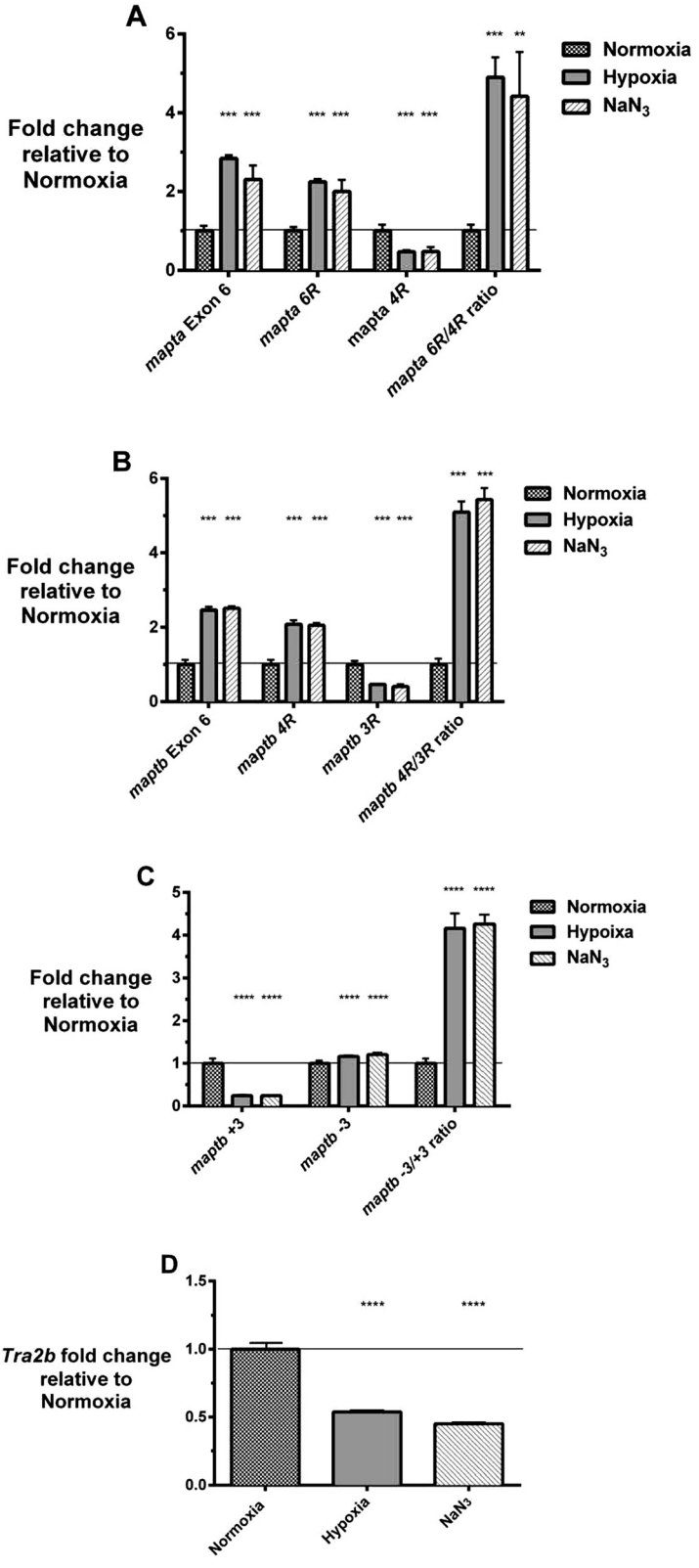

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 qPCR analyses of the expression of A) Measurement of *mapta* exon 6 levels gives the combined expression of all *mapta* transcripts in zebrafish brains. qPCRs to determine relative *mapta* 6R and 4R isoform levels show increased and decreased expression under hypoxia respectively. **B)** Measurement of *maptb* exon 6 levels gives the combined expression of all *maptb* transcripts in zebrafish brains. qPCRs to determine relative *maptb* 4R and 3R isoform levels show increased and decreased expression under hypoxia respectively. **C)** *maptb +3* ("big tau") is decreased relative to *maptb −3* under hypoxia. **D)** *tra2b* transcript levels under normoxia are higher relative to those under hypoxia or chemical mimicry of hypoxia (sodium azide exposure). Expression ratios for *mapta and maptb* are shown relative to normoxia (the normoxia expression level is normalized to *eef1a1l1*). ***$P \leq 0.0001$; **$P \leq 0.001$; ****$P \leq 0.00001$. Error bars represent standard error of the mean.

tau isoform ratio and phosphorylation status can cause defects in the central nervous system by affecting microtubule dynamics and axonal transport resulting in neuronal loss [4]. Therefore, it is conceivable that an alteration of tau isoform ratio and increased tau hyperphosphorylation after brain ischemic insult may contribute to the prevalence of AD in stroke patients [36,37].

Exon 10 of the human *MAPT* gene, is flanked by a large intron 9 (13.6 kb) and intron 10 (3.8 kb), and has a stem-loop structure which spans the 5′ splice sites, which can sequester the 5′ splice site and leads to the use of alternative 5′ splice sites [38]. Thus exon 10 can be included or skipped to produce tau proteins with or without exon 10, depending on the action of *trans*-acting or *cis*-elements located in exon 10. Hutton M, 1998 [8] The pre-mRNA splicing factor Tra2b was shown to promote *MAPT* exon 10 splicing [39]. Levels of Tra2b protein were found to be reduced in AD brains [12]. Decreased levels of this splicing factor were also observed by Suh *et al*. [28] in cortical neurons and in mouse cerebral cortex following hypoxic-ischemic injury. Thus, decreased Tra2b expression under hypoxia may contribute to a shift in 4R-3R tau isoform ratio by increasing incorporation of exon 10 into mature *MAPT* mRNA. Consistent with this we detected putative Tra2b-binding sites in exon 8 of *mapta* and exon 9 of *maptb.* We also saw decreased expression of *tra2b* mRNA under hypoxic conditions.

High molecular weight (HMW) tau isoforms "big tau" have been detected in the neurons of the adult rat peripheral nervous system (PNS), optic nerve, spinal cord, several neuronal cell lines including PC12 and neuroblastoma N115 [24] and non-neuronal tissues [25,26]. "Big tau" appears to be the only tau isoform expressed in adult dorsal root ganglia (DRG) [24,40]. "Big tau" is encoded by an 8 kb mRNA containing an additional exon 4a that is not present in any other tau isoforms. "Big tau" expression is developmentally regulated. It is expressed late in fetal life and its expression increases postnatally [24]. Its presence has been correlated with increased neurite stability in adult DRG [40]. Several studies have investigated "big tau" expression in non-neuronal tissues in AD patients but did not observe any significant changes [25,26]. Chen *et al.* [23] described an alternative splicing event involving *maptb* exon 3, which appears to be equivalent to human *MAPT* exon 4a. In our experiments we observed that hypoxia significantly increases the level of *maptb* transcripts from which exon 3 sequence is excluded but does not appear to change levels of the "big tau" form of *maptb* transcripts. However, we cannot exclude the possibility that this apparent increase in *maptb* expression with decreased exon 3 inclusion may be due to increased expression of the shorter transcript isoform in cells that do not express big tau, rather than a change in the ratio of splicing to form shorter transcript relative to "big tau" transcript within cells expressing both transcripts.

Conclusion

Overall, our findings show that exposure of zebrafish brains to actual hypoxia or chemical mimicry of hypoxia can produce changes in the expression ratio of different tau isoforms. These changes are similar to those observed in a number of neurodegenerative diseases and thus support the use of zebrafish as a model for providing further insight into the mechanisms underlying these disease processes.

Human *MAPT* exon 10 (5'→3')

GTGCAGATAATTAAT**AAGAAGCTG**GATCTTAGCAACGTCCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACGTCCCGGGAGGCGGCAGT

Zebrafish *mapta* exon 8 (5'→3')

ATCCAAATCCTTGAT**CAGAAGGTG**GATTTCAGCAATGTCCAGTCAAAGTGTGGCTCCAAAGCCAACCTGAAGCATACACCAGGAGGTGGAAAT

Zebrafish *maptb* exon 9 (5'→3')

GGGGTGGAAATGTCGAGATCAAATCT**GAGAAGTTG**GATTTCAAAGCCCAGTCAAAGGTTGGATCTCTGGAAAACATTGGACATGTCCCTGGGGGTGGACAGAGGAGG

Figure 3 Sequences from human *MAPT* and zebrafish *mapta* and *maptb* were analysed for the presence of possible Tra2B binding sites using the online software ESE finder (http://genes.mit.edu/burgelab/rescue-ese/). Bold, underlined letters are putative Tra2b-binding sites.

Methods

Ethics

This work was conducted under the auspices of The Animal Ethics Committee of The University of Adelaide and in accordance with EC Directive 86/609/EEC for animal experiments and the Uniform Requirements for Manuscripts Submitted to Biomedical Journals.

Zebrafish husbandry and experimental procedures

Danio rerio were bred and maintained at 28°C on a 14 h light/10 h dark cycle [41]. Adult zebrafish (AB strain) at approximately 1 year of age were used for all experiments (n = 12). Fish for analysis were not selected on the basis of sex. For chemical mimicry of hypoxia adult explant brain tissue was exposed to 100 μM of sodium azide (NaN_3, Sigma-Aldrich CHEMIE Gmbh, Steinheim, Germany) in DMEM medium for 3 hours. Untreated adult zebrafish brain explants that were dissected from zebrafish in the same way as for the treated adult zebrafish brains were used as *in vitro* controls. In the experiments conducted under low oxygen conditions, oxygen was depleted by bubbling nitrogen gas through the medium. Oxygen concentrations were then measured using a dissolved oxygen meter (DO 6+, EUTECH instruments, Singapore). The dissolved oxygen level in the actual hypoxia group was measured to be 1.15 ± 0.6 mg/l; whereas the normal ambient oxygen level was 6.6 ± 0.45 mg/l [27,42]. Zebrafish were exposed to actual hypoxia for 3 hours. Briefly, after each hypoxia trial, the animals were euthanized by hypothermic shock and then decapitated to remove the brain. Total RNA was extracted from samples mentioned above using the QIAGEN RNeasy mini kit (QIAGEN, GmbH, Hilden, Germany) and stored at −80°C for further analysis. RNA concentration was determined with a NanoVue™ UV−vis spectrophotometer (GE Healthcare Life Sciences, Fairfield, USA). To insure quality of RNA, RNA samples were electrophoresed on 1% TBE agarose gels. 700 ng of total RNA were used to synthesize 25 μL of first-strand cDNA by reverse transcription (SuperScript® III First-Strand DNA synthesis kit; Invitrogen, Camarillo, USA).

Quantitative real-time PCR for detection

The relative standard curve method for quantification was used to determine the expression of experimental samples compared to a basis sample. For experimental samples, target quantity was determined from the standard curve and then compared to the basis sample to determine fold changes in expression. Gene-specific primers were designed for amplification of target cDNA and the cDNA from the ubiquitously expressed control gene *eef1a1a*. The reaction mixture consisted of 50 ng/μl of cDNA, 18 μM of forward and reverse primers and *Power* SYBR green master mix PCR solution (Applied Biosystems, Warrington, UK).

Table 1 Gene specific primers used for qPCR

Gene/transcript isoform	Accession number	Sequence	Amplicon size
eef1a1l1 (F)	**NM_131263.1**	5′-CTGGAGGCCAGCTCAAACAT-3′	**87 bp**
eef1a1l1 (R)		5′-ATCAAGAAGAGTAGTACCGCTAGC-3′	
tra2b (F)	**NM_201197**	5′-GCAGACGACATATTGGTGACC-3′	**155 bp**
tra2b (R)		5′-TGACTGCTGGTCGTACACAATG-3′	
maptb 4R (F)	**XM_005171601**	5′-AAGATCGGCTCCACTGAGAACC-3′	**194 bp**
maptb 4R (R)		5′-GATCCAACCTTTGACTGGGCTT-3′	
maptb 3R (F)	**XM_005171601**	5′-GGGAAGGGGTGGAAATGTC-3′	**140 bp**
maptb 3R (R)		5′-GATCCAACCTTTGACTGGGCTT-3′	
mapta 6R (F)	**XM_001340530**	5′-TCGTCACAAACCAGGTGGAG-3′	**152 bp**
mapta 6R (R)		5′-GCTCACGGAACGTCAGTTTG-3′	
mapta 4R (F)	**XM_001340530**	5′-CGGAGGTGGAAAATTGAGTCAC-3′	**100 bp**
mapta 4R (F)		5′-CTCCTCCAGGGACACAATTTCT-3′	
maptb −3 (F)	**XM_005171601**	5′-GAAGCCAAGGCTGGAGCA-3′	**120 bp**
maptb −3 (R)		5′-CTGGGGATGCCTGTGACTGA-3′	
maptb +3 (F)	**XM_005171601**	5′-CCGGCAACAACATAGCATCTG-3′	**140 bp**
maptb +3 (R)		5′-CACCGGGAGTGAATGTGGC-3′	
mapta Ex.6 (F)	**XM_001340530**	5′-CCTAAATCTCCTGCCAGCAAG-3′	**117 bp**
mapta Ex.6 (R)		5′-TGTGGGCGAACGGTTCTT-3′	
maptb Ex.6 (F)	**XM_005171601**	5′-CAAATCACCTGGCTCGCTG-3′	**114 bp**
maptb Ex.6(R)		5′-GGTTGGTGTTTGAGGTTCTCAGTG-3′	

To generate the standard curve cDNA was serially diluted (100 ng, 50 ng, 25 ng, 12.5 ng). Each sample and standard curve reaction was performed in triplicate for the control gene and experimental genes. Amplification conditions were 2 min at 50°C followed by 10 min at 95°C and then 40–45 cycles of 15 s at 95°C and 1 min at 60°C. Amplification was performed on an ABI 7000 Sequence Detection System (Applied Biosystems) using 96 well plates. Cycle thresholds obtained from each triplicate were averaged and normalized against the expression of *eef1a1l1*, which has previously been demonstrated to show unchanged levels of expression under hypoxia in embryos at 6, 12, 48 and 72 hpf and in adult gills [43]. Each experimental sample was then compared to the basis sample to determine the fold change of expression. The primers used for quantitative real-time PCR analysis of relative zebrafish *mapta/b* mRNA levels are shown in Table 1. To reduce possible interference from unspliced RNA and/or contaminating genomic DNA primers were designed to bind in cDNA over exon-exon boundaries. All qPCRs were performed according to MIQE guidelines [44].

Statistical analysis of data

Means and standard deviations were calculated for all variables using conventional methods. Two-way ANOVA was used to evaluate significant differences between normoxia and samples from actual hypoxia or chemical mimicry of hypoxia. *p*-Values are shown in the figure legends, a criterion alpha level of $P < 0.05$ was used for all statistical comparisons. All qPCR assays were done in three biological replicates with three qPCRs per biological replicate). All the data were analysed using GraphPad Prism version 6.0 (GraphPad Prism, La Jolla, CA).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SHMN completed experiments, participated in the design of the study and data analysis and drafted the manuscript. MN participated in the design of the study and revisions of the manuscript. SG completed experiments. MC participated in revision of the manuscript. RM participated in the revision of the manuscript. GV participated in the design of the study and the revision of the manuscript. ML predominantly designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Chapter VII

DISCUSSION

Correct neuronal activity is crucial for the regulation of physiological functions throughout the entire organism, and any disruption of neuronal function has potentially devastating consequences. Neurons are extremely dependent on oxygen availability and demonstrate a high vulnerability during conditions that result in alterations in oxygen supply. Just a few minutes of oxygen deprivation results in significant dysfunction, and longer episodes can eventually result in the induction of cell death [272].

As result of aging, oxygen delivery to cells and tissues is somewhat impaired, therefore increasing the vulnerability of neurons to damage. In addition, cellular adaptation to hypoxia is significantly compromised with increasing age and, at the same time, an ischemic episode has a more dramatic outcome in old versus young people [273, 274]. In line with these findings, the risk of stroke increases with each decade after the age of 55 years, and majority of cerebral infarctions are reported in people over the age of 65 [275]. Increased neuronal vulnerability is also very evident in the sharp rise in the incidence of neurodegenerative diseases that occur in the later stages of life and as the lifespan continues to increase (293).

Alzheimer's disease (AD) is a complex disease in which genetic and environmental factors lead to disease progression. It is now evident that genetic predisposition is responsible for only a small number of AD cases [276]. Although most cases have no known genetic basis, exposure to other pathogenic conditions, including chronic inflammation, traumatic brain injury, cerebrovascular disease, hypoxia/ischemia can also be contributing factors [276-278]. Although AD is characterized by Aβ plaques and NFTs, other pathologies including cerebral infarcts, accumulation of Aβ in cerebrovasculature, white matter changes, and even hemorrhages are often present [279, 280]. Recent findings demonstrate that over 30% of AD patients also bear evidence of cerebral infarcts [281]. Clinical studies demonstrated that strokes or cerebrovascular events worsen impairment and lead to a more rapid decline in patients diagnosed with AD [282-284]. It is estimated that AD is 3 times more likely to precipitate in the elderly after a stroke episode [283]. In addition, it has been reported that AD pathology (Aβ plaques and NFTs) is increased in patients with coexisting evidence of cerebral infarcts [285, 286]. Recent studies have shown that AβPP expression is elevated in post-ischemic brain and ischemia may facilitate the amyloidogenic cleavage of AβPP [287-289]. Ischemic conditions may not only affect the processing of AβPP and the accumulation of Aβ, but also the formation of NFTs as suggested by studies on adult female rats subjected to transient cerebral ischemia [290].

Ischemic brain injury results from a multiple events that develop over time and eventually cause neurons to degenerate. Decrease in blood flow leads to a reduction in adenosine triphosphate (ATP) that is required for maintaining ionic gradients [291]. The disruption of ionic gradients in neurons and glial cells is characterized by influx of Na^+ and Ca^{2+}, efflux of K^+ from cells and cellular depolarization [292]. Increase in intracellular Ca^{2+} causes mitochondrial Ca^{2+} overload leading to the leakage of the mitochondrial membrane, cessation of already compromised ATP production, and a burst of oxygen free

radicals [293]. Accumulation of oxygen free radicals increases oxidative stress and contributes to membrane lipid peroxidation, damage to DNA and proteins, and production of inflammatory mediators [294]. Markesbery *et al*. (2005) and Pratico *et al*. (2002) showed that levels of lipid peroxidation increased significantly in brains of patients with AD when compared with controls [295, 296]. In our findings we demonstrated that exposure of zebrafish brain to chemical hypoxia (using sodium azide, NaN_3) or actual hypoxia leads to an increase in accumulation of oxygen free radicals which consequently increase oxidative stress and an increase in lipid peroxidation. Our results are consistent with the idea that insufficient oxygen supply may cause oxidative stress and alterations in antioxidant enzymes observed early in the development of AD.

One phenomenon commonly observed in sporadic AD brains is an increase in alternative splicing of Presenilin 2 (*PSEN2)* transcripts to produce a truncated isoform lacking exon 5 sequence and thus named "PS2V" [12]. PS2V protein accumulates in intracellular inclusion bodies termed PS2V bodies. PS2V encoding proteins are expressed mainly in the hippocampal CA1 region and temporal cortex in AD patients [11]. Using human neuoblastoma SK-N-SH cells, Sato *et al.* (1999) showed that the exclusion of exon 5 from *PSEN2* transcripts that forms PS2V mRNA is induced by hypoxia but not other forms of cellular stress [12]. This isoform has been described as "aberrant", probably because it had only been detected in human brain and not in mice or rats. PS2V has the remarkable property that it can increase γ-secretase activity and production of Aβ peptide from AβPP [11]. We identified the zebrafish and guinea pig orthologue of the PS2V isoform, which disproves the idea that the PS2V protein is aberrant. We have demonstrated that like human PS2V, zebrafish PS1IV is produced in response to hypoxia as a result of induction of zebrafish Hmga1a protein that binds in transcribed *psen1* sequence cognate with exon 5 of human *PSEN2*. We have also demonstrated that by using a morpholino antisense oligonucleotide we can block the formation of this alternative spliced transcript under hypoxic conditions. The resultant very short PS1IV peptide appears, nevertheless, to be able to stimulate γ-secretase activity.

The unfolded protein response (UPR) is a stress response activated under conditions associated with an accumulation of unfolded or misfolded proteins in the lumen of the ER [297]. The main players in UPR activation are three ER transmembrane proteins: the double stranded RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK)[298], the inositol-requiring enzyme 1 (IRE1)[299] and the activating transcription factor 6 (ATF6) [300]. In the normal state, PERK, IRE1 and ATF6 are inactive. The ER chaperon glucose-regulated protein (GRP78) is bound to these proteins and blocks their activation. Under ER stress such as when unfolded proteins accumulate in the ER lumen, GRP78 is required to help refolding of these proteins. Therefore it dissociates from PERK, IRE1 and ATF6 and binds to the unfolded proteins. Dissociation of GRP78 results in oligomerization, autophosphorylation and activation of PERK and IRE1. After being activated by autophosphorylation, IRE1 is transformed into an active endonuclease that cleaves the X-box protein 1 (XBP1). The spliced XBP1 mRNA (XBP1s) functions as an active transcription factor. It binds to ER stress response

elements (ERSE) of UPR stress genes, including GRP78 and GRP94, and activates their expression (reviewed in [301]). Cells can cope with mild ER stress by activation of UPR, while severe ER stress results in activation of apoptosis where caspase-12 could play a key role [302]. The ER has been identified as the site where the highly toxic amyloidogenic $A\beta_{42}$ is generated [303]. PS-1 mutations linked to Alzheimer's disease impair UPR signaling by inhibiting activation of PERK and IRE1 [304, 305] while phosphorylation of PERK and eIF2α has been also seen in neurons of Alzheimer's disease patients, suggesting activation of UPR [306]. The notion that impairment of ER function may contribute to AD pathology is supported by several studies in which disturbances of ER function and calcium homeostasis are reported in cells expressing mutant PSENs [307-309]. PS2V has also been shown to disrupt the UPR signalling pathway and sensitizes cells to ER stress [11]. PS2V protein increases cells vulnerability to ER stress by inhibiting of GRP78 mRNA induction. The decrease in GRP78 mRNA is caused by the impaired phosphorylation of IRE1α due to binding of PS2V to IRE1α [11]. In our study we have demonstrated that forced expression of PS1IV mRNA can result in the decrease of XBP1s mRNA. We suggest that zebrafish PS1IV is also able to bind to IRE1α and impair its phosphorylation, which leads to the downstream effects seen in PS2V. However, the nature of PS2V binding to the IRE1a and the overall function of PS2V is still yet not properly understood.

To understand the functional genomics changes modulated by PS1IV and to understand the possible roles of PS2V in Alzheimer's disease, we used microarray analysis to identify genes with significantly increased or decreased transcript levels when PS1IV expression is blocked. We also used network analysis to gain greater insight into the function of these transcripts in the presence of PS1IV under hypoxic conditions. Initial analysis of microarray data identified significantly increased and decreased transcription of genes after loss of PS1IV in zebrafish under hypoxic conditions. These included candidate genes linked to the immune response, regulation of TOR signalling, regulation of blood pressure and formation of vascular structures, cell proliferation, ER function and ribosomal protein function. Using network analysis we identified that PS1IV is important for the upregulation of *1L1B* that, in humans, has been strongly implicated in the inflammation that characterises AD brains [310-312]. These studies support a proinflammatory role of IL-1β in the pathogenesis of AD.

In the central nervous system, an upregulation in inflammatory signalling (i.e. neuroinflammation) is represented by the activation of astrocytes and microglia, and the release of proinflammatory regulators [313]. Neuroinflammation is seen in many neurodegenerative diseases, such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, psychiatric disorders, as well as AD [314-316]. Overwhelming evidence suggests an upregulation in the inflammatory molecules and activated glial cells surrounding the senile plaques in brains of AD patients and AD transgenic animal models [317, 318]. The cytokines, in particular IL-1β, IL-6 and TNF α (tumor necrosis factor α), are the major effectors of the neuroinflammatory

signals, and can affect neurophysiologic mechanisms regarding cognition and memory [319, 320]

Conditions such as cerebral ischemia and stroke that result in hypoxic conditions in affected brain areas can promote tau hyperphosphorylation and formation of NFTs. Acute hypoxic conditions have been shown to activate kinases that phosphorylate tau resulting in accumulation of phosphorylated tau in neurons [321]. Suh *et al.* (2010) reported that cerebral ischemia changes the ratio of 4R-3R tau mRNAs and protein levels as well as causing tau hyperphosphorylation [322]. Changes in tau isoform ratio and phosphorylation status can cause defects in the central nervous system by affecting microtubule dynamics and axonal transport resulting in neuronal loss [323]. Therefore, it is conceivable that an altered tau isoform ratio and increased tau hyperphosphorylation after brain ischemic insult may contribute to the prevalence of AD in stroke patients [324, 325].

As mentioned above cerebral ischemia can induce ER stress in neurons, which leads to neuronal loss. Recent studies have shown that stimulation of UPR signalling can induce Tau phosphorylation, possibly through the activation of glycogen synthase kinase 3β (GSK-3β) [326]. Interestingly, neurons exhibiting activated PERK co-express active GSK-3β in affected neurons of AD brain [327]. The functional connection between UPR signalling and tau might be through the expression of PS2V. PS2V has been shown to disrupt UPR signalling which makes neurons susceptible to ER stress [11]. It has been suggested that PS2V can facilitate the aggregation and phosphorylation of tau proteins by blocking that UPR response [212].

We have previously identified two paralogues (co-orthologues) of human *MAPT* in zebrafish, denoted *mapta* and *maptb* and have shown that both genes are expressed in the developing central nervous system. Similar to human *MAPT*, a complex pattern of alternative splicing of the *mapta* and *maptb* transcripts occurs [271]. However, whether hypoxic conditions lead to changes in tau isoform expression has not been widely explored in zebrafish. Our results in this thesis revealed that exposure to acute levels of actual hypoxia or chemical mimicry of hypoxia shifts the production of the predominantly expressed 3R transcript isoform of *maptb* towards formation of the 4R isoform, thus altering the 3R to 4R ratio. We also observed a significant increase in expression of the 6R transcript isoform of zebrafish *mapta* relative to the *mapta* 4R transcript. These changes in tau isoform expression are due to a decrease in the Tra2b splicing factor under hypoxic condition, which has been previously been shown to increase the incorporation of exon 10 into mature *MAPT* mRNA [328]. As far as the behavior in alternative splicing of exons coding for tubulin-binding domain sequences is concerned, our data are in agreement with those of Conrad *et al*. (2007) and Ichihara *et al*. (2009) showing that, in AD brains, the expression level of exon 10 is altered [329, 330]. However, due to the lack of an efficient antibody to detect the hyperphosphorylation of these isoforms in zebrafish we were unable to show that hypoxia also increases the hyperphosphorylation of tau isoforms or zebrafish PS1IV has any effect

on tau phosphorylation. Future work would be to generate an antibody to detect the phosphorylated form of tau in zebrafish.

A major obstacle to AD research is the lack of an animal model that replicates all the characteristic of the human disease. Transgenic animal models have yielded a number of important insights into disease mechanisms and are a useful tool for therapeutic design and evaluation. However, the genetic manipulation required to produce them does not accurately reflect the human disease state, and consequently limits at least some of the conclusions that can be drawn. An alternative strategy may be to investigate potential risk factors in species with similar Aβ sequence to humans. One candidate is the guinea pig [331]. *In vitro* studies using guinea pig neuronal cells have demonstrated that AβPP processing in this species is very similar to that in humans [332, 333]. Guinea pigs have also been used in a small number of studies to investigate the effect of glutamate [334], serotonin [335], β-secretase inhibitors [336], and cholesterol on Aβ production [78].

Studies utilising animal models of AD, including rabbits [337] and transgenic mice [77], have all shown a strong correlation between serum cholesterol levels and cerebral Aβ production. In this thesis we show a similar correlation in guinea pig brain. We showed that, in guinea pigs, cholesterol upregulates BACE1 and down-regulates ADAM10 expression, which would contribute to the promotion of amyloidogenic processing of AβPP to generate Aβ. These results are consistent with previous findings where modulating cholesterol alters the expression of BACE1 and ADAM10 *in vitro* and *in vivo* in animal models [79, 80, 82, 338].

Guinea pigs are the only small animal mammalian model in which formation of PS2V has been confirmed. Our results showed, for the first time, that an additional stimuli, hypercholesterolemia, simulates PS2V production in addition to up-regulating Aβ synthesis. The up-regulation of PS2V could be a contributing factor modulating Aβ in hypercholesterolemia. Hypercholesterolemia can lead to vessel wall changes in the brain, leading to hypoperfusion, ischemia and hypoxia and evidence indicates that this can contribute to AD pathogenesis [339]. Hypoxia induced by cerebrovascular hypoperfusion in rats leads to accumulation of cerebral Aβ and cognitive deficits [340]. Hypoxia has also been shown to up-regulate the genes required for Aβ production and here we have shown it to up-regulate PS2V in guinea pig brain [341, 342]. Whether cholesterol upregulates PS2V, Aβ and genes encoding enzymes involved in Aβ production via impacting on cerebrovascualture, promoting ischemia or hypoxia could not be determined from our data, but could be addressed in *in vitro* or *in vivo* follow up studies by assessing vasculature/hypoxic markers under cholesterol loading conditions. Our results indicate that guinea pigs represent the alternative *in vivo* model to mice and rats for studying the effects of AD risk factors such as cholesterol on Aβ metabolism and PS2V generation and for evaluating dietary interventions that may have beneficial outcomes in AD.

In summary, we have used zebrafish as a model organism for the investigation of molecular pathology of Alzheimer's disease (AD). We have shown that a decrease in

oxygen levels in the zebrafish brain can lead to an increase in accumulation of oxygen free radicals, which consequently increase oxidative stress. This is consistent with the idea that insufficient oxygen supply may cause oxidative stress and alterations in antioxidant enzymes observed early in the development of AD. We also show that an orthologue of the PS2V isoform exists in zebrafish and guinea pigs. Like human PS2V, zebrafish PS1IV is produced in response to hypoxia. Further characterization of this splice variant in zebrafish revealed that PS1IV is able to stimulate γ- secretase activity and suppresses the UPR in a similar fashion to PS2V. We also observed that hypoxia can alter the expression and splicing of zebrafish tau (*mapta* and *maptb*) transcripst. Using microarray and network analysis we found that PS1IV can up-regulate pathways involved in the innate immune response, which have been previously implicated in AD. Our results suggest PS2V plays an important role in the development of sporadic AD pathology. Since hypoxia is important for induction of PS2V formation this supports that insufficient brain oxygenation may be a fundamental driver of such pathology. Thus, PS2V may form a facilitating link between hypoxia and a number of pathological processes in AD brains. It is possible that blockage of PS2V action in AD brains might inhibit some of these pathological processes such as inflammation or might relieve suppression of the UPR that normally restricts generation of inappropriately folded, aggregation-prone proteins.

Currently there is no cure for AD; while current therapies may temporarily decrease the symptoms, death usually occurs approximately 8 years after diagnosis. These alarming statistics emphasise the importance of gaining a greater understanding of the pathophysiology of AD. Attention is now being directed to the discovery of biomarkers, which may not only facilitate pre-symptomatic diagnosis, but also provide an insight into aberrant biochemical pathways that may reveal potential therapeutic targets, including nutritional targets, which could slow or stop disease progression well before any clinical symptoms manifest.

A nutritional approach to prevent, slow, or halt the progression of disease is a promising strategy that has been widely investigated. Several epidemiologic studies indicate that nutritional intake may have an impact on the development and progression of AD [343]. Modifiable, environmental causes of AD include potential metabolic derangements caused by dietary insufficiency [344, 345]. Furthermore, many nutritional supplements and dietary changes may directly influence the pathological contributions of increased oxidative stress, defects in mitochondrial dysfunction and cellular energy production, chronic inflammatory mechanisms, and even direct pathways to amyloid accumulation and neurofibrillary degeneration that contribute to the degenerative cascade in AD [346, 347].

Nutritional changes have the advantage of being cost effective, easy to implement, socially acceptable and generally safe and lack any significant adverse events in most cases. Many nutritional interventions have been studied and continue to be evaluated in hopes of finding a successful compound that can be used for the prevention and or treatment of AD [348].

There is much debate as to the type of antioxidant that may afford the most protection. Some antioxidants preferentially target cytosolic oxidative stress pathways whereas others preferentially serve as mitochondrial cofactors that may reduce intrinsic oxidative stress mechanisms [349]. Recent studies have shown that cytosolic antioxidants, such as vitamin E (α-tocopherol), can prevent AD-like changes in the brains of genetic AD mouse models [350]. Other antioxidants such as selegiline, that also inhibits oxidative deamination, have also shown neuroprotective properties in animal models of degenerative disease [351]. Treatment with selegiline (15mg twice daily) and vitamin E (1000IU twice daily) increased median survival by 215 and 230 days over the placebo [352]. Vitamin E should be taken in conjunction with vitamin C as a recharging antioxidant that maximizes the dose of vitamin E. Wheat germ, sunflower, and safflower oils, leafy green vegetables, and asparagus are among the best food sources of vitamin E. Apart from its effects in correlation with vitamin E, vitamin C (ascorbic acid) has been extensively studied for the prevention and treatment of AD [353]. Vitamin C is an essential vitamin that cannot be produced by humans from glucose or other substrates. Fortunately dietary sources of vitamin C are common and include citrus fruits, berries, and many vegetables that are a common part of most human diets worldwide. Several epidemiologic and cohort studies have investigated the association of vitamin C dietary intake and supplementation with AD and cognitive function in elderly humans [353].

Several studies have shown some of the advantages from mitochondrial cofactors that may function to reduce oxidative stress for example coenzyme Q10 (CoQ10) in reducing amyloid plaque deposition in mice models [354]. CoQ10 is part of the ubiquitin-proteasome complex pathway in the mitochondria and the cellular breakdown product disposal, which is suggested to contribute to amyloid plaque formation and accumulation [354]. Other molecules of recent interest include Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) which are major omega- 3 fatty acids [355]. The fatty acids like DHA and EPA allow for lipid raft formation across their unsaturated moieties to absorb the oxidative stress of free radicals and increase cell membrane fluidity necessary for lipid raft creation and formation of effective synaptic contacts [355]. Fish oil, rich in omega-3 fatty acids, when consumed regularly has been found to lower the incidence of AD in epidemiologic studies [355]. In addition to potential direct effects on neurodegeneration in AD, the benefits of omega-3 fatty acids for reducing cerebrovascular disease are widely recognized and may provide additional benefit in those suffering from AD, in whom cerebrovascular disease may be the most common comorbidity. Omega-3 fatty acids reduce circulating cholesterol levels, inhibit systemic inflammation in the circulatory system and vasculature, and inhibit platelet aggregation [355].

Development of PS2V blood biomarkers for Alzheimer's disease diagnosis

There has been an extensive search for AD-specific biomarkers over the past decade. Diagnosis of familial AD can be achieved by DNA sequencing and analysis of known genetic mutations that cause AD. These mutations occur in three genes encoding *APP, PSEN1*, and *PSEN2* [356]. An increased risk of sporadic AD is associated with environmental factors and genetic factors. Imaging techniques are relatively non-invasive, however, they are limited by availability and high cost. In addition, the accuracy of these techniques is still under debate [357-359].

For the growing AD population, the collection of blood by venepuncture is a simple, noninvasive, inexpensive and time-saving method. Thus, a blood-based biomarker would have more potential for routine screenings with repeatable measurements. This type of screening would provide a good chance for early detection, diagnosis and monitoring of the disease, and treatment. Peripheral blood has no direct contact with the brain and its delimitation by the blood-brain barrier (BBB) limits the usefulness of markers [360]. However, in humans, CSF is constantly exchanged and cleared *via* the blood [361] suggesting blood could reflect pathological changes in the brain and thus provides a good source of AD biomarkers. In plasma, serum and blood cells (i.e. erythrocytes, leukocytes, platelets) various proteins, lipids and other metabolic products can be examined. Plasma is a highly complex fluid with thousands of proteins available for potential biomarker evaluation. Several candidate biomarkers in blood and blood cells have been introduced, but their lack of sensitivity, specificity, and true relation to brain mechanisms remain unclear. Altogether, the discovery of a single blood-based biomarker in AD has thus far failed and further intense investigations are needed. We therefore set up a study to explore the possibility that the blood biomarkers that are associated with the pathology of AD such as PS2V which is capable of discriminating MCI group from Human Control (HC) group, AD group from HC group, or AD group from MCI group. In our preliminary result the blood PS2V biomarker was able to distinguish between MCI and HC groups. However, the blood biomarkers in the present research were not able to distinguish between ADI and HC groups due to small sample size.

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Appendix I

DIFFERENTIAL, DOMINANT ACTIVATION AND INHIBITION OF NOTCH SIGNALLING AND APP CLEAVAGE BY TRUNCATIONS OF PSEN1 IN HUMAN DISEASE

Newman, M., Wilson, L., Verdile, G., Lim, A., Khan, I., Moussavi Nik, S.H., Pursglove, S., Chapman, G., Martins, R.N. & Lardelli, M. (2014). Differential, dominant activation and inhibition of Notch signalling and APP cleavage by truncations of PSEN1 in human disease.
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Appendix II

IDENTIFICATION AND EXPRESSION ANALYSIS OF THE ZEBRAFISH ORTHOLOGUES OF THE MAMMALIAN MAP1LC3 GENE FAMILY

Ganesann, S., Moussavi Nik, S.H., Newman, M. & Lardelli, M. (2014). Identification and expression analysis of the zebrafish orthologues of the mammalian *MAP1LC3* gene family.
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