AGE-RELATED EFFECTS OF APOLIPOPROTEIN E GENOTYPES ON CHOLESTEROL METABOLISM AND INSULIN SIGNALING

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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13 Nov 2012

Summary

Apolipoprotein E (ApoE) plays an important role in the regulation of lipid metabolism in the hepatic and central nervous system (CNS). HuApoE4 carriers have been associated with higher peripheral cholesterol level and increased risk for atherosclerosis. Surprisingly, my data showed that huApoE4 has little impact on brain cholesterol metabolism. HuApoE4 isoform is also known to accelerate memory decline in ageing and certain neurological diseases but the molecular mechanism remains elusive.

Transgenic mouse models bearing phenotypic resemblance to human diseases are commonly used to study and dissect molecular relevant pathways. My data showed that an established mouse model for the human Niemann-Pick type C (NPC) disease exhibits varying ApoE present in its CNS. Extensive neurodegeneration and abnormal metabolic profiles in the CNS have been reported. My results showed that the PI3K/AKT signalling pathway was disrupted in the CNS of NPC mouse model. The PI3K/AKT pathway plays a key role in multiple cellular processes such as glucose metabolism and GSK3β activity.

Genetic modified huApoE3/E4 mouse models were used to study the effects of huApoE genetic polymorphism. These animals exhibited distinct glucose metabolic profiles and changes in the PI3K/AKT signalling pathway. Similar observations were made in the peripheral and CNS. These findings uncovered potential molecular pathways associated with glucose dysfunction. I have also noticed that most of these molecular changes were evident in older mice.

These strongly suggest that ageing plays an important role together with the huApoE. Collectively, my data suggests a novel role for huApoE in regulating brain insulin signalling.

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List of abbreviations

AD	Alzheimer's disease
AEBP1	AE binding protein 1
AKT	Protein kinase B
ApoE	Apolipoprotein E
ApoER2	ApoE receptor 2
BBB	Blood brain barrier
Cdk5	Cyclin-dependent kinase 5
CE	Cholesteryl esters
CNS	Central nervous system
CSF	Cerebrospinal fluid
FoxO1	Forkhead box O1
GluT	Glucose transporters
GS	Glycogen synthase
GSK	Glycogen synthase kinase 3
HDL	High-density lipoproteins
IGF1Rβ	Insulin growth factor 1 receptor-beta
IR	Insulin receptor
IRS	Insulin receptor substrates
LDLR	Low density lipoprotein receptor
LDLs	Low-density lipoproteins
LRP1	LDLR related protein 1
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Ras/mitogen-activated protein kinase

NPC	Niemann-Pick type C
PD	Parkinson disease
PDK1	3-phosphoinositide dependent protein kinase-1
PH-	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinases
PIP2	Phosphatidylinositol (4, 5)-bisphosphate
PIP3	Phosphatidylinositol (3, 4, 5)-trisphosphate
PKC	Protein kinase C
PL	Phospholipid
PTEN	Phosphatase and tensin homolog
RCT	Reverse cholesterol transport
RTK	Receptor tyrosine kinase
SH2-	Src homology 2
SNP	Single nucleotide polymorphism
T2DM	Type 2 diabetic mellitus
TG	Triglyceride
TNFα	Tumor necrosis factor-alpha
VLDLs	Very low-density lipoproteins

List of publications

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CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Apolipoprotein E (ApoE)

1.1.1 Characteristics of Apolipoprotein E

Human ApoE is located on chromosome 19 encoding a 35 kDa glycoprotein(Rall, Weisgraber et al. 1982) that exists in 3 isoforms, E2, E3 and E4 (Mahley 1988; Zannis, Kardassis et al. 1993). Although sequence analysis has shown that ApoE4 is the ancestral state in humans (Hanlon and Rubinsztein 1995), ApoE3 had increased in frequency through evolution as the most common isoform. This is followed by ApoE4 and ApoE2. They differ with a single amino acid at position 112 and 158 (Zannis, Breslow et al. 1982). These crucial amino acid changes alter the charge and stability of ApoE, contributing to its distinctive physiological functions (Table 1).

Table 1 Different ApoE isoforms and its allelic frequency in the population (Hauser, Narayanaswami et al. 2011).

ApoE3 is the most common allele among the population. ApoE2 and ApoE4 differ from ApoE3 by one amino acid at either 112 or 158 position.

	Isoform amino acid differences		Allelic Frequency			
				E2	E3	E4
ApoE2	Cys 112	Cys 158	E2	1-2%	~15%	1-2%
ApoE3	Cys 112	Arg 158	E3		~55%	~25%
ApoE4	Arg 112	Arg 158	E4			1-2%

ApoE belongs to a group of lipid carrier molecules that is vital in the cholesterol homeostasis of the body, both the peripheral and the central nervous system (CNS) (Brown and Goldstein 1986). It is one of the key constituent of lipoproteins that regulates the metabolism of lipids in the body

through ApoE receptors and related proteins. ApoE is widely expressed in various tissues with the highest expression in liver and brain. Emerging studies have suggested that its functions may extend beyond lipid metabolism to include maintenance of normal brain function and possible involvement in neurological diseases (Mahley and Rall 2000; Mahley, Weisgraber et al. 2009). Structural variations in ApoE isoforms might affect its preferential binding to lipoprotein receptors in the peripheral and CNS which in turn could potentially remodel the lipid metabolism and/or neuronal signalling respectively.

1.1.2 Functions of Apolipoprotein E

1.1.2.1 Peripheral system

Cholesterol is an essential component of cell structure and source of steroid hormones in the cell. The hydrophobic nature of cholesterol presents an obstacle to its distribution in the body. Henceforth robust and balance mechanisms are in place to maintain the homeostasis of cholesterol in the body. Lipid carriers such as apolipoproteins are deployed to package cholesterol into lipoproteins in order to be transported around the body.

Cholesterol is delivered to the peripheral tissues from the liver in the form of low-density lipoproteins (LDLs) and very low-density lipoproteins (VLDLs). The cell cholesterol requirements are met through in situ synthesis and absorption of VLDLs and LDLs. Excess cholesterol are subsequently excreted from the peripheral tissue in the form of high-density lipoproteins (HDLs) into the circulatory system and back to the liver. At the liver, the cholesterol are

released from the bile into the intestinal tract to be excreted as faeces or reabsorbed. This is known as the reverse cholesterol transport (RCT).

There are six major classes of apolipoproteins and our focus falls on ApoE. ApoE is an integral component of chylomicrons, VLDLs and HDLs in the peripheral system (Table 2). It operates as part of an anchoring mechanism that aids in the transport of triglyceride (TG), phospholipid (PL), cholesteryl esters (CE) and cholesterol into cells by mediating the binding and internalization of these lipoprotein particles. ApoE has a strong affinity and is the main ligand for members of the low density lipoprotein receptor (LDLR) family found on liver and other tissues. This super family includes the LDLR, LDLR related protein 1 (LRP1), VLDL receptor and ApoE receptor 2 (apoER2). Interaction of ApoE with LDLR mediates the removal of ApoE-containing lipoproteins and modulates the homeostasis of lipids in the peripheral system.

Table 2 Plasma lipoproteins containing ApoE (Smith, Pownall et al. 1978).

ApoE is present in three out of the four classes of lipoproteins in the peripheral system.

Properties	Chylomicrons	VLDL	LDL	HDL
Major apolipoproteins	ApoA-I ApoB ApoC	ApoB ApoC-I ApoC-II ApoC-III **ApoE**	ApoB	ApoA-I ApoA-II
Minor apolipoproteins	ApoA-II **ApoE**	ApoA-I ApoA-II ApoD	ApoC	ApoC-I ApoC-II ApoC-III ApoD **ApoE**

ApoE polymorphism has an influence in the plasma cholesterol level (Boerwinkle, Visvikis et al. 1987). Clinical studies have shown that ApoE4 is associated with higher plasma total cholesterol and LDL, followed by ApoE3 and ApoE2 (Ehnholm, Lukka et al. 1986; Eichner, Dunn et al. 2002). This is largely attributed with ApoE4 preferential binding to VLDL and ApoE3 to HDL (Nguyen, Dhanasekaran et al. 2010). Nonetheless, these observations may be challenged by individual's dietary fat intake and other lifestyle behaviours (Petot, Traore et al. 2003).

The importance of ApoE in lipid metabolism is asserted with the extensive accumulation of lipoproteins in the circulatory system of ApoE-null mouse (Plump, Smith et al. 1992). In addition, a Western high fat diet further elevates plasma cholesterol and aggravates atherosclerotic lesions (Zhang, Reddick et al. 1992). On the other hand, overexpression of ApoE in transgenic mouse drastically reduces plasma cholesterol and TG level, consequently eliminating diet-induced hypercholesterolemia (Shimano, Yamada et al. 1992).

1.1.2.2 Central nervous system (CNS)

The human brain contains up to 25% of cholesterol that is essential for myelin production, function and integrity. It is vital to maintain the cholesterol homeostasis in the CNS (Dietschy and Turley 2004; Saher, Brugger et al. 2005). The CNS cholesterol is independently regulated from the peripheral system. It is believed to be mainly synthesized by glial cells for the neurons (Quan, Xie et al. 2003). The process to synthesize cholesterol is energy intensive and thus it is more efficient for the astrocytes to carry out this role

(Brecht, Harris et al. 2004; Xu, Bernardo et al. 2006). Cholesterol dysfunction in the CNS has been associated with ageing and the development of certain neurodegenerative diseases such as AD, Parkinson disease (PD) and NPC (Simons and Ehehalt 2002; Karten, Hayashi et al. 2005).

The blood brain barrier (BBB) restricts the exchange of lipoproteins and ApoE between the central nervous and peripheral systems. Henceforth, the lipid composition and regulation in both systems function independently. In the CNS, lipoproteins are primary synthesized by glial cells (Roheim, Carey et al. 1979; Pitas, Boyles et al. 1987) and ApoE may play a pivotal role in the transportation of these packages (Boyles, Zoellner et al. 1989; Goodrum 1991). An early study had shown that injury to the brain resulted in increased ApoE protein in the brain (Poirier, Hess et al. 1991). It was proposed that ApoE-containing lipoproteins were taken up by ApoE receptor-rich neurons for repair (Ignatius, Gebicke-Harter et al. 1986; Boyles, Zoellner et al. 1989).

HuApoE isoforms have been associated with risks to neurological diseases (Corder, Saunders et al. 1993; Saunders, Strittmatter et al. 1993; Roses 1996; Ashford 2004, Raber, 2004 #296). Other than huApoE isoforms, differential expression level of huApoE proteins in the CNS is also linked to neurological symptoms such as NPC and AD (Ramaswamy, Xu et al. 2005; Riddell, Zhou et al. 2008; Sullivan, Han et al. 2011). More recently, there have been two reports suggesting that brain huApoE regulates the clearance of Aβ which is a

common hallmark for some neurological diseases, such as Alzheimer's disease (Kim, Jiang et al. 2011; Bien-Ly, Gillespie et al. 2012).

1.2 Glucose metabolism in the CNS

Glucose is the most common source of energy for the body and it is important to maintain its regulation. In the peripheral system, an overdose of blood glucose may lead to diabetes mellitus while low blood glucose can result in hypoglycaemia (Weyer, Bogardus et al. 1999; Schwartz and Porte 2005). Abundant amount of blood glucose will lead to increase metabolism in the pancreatic β cells. This leads to an elevation of insulin and kicks off a series of insulin signalling linked pathways.

1.2.1 Glucose hypometabolism in diabetic and Alzheimer's disease patients

Type 2 diabetic mellitus (T2DM) patients typically suffer from glucose dysfunction due to impairments in the insulin signalling pathway (Niswender, Morrison et al. 2003; Plum, Belgardt et al. 2006). The human brain consumes up to 30% of the total body glucose, thus glucose regulation plays an important role in the CNS. Any disruption to glucose regulation may affect the health of the CNS. It is widely recognized that hypometabolism occurs in certain regions of the brain for AD patients and also elderly population (Mosconi, Sorbi et al. 2004; Samuraki, Matsunari et al. 2012). It is still unclear if hypometabolism is associated to neurodegeneration in the CNS. (Jack, Knopman et al. 2010).

Emerging studies in human and animal populations support the notion that lower brain glucose metabolism may be indicative of cognitive declination later in life (Reiman, Caselli et al. 1996; Drzezga, Riemenschneider et al. 2005; Reiman, Chen et al. 2005; Caselli, Dueck et al. 2009; Kalpouzos, Chetelat et al. 2009). It is also noteworthy that most of these studies have associated ApoE4 as a major genetic risk factor for AD with glucose dysfunction in the CNS.

Epidemiological studies have shown that T2DM is a risk factor for memory and learning impairment diseases such as AD (Gispen and Biessels 2000; Kopf and Frolich 2009; Sims-Robinson, Kim et al. 2010). In line with these, a recent clinical publication cited significantly decrease in the activity of PI3K-AKT signalling pathway in T2DM and AD patients. This may potentially lead to activation of glycogen synthase kinase-3β (GSK3β), the major tau kinase (Liu, Liu et al. 2011).

It is also largely believed that glucose transporters 1, 3 and 4 (GluT1, GluT3 and GluT4) which are found in the CNS may be reduced or desensitized to the effects of insulin. This subsequently leads to lower brain glucose metabolic rate (Hooijmans, Graven et al. 2007). In light of several other evidences (Schubert, Gautam et al. 2004; Watson and Craft 2004; Cole and Frautschy 2007), the insulin signalling pathway is likely to be defective in neurological diseases.

1.2.2 Role of insulin in the central nervous system

Insulin is a hormone that is responsible for the regulation of the blood glucose level in the body. It consists of 51 amino acids and is produced by the β-cells in the islets of Langerhans of the pancreas. It was believed that pancreas was the sole source of insulin for the body and it can be transported across the blood brain barrier to the brain only via specialized insulin transporters (Banks 2004). Recently, more reports seem to favour the idea that the brain itself is also capable of synthesizing insulin and this is associated with the survival of brain cells (Devaskar, Giddings et al. 1994; Steen, Terry et al. 2005). Brain insulin is responsible for the regulation of food intake and body weight (Schwartz, Baskin et al. 1999). It has also has been shown to regulate neural development and possibly cognition related functions (Zhao, Chen et al. 1999; Zhao and Alkon 2001; Gerozissis 2008).

Insulin receptor (IR) belongs to the receptor tyrosine kinase (RTK) family and consists of two (α- and β-) subunits. The alpha subunit of 135 kDa forms the extracellular ligand binding protein, while the beta subunit of 95 kDa contains the kinase catalytic domain (Taylor, Cama et al. 1992; De Meyts and Whittaker 2002). These subunits form a tetrameric structure held together by disulphide bonds and span across cell plasma membrane (Olefsky 1990). When insulin binds and activates the alpha subunit, it triggers a rapid autophosphorylation of IR. This is followed by a cascade of phosphorylation events that leads to different biological functions.

Insulin receptor is widely expressed in the brain with higher concentration in the olfactory bulb, cerebral cortex, hypothalamus and hippocampus. The expression of IR appears to be developmentally regulated, with higher expression in the early stage and declines with age. It is also noteworthy that IR is highly expressed in neurons relative to the glial cells (Havrankova, Roth et al. 1978; van Houten, Posner et al. 1979; Werther, Hogg et al. 1987). In the CNS, both the peripheral and brain IR can be found in the glial and neuronal cells respectively (Adamo, Lowe et al. 1989). Brain IR has a lower molecular weight as to peripheral IR due to alternate splicing that results in the deletion of its exon 11, otherwise they are largely similar (Heidenreich, Zahniser et al. 1983; Wozniak, Rydzewski et al. 1993)

Since the discovery of IR in the brain by use of ligand autoradiography 30 years ago (Havrankova, Roth et al. 1978), researchers have attempted to understand the intracellular molecular mechanisms and proteins involved in insulin signalling. Valuable information gathered on these pathways served as a database for pharmaceutical companies to identify potential therapeutic targets.

1.2.3 PI3K/AKT signalling pathway in the central nervous system

Upon activation of IR, the tyrosine residues within the β-subunit are phosphorylated and target insulin receptor substrates (IRS) (White 1997). Generally, the two main pathways that are activated by insulin receptor include the PI3K-protein kinase B (AKT) and the Ras/mitogen-activated protein kinase (MAPK) pathways. Our research focus will remain with PI3K-

AKT and it will be further elaborated. Unlike most receptor tyrosine kinases, IRS serves as accessory platform for src homology 2 (SH2-) domain containing molecules, such as phosphatidylinositol 3-kinases (PI3K) (White 2002). This cascade of events plays in important part in the insulin signalling pathway. Defective IRS has been observed in skeletal muscle of obese and type 2 diabetes patients (Danielsson, Ost et al. 2005).

With the recruitment of PI3K, it produces phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) from phosphatidylinositol (4, 5)-bisphosphate (PIP2). Dysregulation of IRS and PI3K have been observed in the brain samples of post mortem AD patients (Moloney, Griffin et al. 2008). This further strengthens the link between neurological disease and insulin/IR activation. Subsequently, PI3K kicks off another round of events that involves the serine/threonine phosphorylation of pleckstrin homology (PH-) domain containing proteins which include 3-phosphoinositide dependent protein kinase-1 (PDK1), AKT and protein kinase C (PKC) (Alessi and Downes 1998). Phosphorylated AKT (p-AKT) has been actively linked to several glucose associated functions.

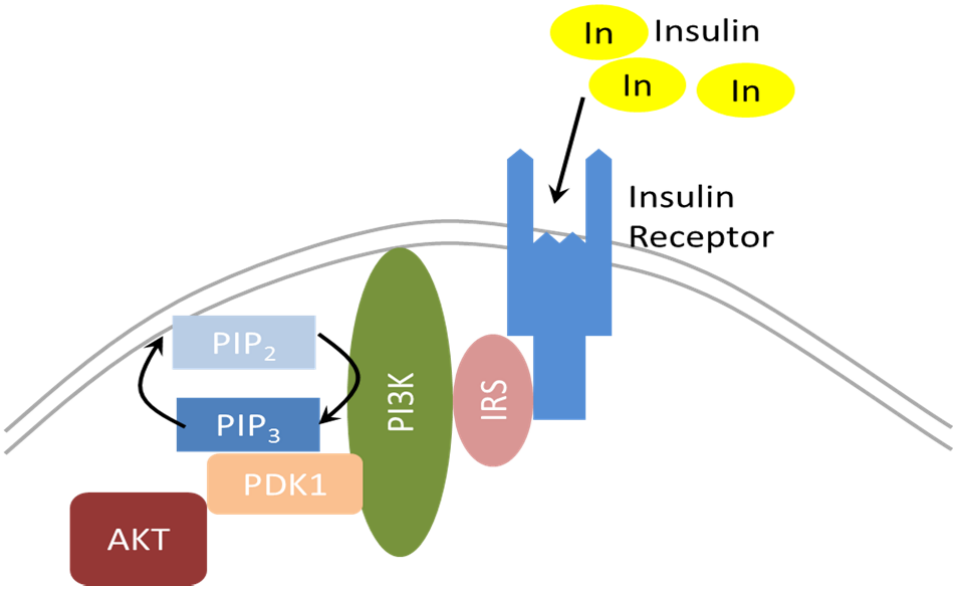

Figure 1 Schematic diagram of insulin driven AKT activation

Insulin binds to extracellular alpha subunit of insulin receptor and triggers autophosphorylation the tyrosine residues within the β-subunit of insulin receptor. This leads to the activation of IRS. Following the recruitment of PI3K, these proteins facilitate the production of PIP3 from PIP2. This protein structure then engage PDK1 leading to the activation of AKT.

p-AKT targets GSK3 (Glycogen synthase kinase 3) (Cross, Alessi et al. 1995) and AS160 (Kane, Sano et al. 2002) which are involved in glycogen synthesis and translocation of glucose transporter to the plasma membrane respectively (Sano, Kane et al. 2003). GSK3 activity is inhibited when it is phosphorylated at Ser21 and Ser9 of GSK3α and GSK3β respectively. GSK3 activity is also associated with the phosphorylation of glycogen synthase (GS) and glycogen synthesis activity (Brady, Bourbonais et al. 1998). Phosphorylated AKT has also been shown to mediate glucose synthesis through the inhibition of FoxO1 (Forkhead box O1) (Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999). Majority of these information are collected from the peripheral system, it is presumed that the insulin signalling pathway in the CNS functions in a similar manner. Insulin activation triggers several events and proteins, however only a

segment has been briefly elaborated. Insulin signalling dysfunction may be the result of disruption at any one or more points in the pathway.

1.2.4 Insulin affects cognitive performance

The association between diabetes and cognitive impairment in human has been well documented (Perlmuter, Hakami et al. 1984; Yoshitake, Kiyohara et al. 1995; Curb, Rodriguez et al. 1999; Gispen and Biessels 2000; Bruce, Casey et al. 2003; Munshi, Grande et al. 2006). These data include epidemiological studies highlighting hyperinsulinemia as a risk factor for dementia (Ott, Stolk et al. 1999; Biessels, Staekenborg et al. 2006). Clinical investigation showed improvement in cognitive impaired patients that underwent with insulin therapy but these positive effects diminished with prolong usage. However, this study is sufficient to demonstrate that the insulin signalling pathway is involved in memory and learning (Kern, Peters et al. 2001; Van den Berghe, Schoonheydt et al. 2005; Reger, Watson et al. 2008). Interestingly, elderly patients with higher insulin levels have higher risk to develop cognitive impairment later in life (Stolk, Breteler et al. 1997; Stolk, Pols et al. 1997). Significantly lower CSF (Cerebrospinal fluid) insulin levels were measured in AD patients in contrast to healthy patients (Craft, Peskind et al. 1998). Reduced mRNA and protein levels of insulin have also been reported in patients suffering from neurodegenerative disorders such as AD (Steen, Terry et al. 2005; Zhao, De Felice et al. 2008). These observational reports suggest links between insulin level and cognitive performance, however the molecular details of this relationship remains elusive.

Several *in-vitro* studies have been conducted to support earlier clinical observations. The addition of insulin protects hippocampal neurons against Aβ induced cytoxicity (Takadera, Sakura et al. 1993; Rensink, Otte-Holler et al. 2004; De Felice, Vieira et al. 2009). Insulin has also been shown to compete with Aβ for insulin receptors in neuronal cells. This process results in a decrease in the IR/PI3K/AKT signalling pathway and prevents Aβ from damaging the neurons (Xie, Helmerhorst et al. 2002; De Felice, Vieira et al. 2009). Parallel to clinical injection of insulin to the CNS, insulin was able to promote dendritic spine formation in primary culture studies . This is possibly via the insulin-induced AKT signalling pathway (Lee, Huang et al. 2011). The molecular mechanism underlying these observations are not clearly understood but it is likely to involve the PI3K/AKT signalling pathway. In summary, the balance of insulin plays an important role in cognitive performance, particularly in elderly. Other parameters such as the regulation and metabolism of glucose in the CNS may contribute to these insulin-associated effects.

1.3 Apolipoprotein E and neurological diseases

1.3.1 Alzheimer's disease

Alzheimer's disease is a widespread neurodegenerative illness and also the leading cause of dementia. Currently AD affects approximately 40% of the population over 80 years of age and the loss of memory can be very costly to the psychological and economic health of the society. This is especially prevalent in developed countries with higher life expectancy. The estimated health cost of AD in US is set to exceed 100 billion dollars (Ernst and Hay 1994) and this is a growing problem in the fast-aging society of Singapore.

The most prominent symptom of AD is declination of recent memory. As the disease develops, other cognitive abilities such as language, movement and sightedness begin to deteriorate. The disease eventually results in global cognitive decline. The classical hallmarks of AD include extracellular amyloid plaques consisting of amyloid beta aggregates and intracellular neurofibrillary tangles of hyperphosphorylated tau protein (Morris 1997). Clinical diagnosis of AD is still in its infancy and there is a lack of established and non-invasive approaches to accurately determine the severity or progression of the illness. Existing cognitive performance tests are only able to determine the severity of the cognitive impairment. To date, confirmed AD patients can only be identified through post-mortem brain pathology.

In addition to plaques and tangles, correlation studies have identified ApoE as a major risk factor and the most important genetic factor to AD (Corder, Saunders et al. 1993; Saunders, Strittmatter et al. 1993; Roses 1996; Ashford

2004, Raber, 2004 #296). These associations are supported by population studies manifesting severe cognitive declination with the presence of ApoE4 allele (Jonker, Schmand et al. 1998). However, there is still much discrepancy on the potential risks that ApoE4 carriers are exposed to. They include earlier onset of AD (Blacker, Haines et al. 1997; Meyer, Tschanz et al. 1998; van der Vlies, Koedam et al. 2009), higher rate of developing AD (Kivipelto, Helkala et al. 2001; Whitmer, Sidney et al. 2005) and/or accelerated rate of progression of AD (Saunders, Strittmatter et al. 1993).

Other than polymorphic differences in ApoE, it is widely agreed that the expression level of ApoE may be associated to AD. Earlier evidences indicate that huApoE4 protein is less stable as compared to huApoE3 protein (Huang, Liu et al. 2001). This instability contributes to lower huApoE4 protein in the body due to degradation. This is supported with observations in patients (Eto, Watanabe et al. 1986; Gregg, Zech et al. 1986) and animal models (Riddell, Zhou et al. 2008).

Interestingly, conflicting clinical data of ApoE level in the CNS have been published. No distinct ApoE level change has been observed in patients' CNS (Lehtimaki, Pirttila et al. 1995; Landen, Hesse et al. 1996; Lefranc, Vermersch et al. 1996; Pirttila, Soininen et al. 1996; Lindh, Blomberg et al. 1997). Significantly lower (Bertrand, Poirier et al. 1995; Beffert, Cohn et al. 1999) and higher levels (Harr, Uint et al. 1996; Lambert, Perez-Tur et al. 1997; Lindh, Blomberg et al. 1997; Fukumoto, Ingelsson et al. 2003; Bray, Jehu et al. 2004; Sihlbom, Davidsson et al. 2008) of ApoE were also observed in the

CNS of ApoE4 carriers. The varying ApoE level in these clinical samples may be due to different sample preparation methods or the specific regions that were studied.

In spite of all these, ApoE4 allele is not sufficient for the development of AD but more of a supportive role in the disease progression. A multitude of factors contributes to AD, they include gender, age, amyloid beta and other related diseases such as atherosclerosis and type 2 diabetes. Since the genetic impact of ApoE4 is not the principle and sole factor, it is not practical as a form of diagnosis for AD. In summary, ApoE is classified as a risk factor for AD but the molecular events that precede dementia remain elusive.

1.3.2 Niemann-Pick type C (NPC) disease

NPC is an inherited autosomal recessive disorder caused by a failure in cholesterol trafficking due to a mutation in NPC1 (95% of cases) or NPC2 protein. NPC1 is a large transmembrane protein of 1278 amino acids. It is localized to the late endosomal membrane and has been associated with cholesterol trafficking (Higgins, Davies et al. 1999; Wiegand, Chang et al. 2003). NPC patients exhibit accumulation of unesterified cholesterol and other lipids in the peripheral tissues, particularly in the liver and spleen (Beltroy, Richardson et al. 2005). The lipid accumulation results in neonatal jaundice and liver enlargement which can lead to acute liver failure.

NPC1 deficiency in patients have little effect on the plasma cholesterol whereby only an increase in the plasma triglyceride was recorded (Shamburek,

Pentchev et al. 1997; Garver, Jelinek et al. 2009). Oddly, the CNS is uniquely spared from similar lipid accumulation and a significant reduction in the cholesterol is observed (Vanier 1999). As the disease progresses, patients further develop extensive neurodegeneration of the cerebellum, especially in the thalamus and the purkinje cell layer (Vanier and Millat 2003). Since no effective treatment is available for NPC patients, death typically occurs in their teenage years. It is interesting to note that both AD and NPC bear strong pathological resemblances such as neurofibrillary tangles, tau pathology and increased Aβ generation. (Nixon 2004).

It has been reported that NPC patients with an ApoE4 allele suffer from an accelerated form of NPC symptoms (Saito, Suzuki et al. 2002). Notable increase in the expression of ApoE was also observed in the CNS of NPC transgenic mice (Burns, Gaynor et al. 2003; Li, Repa et al. 2005). These data suggest that ApoE may have a role in dysregulation of cholesterol and indirectly associated with the progression of neurodegeneration in NPC patients.

CHAPTER 2

MATERIALS AND METHODS

2 Materials and methods

2.1 Animal models

Experimental protocols involving the maintenance and euthanasia of laboratory mice were in accordance with guidelines approved by the Institutional Animal Care and Use Committees (IACUC) at the National University of Singapore.

Mice used in the NPC study were homozygous mutant BALB/cNctr-Npc1m1N/J (NPC^{NIH}) mice (Loftus et al. 1997) from Jackson Immuno-Research (West Grove, PA, USA) and their wildtype littermates as controls. Homozygous NPC mice developed neurological abnormalities at 6–7 weeks of age and died within 10–12 weeks of age. Both homozygous NPC^{NIH} and control mice were euthanized. Brains were harvested at week 5 and 9, corresponding to before and after the onset of neuropathology.

Mice used in the ApoE study were homozygous mutant B6.129P2-Apoetm3 (APOE*3) Mae N8 mice and B6.129P2-Apoetm3 (APOE*4)Mae N8 mice with defined C57BL6/J background (Knouff C et al. 1999) from Taconic Farms, Inc (Germantown, NY, USA). Homozygous ApoE mice developed abnormal serum lipid profiles under high fat diet according to the huApoE isoform which is expressed in the animal. The animals were raised up to 72 weeks. Both mouse models were euthanized with accordance to IACUC guidelines. They were harvested at 12^{th}, 32^{nd} and 72^{nd} week to study the effects of huApoE genotype with relevance to ageing. $n = 5$ were used from each time point in each animal group for all analysis, where possible.

Animals were fasted for about 4 hours or more prior to harvest. About 0.7 mL of mouse anesthesia from Animal House Unit (AHU) was used via intraperitoneal injection. Cardiac puncture were performed with 22G needle and the blood were dispensed into EDTA tubes. The blood tubes were centrifuged at 1,000 x *g* for 10 minutes. The plasma were collected and stored at -80℃. The liver, left and right brains were harvested and flushed with sterile PBS. They were subsequently stored under -80℃ separately.

2.2 Preparation of brain homogenates

The whole left hemisphere of the mouse brain was snapped frozen in liquid nitrogen. The wet weight of the tissues (in mg) was measured and 1X cell lysis buffer (Cell Signalling Technology, Danvers, USA) with Roche protease and phosphatase inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, IN, USA). The tissue was prepared at 20% (weight: volume) ratio.

The mix was then homogenized with a handheld homogenizer with 3 pulses of 20 seconds each, with 10 seconds interval on ice to minimize heat degradation to proteins. These tissue lysates were then placed on ice for 30 minutes before centrifuging them at 6,000 x *g* for 5 minutes at 4℃. The soluble portions of the lysates were harvested while the insoluble portions were stored at -80℃ freezer. The soluble lysates were further distributed into aliquots to minimize freeze-thaw over the period of usage.

2.3 Preparation of liver homogenates

The whole liver of the mouse was harvested and snapped frozen in liquid nitrogen. The wet weight of the tissues (in mg) was measured and 1X cell lysis buffer (Cell Signalling Technology, Danvers, USA) with Roche protease and phosphatase inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, IN, USA). The tissue was prepared at 20% (weight: volume) ratio.

The mix was then homogenized with a handheld homogenizer with 3 pulses of 20 seconds each, with 10 seconds interval on ice to minimize heat degradation to proteins. These tissue lysates were then placed on ice for 30 minutes before centrifuging them at 6,000 x *g* for 5 minutes at 4℃. The soluble portions of the lysates were harvested while the insoluble portions were stored at -80℃ freezer. The soluble lysates were further distributed into aliquots to minimize freeze-thaw over the period of usage.

2.4 Protein quantification of lysates

PierceTM MicroBCA Assay kit (Thermofisher Scientific, Waltham, USA) was used to quantify the protein concentration of tissue lysates (2 mg lysed in 100 µl lysis buffer). Lysates from brain and liver tissues were diluted with PBS by 50 and 150 folds respectively. 25 µL of diluted samples alongside with the BSA standards were pipetted into microplate wells in duplicates. 200 µL of working reagent was added to each well and incubated for 30 minutes at 37℃ before measurements were taken at 562 nm on a Tecan microplate reader.

Protein concentrations of samples were then calculated based on the standard curve constructed from BSA standards.

2.5 SDS-PAGE and Western blot analysis

The final tissue lysates were quantitated and adjusted to 30 - 70 µg of proteins per lane with PBS. These protein samples were added with 4X loading buffer (42 µL) and subjected to heating at 95°C for 5 minutes. These protein samples were then loaded onto a 7.5 - 10% Tris-glycine polyacrylamide gel. 5 µL of Precision Plus ProteinTM standard (Bio-Rad Laboratories, Hercules, California USA) was used as a molecular weight standard and ran alongside with samples in individual lanes.

Gel resolution was performed in Mini-PROTEAN Tetra electrophoretic system (Bio-Rad Laboratories). The stacking gel was subjected to 60-90 V for approximately 30 minutes, after which the resolving gel was subjected to 90-120 V until the dye front had reached the bottom of the protein gel.

The gel was removed and the proteins were transferred onto a nitrocellulose membrane using Mini Trans-Blot cell (Bio-Rad Laboratories, Hercules, California USA) either overnight at 20V or at 110V for 60 mins. The transfer efficiency was verified with Ponceau S (Sigma-Aldrich, St-Louis, USA) staining and rinsed 3 times with PBSt (PBS with 0.1% Tween 20) to wash off Ponceau S stains. The blot was blocked with 5% non-fat milk in PBSt for 30 minutes with gentle agitation and rinsed twice with PBSt, with agitation of 5 minutes each time.

The blot was then incubated with the intended primary antibody overnight, with constant gentle agitation. The HRP-conjugated secondary antibody (dissolved in 3% non-fat milk in PBSt) was introduced after the blot has been rinsed 3 times with PBSt, and left to incubate for 1 hour under gentle agitation. The blot was then rinsed with PBSt 3 times and intended bands were visualized using chemiluminescence method with either SuperSignal West Dura or SuperSignal West Femto (ThermoFisher Scientific, Waltham, USA) based on the protein abundance and sensitivity of the substrate.

The blot was stripped with Restore Western Blot stripping buffer (ThermoFisher Scientific, Waltham, USA) and blocked with 5% non-fat milk in PBSt prior to incubation with anti-actin primary antibody and the respective secondary antibody for final visualization,

All blots were developed using Image Station 4000R (Carestream Health Inc, New York, USA).

Table 3 Primary antibodies used in immunoblotting analysis.

Table includes the name of the targetted protein and its size. The animal source, name of the commercial company, catalog number and the dilution factor used are also provided.

Protein target	Size	Ab source		Ab coy	Ab cat no.	Dilution
Actin	42kDa	Mouse	Monoclonal	Sigma	A5316	15,000X
Actin	42kDa	Rabbit	Monoclonal	Sigma	A2066	2,000X
AEBP1	103kDa	Rabbit	Polycolonal	Abbiotec	250461	200X
AKT	60kDa	Rabbit	Monoclonal	Cell Signaling Technology	4691	1000X
GluT1	45 + 55kDa	Rabbit	Polycolonal	Millipore	07-1401	500X

GluT3	46 + 55kDa	Rabbit	Polycolonal	Millipore	1344	1000X
GluT4	50kDa	Mouse	Monoclonal	Cell Signaling Technology	2213	1000X
GSK3β	46kDa	Rabbit	Monoclonal	Cell Signaling Technology	9315	1000X
huApoE	36kDa	Mouse	Monoclonal	Santa Cruz	13521	200X
IGF-I Receptor β	95kDa	Rabbit	Monoclonal	Cell Signaling Technology	3018	1000X
Insulin Receptor β	95kDa	Mouse	Monoclonal	Cell Signaling Technology	3020	1000X
IRS1	180kDa	Rabbit	Monoclonal	Cell Signaling Technology	3407	1000X
IRS2	180kDa	Rabbit	Monoclonal	Cell Signaling Technology	3089	1000X
p-AKT (Ser473)	60kDa	Rabbit	Monoclonal	Cell Signaling Technology	4060	1000X
p-AKT (Thr308)	60kDa	Rabbit	Monoclonal	Cell Signaling Technology	2965	1000X
PDK1	58-68kDa	Rabbit	Polycolonal	Cell Signaling Technology	3062	1000X
Phospho-GSK-3β (Ser9)	46 kDa	Rabbit	Monoclonal	Cell Signaling Technology	9323	1000X
PI3Kp110	110kDa	Rabbit	Monoclonal	Cell Signaling Technology	4249	1000X
PI3Kp85	85kDa	Rabbit	Monoclonal	Cell Signaling Technology	4257	1000X
p-IGF1R / p-IRb	95kDa	Rabbit	Polycolonal	Cell Signaling Technology	3021	1000X
p-PDK1 (Ser241)	58-68kDa	Rabbit	Polycolonal	Cell Signaling Technology	3061	1000X
PTEN	54kDa	Mouse	Monoclonal	Cell Signaling Technology	9556	1000X

Primary antibodies from Sigma-Aldrich (St Louis, MO, USA), Merck-Millipore (Massachusetts, USA), Abbiotec (San Diego, CA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, USA) were used for the experiments. The respective catalogue number and dilution factor for immunoblotting assays are stated above.

Secondary antibodies used include: HRP-conjugated goat anti-mouse IgG (Millipore, Massachusetts, USA) and HRP-conjugated goat anti-rabbit IgG (Millipore, Massachusetts, USA). Secondary antibodies were used at a dilution factor of 1: 5, 000 for immunoblotting assays.

Western blots were analyzed using ImageJ which is available under http://rsb.info.nih.gov/ij/. The method of analyzing the Western blot images using ImageJ was featured under http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. This was followed by statistical analysis of the results using the Student's t-test.

2.6 Amplex red glucose assay

The amplex red glucose kit (Life Technologies, Carlsbad, California, USA) was used to determine the glucose content in biological samples. Glucose standards ranging from 0 to 100 μM were used to establish a standard curve. 50 μL of amplex red working solution reagent was added to 50 μL of neat tissue lysate samples and incubated for 30 minutes at room temperature in the dark. The samples were then measured at excitation of 545 nm and emission of 590 nm using a Tecan microplate reader, M200 infinite series (Tecan Group, Mannedorf, Switzerland). Values from no glucose controls were used to correct background fluorescence. Glucose content of samples were then determined in accordance to the kit instruction.

2.7 Amplex red cholesterol assay

The amplex red cholesterol assay kit (Life Technologies, Carlsbad, California, USA) was used to determine the cholesterol content in biological samples. Tissue lysates were diluted 400-folds in the assay buffer prior to analysis. Cholesterol standards ranging from 0-8 μg/ml was used to establish a cholesterol standard curve. 50 μL of working solution (Amplex Red reagent/HRP/cholesterol oxidase/cholesterol esterase) was added to 50 μL of samples/standards. The mixture in 96 microplate format was then incubated for 30 minutes at 37℃ in the dark. The plate was then read at excitation wavelength of 560 nm and emission wavelength of 590 nm using a Tecan microplate reader, M200 infinite series (Tecan group, Mannedorf, Switzerland). Cholesterol content of samples was determined in accordance to the kit instruction.

2.8 Insulin ELISA

The rat or mouse insulin ELISA kit (Millipore, Massachusetts, USA) was used for quantification of insulin. 10 μL of diluted protein lysates along with 7 insulin samples were added to prewashed wells containing 10 μL of assay buffers. 80 μL of detection antibodies was added and the mix was incubated at room temperature for 2 hours under 450 x *g*. The wells were then washed thrice before the addition of 100 μL of substrate. Readings at absorbance of 370 nm were measured after 15 minutes of incubation and constantly measured at 1 minute intervals using a Tecan microplate reader. 100 μL of stop solution was then added when one of the absorbance readings hits 1.8.

The plate was then read at 450 nm and 590 nm. The insulin content of the samples was then determined in accordance to the kit instruction.

2.9 Real-time PCR analysis

2.9.1 Isolation of total RNA

RNA extraction for tissues was done using TRIzol® reagent (Life Technologies, Carlsbad, California USA). In general, less than 50 mg of brain tissue was used which was then homogenized in 500 µL of TRIzol® reagent with a handheld homogenizer (ThermoFisher Scientific, Waltham, USA). To ensure complete lysis of tissue, the resulting lysate was further homogenized by 10 passages through a 22 G needle. These lysates were then processed following manufacturer's instructions provided in the material datasheet up to the RNA precipitation stage.

The precipitated RNA fraction was subjected to an additional clean-up step using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA samples were typically eluted twice in 40 µL of RNase free water provided in the kit before they were quantified using the Nanodrop™ 2000. The ratio of the absorbance at 260 and 280nm (A260/280) is used to assess the purity of nucleic acids. Pure RNA A260/280 is ~2.

2.9.2 Reverse transcription of RNA

RNA samples were reverse transcribed using the Reverse Transcription system (Promega, Madison, USA) with oligo dTs. Typically, 1 or 2 µg of RNA was processed each time. The RNA was first heated at 70°C for 10 minutes and placed on ice before mastermix containing reverse transcriptase

was added. The resulting mixture was incubated at 42°C for 20 minutes to allow for cDNA synthesis. Lastly, the cDNA was heated at 95°C for 5 minutes and placed on ice for another 5 minutes to inactivate the reverse transcriptase enzyme.

2.9.3 Real-time PCR

Each real-time PCR reaction was performed using 50 ng of cDNA. Samples were ran in duplicates in volumes of 20 µL each. Specific TaqManR probes (Life Technologies, Carlsbad, California USA) were used for the detection of various gene products. A typical reaction setup consists of the following components:

Component	Volume (µL)
2× TaqMan® Gene Expression Master Mix	10 uL
20× TaqMan® probe	1 uL
Nuclease free water	7 µL
cDNA	50 ng
Total volume	20 µL

Reactions were then run in a 96-well format on a StepOnePlus™ Real-time PCR system (Life Technologies, Carlsbad, California USA) using the default cycling conditions. For each real-time PCR, a minimum of n=3 set of samples was used and each sample run in duplicates to ensure accuracy. Statistical analysis of the results was done using the Student's t-test.

2.9.4 TaqMan® probes

The expression level of the following genes was investigated using real-time quantitative PCR and TaqMan probe-based chemistry (Life Technologies, Carlsbad, California USA).

a. Human Apolipoprotein E (Hs00171168_m1).
b. Mouse Actin (Mm00607939_s1)

These probes span the exon(s) of the targeted genes and the assays were performed according to the manufacturer's instructions.

CHAPTER 3

IMPAIRED LIPID METABOLISM AND INSULIN SIGNALLING IN NIEMANN-PICK TYPE C ANIMAL MODEL

3 Impaired lipid metabolism and insulin signalling in Niemann-Pick type C animal model

3.1 Introduction

3.1.1 Apolipoproteins and cholesterol linked diseases

Apolipoproteins are the key constituents of cholesterol-rich lipoproteins, hence several studies have been done in this area. Cholesterol dysfunction has been associated with cardiovascular diseases, Niemann-Pick type C and metabolic syndrome. Clinical studies have shown that NPC patients carrying ApoE4 allele has an accelerated accumulation of phosphorylated tau in neurons (Saito, Suzuki et al. 2002). In principle, their data suggested a genotype- and age-dependent elevation in the expression of ApoE and ApoD (Burns, Gaynor et al. 2003; Li, Repa et al. 2005) with NPC. The lab found a similar increase in ApoE at 9 week but not 5 week old mice. Furthermore, glycosylation modification in ApoE had also been associated to the pathological symptoms of these animals (Chua, Lim et al. 2010).

ApoE and ApoD are major lipid carriers in the CNS. Increased expression of ApoE (Iwata, Browne et al. 2005; Karten, Hayashi et al. 2005; Singh and Thakur 2010) and ApoD (Franz, Reindl et al. 1999) has long been associated with brain injury and neuronal repair. It is presumed that the up regulation of these apolipoproteins is part of the body's attempt to repair damaged cells by transporting essential cholesterol to the site. However, little data was able to verify if these observations were the causes or consequences of neurodegeneration. Alternatively, the increment in the expression of ApoE may have been triggered to accelerate the removal of excess cholesterol

present in the cells. Nevertheless, most literature agreed that ApoE does play a significant role in the disease progression NPC^{NIH} mouse. In view of this association, several groups went on to explore the effects of apolipoprotein profiles in the CNS of NPC^{NIH} mice.

3.1.2 NPC transgenic mouse model

Transgenic mouse models with vast phenotypic resemblance to human diseases are commonly used to study and dissect relevant molecular pathways. BALB/cNctr-Npc1m1N/J (NPC^{NIH}) is an established model for the human NPC genetic disorder and it has been widely used over the decade. Mice homozygous for the recessive NIH allele of the Niemann Pick type C1 gene results in premature truncation of the protein.

The average lifespan of NPC^{NIH} mice is about 75 days (Sarna, Larouche et al. 2003). By 5 week old, they develop ataxia and other neurological symptoms similar to those of the human NPC disease. NPC^{NIH} mice suffer from hypercholesterolemia and accumulation of cholesterol in most major organs with exception to the brain (Xie, Turley et al. 1999; Xie, Burns et al. 2000). At 7 week old, gradual losses in myelin cholesterol and brain weight are evident, thus reflecting extensive brain dystrophy (Vance 2006).

Conventional ApoE transgenic mouse models are usually complete ablation or target-replacement of the gene with different human isoform. These animal models do carry some similarities such as cholesterol dysfunction and learning impairment (Raber, Wong et al. 1998). The difference in the expression level

of ApoE in control and NPC^{NIH} mouse models present an excellent platform for our group to investigate how ApoE expression may be associated with neurodegeneration and/or other symptoms presented in NPC mouse model.

3.1.3 Cholesterol dysfunction may have resulted in neurological problems in NPC patients and mouse models

Observations made in NPC^{NIH} mouse model strongly suggest that cholesterol dysfunction is largely responsible for the disease pathological features. Earlier studies hypothesize that impairment in the production of lipoproteins might have been the culprit to the neurological symptoms in NPC disease. However this was proved otherwise as sequestered lipoproteins from NPC1-deficient astrocytes and control cells were found to be structurally and functionally similar (Karten, Hayashi et al. 2005).

Significant loss of cholesterol in the brains of NPC patients were described and well documented (Vanier 1999). Similar loss of cholesterol in NPC^{NIH} mouse brains has also been reported (Xie, Burns et al. 2000). Unlike the peripheral system, extensive loss of cholesterol was observed in the CNS. It was speculated that intracellular cholesterol accumulation existed as reported in animal tissues at preclinical stage (Treiber-Held, Distl et al. 2003) and tissue culture studies (Henderson, Lin et al. 2000). Neurons in the CNS were unable to tolerate the high accumulation of cholesterol and began to die. This is followed by extensive demyelination in the brain. This contributed to the cholesterol loss, since myelin is the major source of cholesterol in the brain.

3.1.4 NPC patients and mouse models develop metabolic symptoms

Genome-wide association study conducted on a group of european populations identified two NPC1 SNP (single nucleotide polymorphism) that were closely linked to obesity (Meyre, Delplanque et al. 2009). In another recent finding, significantly higher levels of plasma glucose, insulin and cholesterol were measured in NPC+/- heterozygous mice in contrast to the control (Jelinek, Millward et al. 2011). These data agree with the author's earlier report that NPC+/- mice were more susceptible to weight gain (Jelinek, Heidenreich et al. 2010). These animals also appeared to exhibit several other metabolic features such as impaired fasting glucose, glucose intolerance, hyperinsulinemia, hyperleptinemia and dyslipidemia. In summary, these studies strongly suggest that NPC1 gene may be involved in the regulation of glucose.

Fatty liver syndrome due to prolonged lipid accumulation in NPC^{NIH} mouse has been associated with severe insulin resistance (Marchesini, Brizi et al. 1999; Michael, Kulkarni et al. 2000; Marchesini, Bugianesi et al. 2003; Vainio, Bykov et al. 2005) and may evolve to a systemic insulin resistance. Under high lipid environment, it is speculated that the lipid rich plasma membrane may be altered thus changing the expression and functionality of insulin receptors (Ikonen and Vainio 2005).

3.1.5 Hypothesis

Growing interest in the interplay between insulin and cholesterol metabolism (Suzuki, Lee et al. 2010) has driven me to investigate the impact of NPC1 deficiency on the regulation of glucose and insulin in the CNS. The lab's

earlier publication has also shown that muApoE expression varies in this mouse model (Chua, Lim et al. 2010).

Current literature do not fully explain how apolipoproteins may impact on abnormal cholesterol and/or glucose regulation, henceforth the NPC mouse model provides an excellent tool to explore insulin and cholesterol associated pathways. I propose that ApoE may affect the regulation of insulin signalling cascade in the CNS of NPC^{NIH} mouse.

3.2 Results

3.2.1 Glucose and insulin profiles of NPC^{NIH} mouse brains

A significantly lower level of insulin ($p \leq 0.05$) was detected in NPC^{NIH} mouse brain at both 5^{th} and 9^{th} week time points as compared to Balb/c wildtype, 20% and 12% loss respectively (Figure 2). No significant change was observed in the glucose level between Balb/c wildtype and NPC^{NIH} mouse brain homogenates at the 5^{th} and 9^{th} week time points.

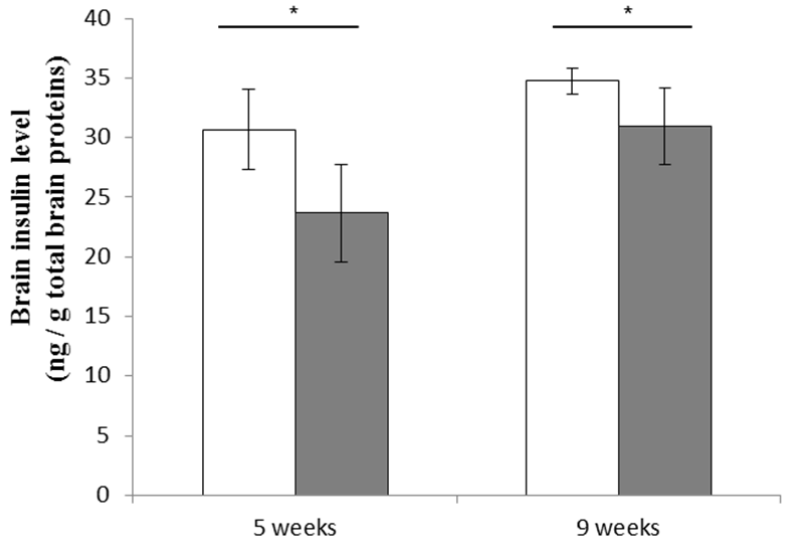

Figure 2 Insulin level of Balb/c wildtype and NPC^{NIH} mouse brain lysates

5 and 9 week old Balb/c wildtype (white bar) and NPC^{NIH} (grey bar) mouse brain lysates were measured using mouse insulin ELISA kit against a set of standard. Absolute amount of insulin was measured and normalized against the protein concentration of brain homogenates. Each value represents the mean ± SD for individual mouse sample ($n \geq 3$ at each time point for each mouse line). * p value ≤ 0.05 using Student's t-test.

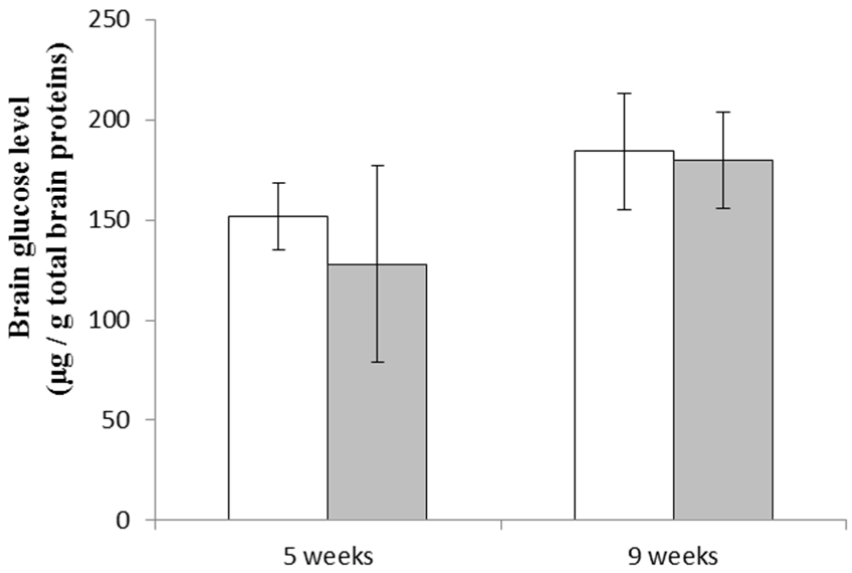

Figure 3 Amplex red glucose assays of Balb/c wildtype and NPC^{NIH} mouse brain lysates

5 and 9 week old Balb/c wildtype (white bar) and NPC^{NIH} (grey bar) mouse brain lysates were measured using Amplex red glucose kit against a set of standard Absolute amount of glucose was measured and normalized against the protein concentration of brain homogenates. Each value represents the mean ± SD for individual mouse brain sample ($n \geq 3$ at each time point for each mouse line). * p value ≤ 0.05 using Student's t-test.

3.2.2 Western blot analysis of PI3K/AKT signalling pathway in NPC^{NIH} mouse brains

As illustrated in figure 4, there is a notable loss in the expression of IRS1 and IRS2 proteins in NPC^{NIH} mouse brain lysates as compared to Balb/c wildtype at both 5th and 9th week time points. Significantly lower PI3Kp85 was observed in 9 weeks old NPC^{NIH} mouse brain lysates. Activated AKT (p-AKT Thr 308 and Ser 473) was also lower in the NPC^{NIH} mouse brain lysate as compared to wildtype Balb/c at 5th and 9th week time points (Figure 5). There was no change in the expression of both phosphorylated and total PDK1 proteins at either time points (results not shown).

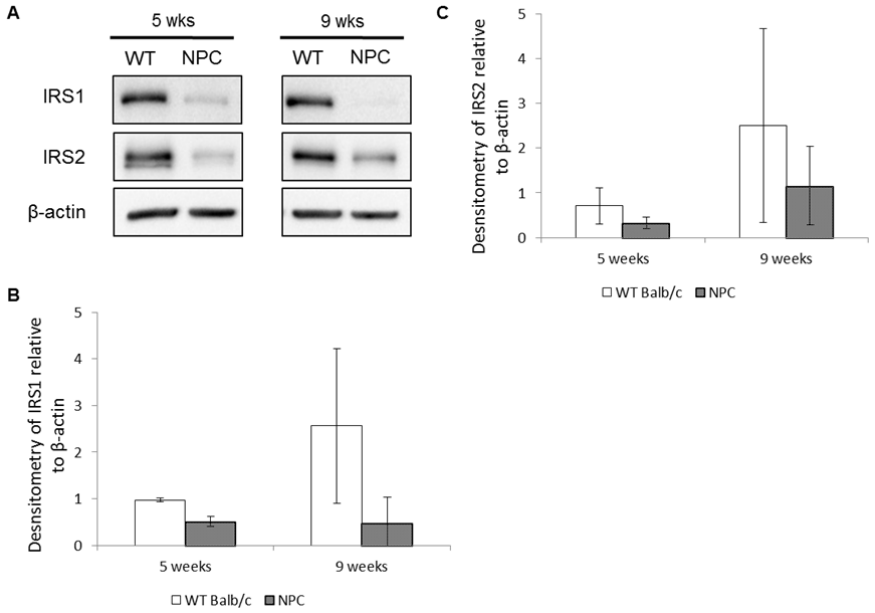

Figure 4 Brain insulin receptor substrate (IRS) protein expression level in Balb/c wildtype and NPC^{NIH} mouse brain lysates

(A) Western blot analysis of insulin receptor β (IRβ), insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) IRS1 and (C) IRS2 in 5 and 9 week old Balb/c wildtype (white bar) and NPC^{NIH} (grey bar) mouse brain lysates were performed using the NIH ImageJ software. Each value represents the mean for individual mouse brain sample (n = 2 at each time point for each mouse line).

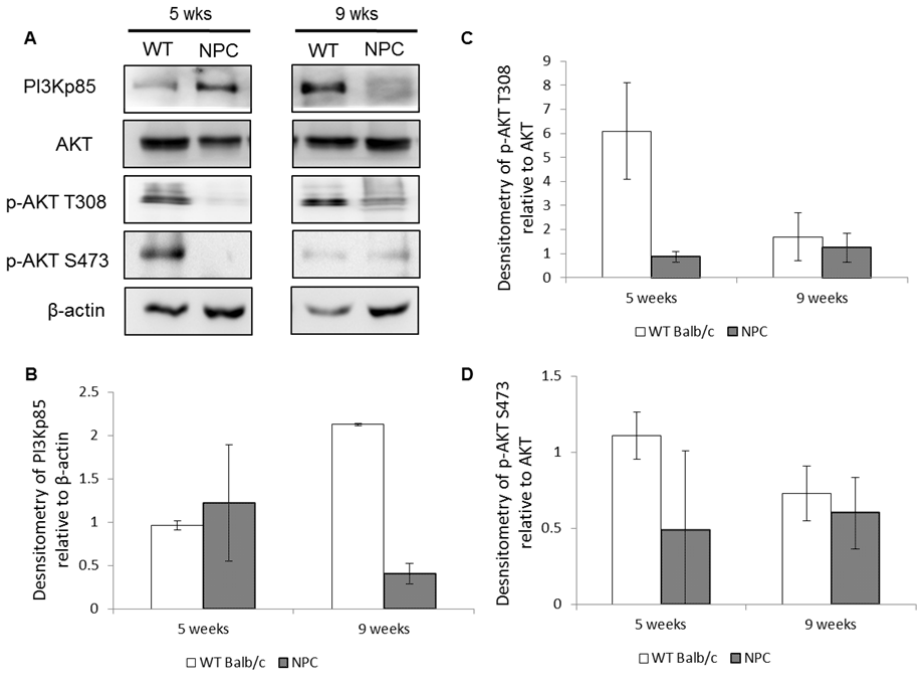

Figure 5 Protein expression of key targets in PI3K/AKT signalling pathway of Balb/c wildtype and NPC^{NIH} mouse brain lysates

(A) Western blot analysis of PI3Kp85, AKT, p-AKT T308 and S473. β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) PI3Kp85, (C) p-AKT T308/AKT and (D) p-AKT S473/AKT in 5 and 9 week old Balb/c wildtype (white bar) and NPC^{NIH} (grey bar) mouse brain lysates were performed using the NIH ImageJ software. Each value represents the mean for individual mouse brain sample (n = 2 at each time point for each mouse line).

3.2.3 Aebp1 activity in the CNS of NPC^{NIH} mouse model

In both 5 and 9 week old mouse brain lysates, significantly lower expression of Aebp1 (Figure 6) was noted. Following up on this finding, increasing expression of PTEN in NPC^{NIH} mice was observed at both time points (Figure 6).

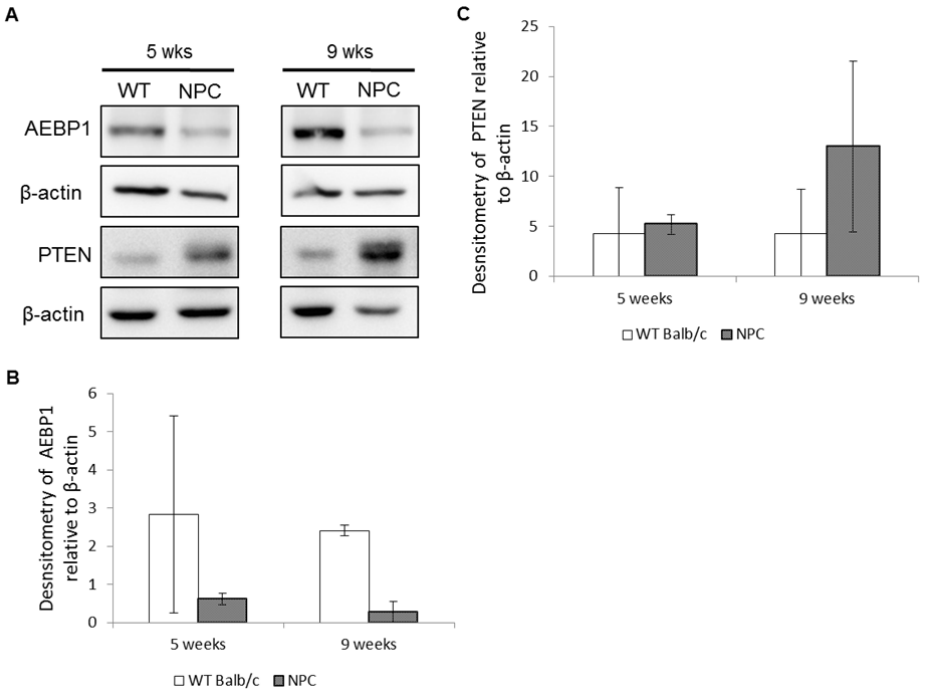

Figure 6 Protein expression of Aebp1 and PTEN in Balb/c wildtype and NPC^{NIH} mouse brain lysates

(A) Western blot analysis of AEBP1 and PTEN. β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) AEBP1 and (C) PTEN in 5 and 9 week old Balb/c wildtype (white bar) and NPC^{NIH} (grey bar) mouse brain lysates were performed using the NIH ImageJ software. Each value represents the mean for individual mouse brain sample (n = 2 at each time point for each mouse line).

3.2.4 GSK3β activity in the CNS of NPC^{NIH} mouse model

With reference to figure 7, the magnitude of phosphorylation in GSK3β was severely diminished in the CNS of NPC^{NIH} mice and this was especially significant at the later 9th week time point.

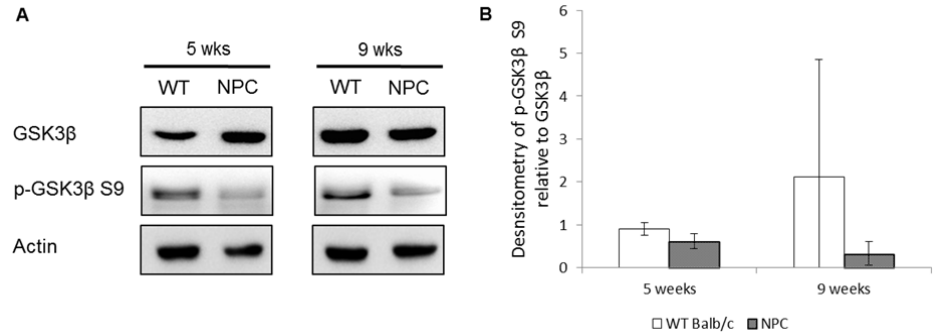

Figure 7 GSK3β activity in Balb/c wildtype and NPC^{NIH} mouse brain lysates

(A) Western blot analysis of GSK3β and p-GSK3β. β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) p- GSK3β in 5 and 9 week old Balb/c wildtype (white bar) and NPC^{NIH} (grey bar) mouse brain lysates were performed using the NIH ImageJ software. Each value represents the mean for individual mouse brain sample (n = 2 at each time point for each mouse line).

3.2.5 Expression of glucose transporters in the CNS of NPC^{NIH} mouse model

GluT1 are ubiquitously expressed in the body while GluT3 are exclusively found in the CNS, in particular the neurons. The results showed that neither glucose transporters were affected by the loss of NPC1 or ageing (Figure 8).

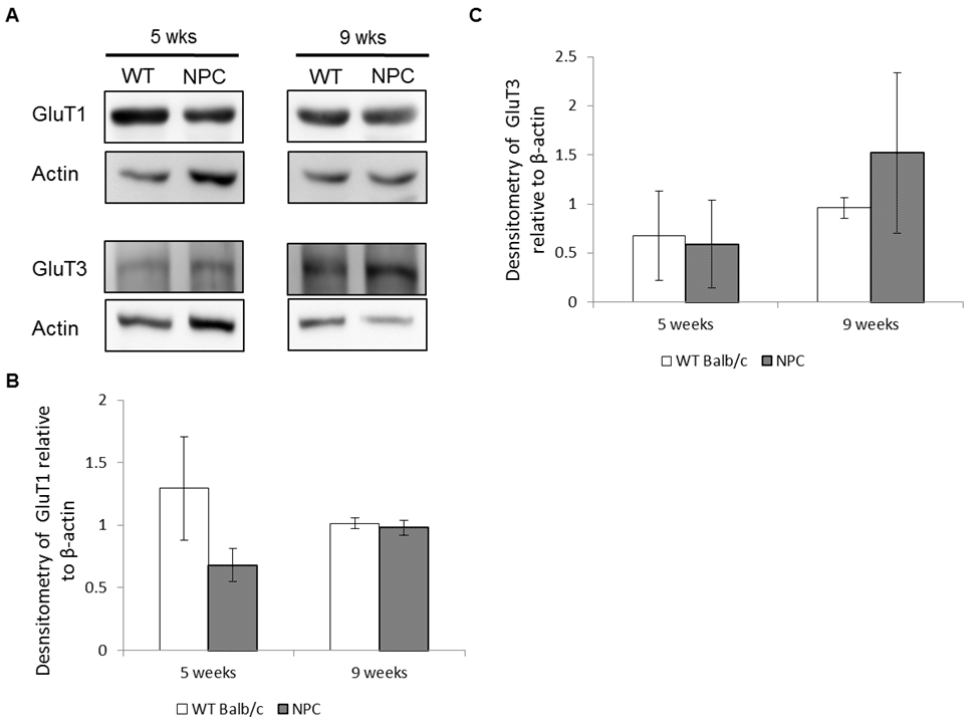

Figure 8 Expression of glucose transporters in Balb/c wildtype and NPC^{NIH} mouse brain lysates

(A) Western blot analysis of GluT1 and GluT4. β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) GluT1 and (C) GluT4 in 5 and 9 week old Balb/c wildtype (white bar) and NPC^{NIH} (grey bar) mouse brain lysates were performed using the NIH ImageJ software. Each value represents the mean for individual mouse brain sample (n = 2 at each time point for each mouse line).

3.3 Discussion

3.3.1 Cholesterol dysfunction and abnormal insulin profiles in the CNS of NPC^{NIH} mouse model

Extensive neurodegeneration accompanied by reduction in the cholesterol in the brain of NPC^{NIH} mice are relatively well established. Other than cholesterol, several groups including our lab had also observed elevations in the expression of ApoE and ApoD in these transgenic mice. (Burns, Gaynor et al. 2003; Karten, Hayashi et al. 2005; Li, Repa et al. 2005; Chua, Lim et al. 2010).

As opposed to conventional hypothesis, Xie et al suggested that the concentration of cholesterol in the brain of NPC^{NIH} transgenic mice was higher than the control. However, this was only observed at an early stage of the disease. It is only later in the disease, due to high rate of myelin loss, the overall loss of cholesterol in the brain of NPC^{NIH} transgenic mice appeared to be significantly higher (Xie, Burns et al. 2000). These speculations were later confirmed, as neurons isolated from postnatal NPC^{NIH} mice showed large quantities of cholesterol accumulated in the vesicles of these neurons (Karten, Vance et al. 2002). Parallel to Xie's concept, our lab supports the hypothesis that cholesterol accumulation took place in the CNS as in all other tissues however the brain tissues are less tolerant to the high lipid environment. This ultimately led to cell deaths in various part of the CNS and loss of myelin-associated cholesterol.

Although the cholesterol link in NPC patients and transgenic mice has been thoroughly explored, limited studies were conducted on other metabolic indicators such as glucose and insulin. As suggested earlier, lipid environment of primary hepatocytes from NPC^{NIH} mice was shown to regulate the expression and functionality of insulin receptor. This is possibly via its plasma membrane (Vainio, Bykov et al. 2005). Traditionally, glucose and cholesterol metabolism have been separately associated with brain related diseases such as Alzheimer's disease. More recently, there is increasing research on possible crosstalk between these two pathways and how they might complement each other leading to neurodegeneration (Vainio, Bykov et al. 2005; Taghibiglou, Bradley et al. 2009; Block, Dorsey et al. 2010; Sima 2010). The lab found significantly lower levels of insulin in 5 and 9 week old NPC^{NIH} mice as compared to the control and no difference in the glucose level. It is noteworthy that insulin and glucose level in the CNS of transgenic NPC^{NIH} mice were not investigated in any previous study. Since insulin profiles from both the peripheral and the CNS were shown to differ (Gerozissis, Orosco et al. 1993), it is likely that insulin found in the CNS is largely produced *in situ*. Unfortunately no one is able to identify the source or the regulatory mechanism of insulin in the CNS yet.

Insulin is a prominent player in the regulation of glucose in the body as it mediates the influx and fate of glucose in the cells via glucose transporter and glycogen synthase respectively. In view of these data, I was also interested to determine the brain glucose levels of these animals and how it may be associated to the current observations. However, brain glucose levels between

NPC^{NIH} and control animals showed little difference at both time points (Figure 3).

3.3.2 Age dependent attenuated PI3K/AKT signalling in NPC^{NIH} mouse model

I have also decided to explore the popular hypothesis; cholesterol metabolism affects the functionality of insulin receptors on the plasma membrane of neurons. The regulation of IR in a lipid rich environment of NPC^{NIH} cultured cells has previously been reported. This study was performed in primary NPC^{NIH} mouse hepatocytes (Vainio, Bykov et al. 2005). It is postulated that increased amount of cholesterol in the plasma membrane of primary NPC^{NIH} mouse hepatocytes altered the composition and fluidity of the plasma membrane lipids. Subsequently intervention study further supports this idea; removal of excess cholesterol to the culture restored the functionality of insulin receptors.

Regulating the cholesterol environment affects the membrane fluidity of NPC^{NIH} cells. This in turn may have tremendous impact on the activity of surface membrane insulin receptors. Lipid rafts are pockets of cholesterol structures that exist in the plasma membrane and the function of proteins (in this case IR) in them are influenced by changes in the concentration of cholesterol and sphingomyelin in the membrane. With increasing membrane cholesterol, the rafts tend to enlarge and vice versa (Henderson, Edwardson et al. 2004).

Cholesterol-induced insulin resistance observations were made later in 2009 whereby primary cortical cultures from rat were treated with cholesterol and this resulted in an attenuated insulin signalling pathway. The author postulated that insulin receptors were recruited into the lipid rafts domains in the plasma membrane. This movement rendered the IR less responsive to insulin as compared to those located in the non-lipid raft regions (Taghibiglou, Bradley et al. 2009).

Significant loss of insulin in NPC^{NIH} mouse brains and possibly reduced functionality of insulin receptors encouraged me to study the insulin signalling pathway. Insulin receptors may activate IRS1 and IRS2 which are responsible for other downstream targets related to insulin and glucose metabolism. My findings demonstrated down regulation of subsequent downstream targets in the PI3K/AKT signalling pathway. The targets include IRS1, IRS2, PI3Kp85 and p-AKTs (Figure 4 and 5). These observations suggested an attenuated PI3K/AKT signalling pathway. Activated IRS recruits and activates PI3K which is capable of converting membrane lipid PIP2 to PIP3. With this conversion, several downstream targets including survival-promoting kinases such as AKT are activated. Increasing amount of evidence has also associated PI3K signalling to neuronal survival, LTP (long-term potentiation), LTD (long-term depression), learning and memory. (Zhao, Chen et al. 1999; Kelly and Lynch 2000; Yamaguchi, Tamatani et al. 2001; Zhao and Alkon 2001; Sanna, Cammalleri et al. 2002; Chiang, Wang et al. 2010).

The full activation of AKT requires PDK1 and PI3K with phosphorylation on Threonine 308 and Serine 473. Since no change was observed in PDK and p-PDK1, it is likely that PDK1 has little or no influence in the diminished level of activated AKT. The lower phosphorylation of AKT at site Threonine 308 and Serine 473 is speculated to be associated with PI3K. In summary, the activity of PI3K/AKT signalling pathway is significantly reduced in NPC^{NIH} mice. This is possibly attributed by the loss of insulin and/or cell surface IRs due to the high lipid environment and/or extensive neuronal cell death.

3.3.3 The effects of attenuated PI3K/AKT signalling pathway in NPC^{NIH} mouse model

AKT is actively involved in several cellular mechanisms such as glucose metabolism, cell proliferation, cell migration, apoptosis and transcription. More importantly, phosphorylated AKT also modulates the activities of glucose transporters, glycogen synthase and tau phosphorylation through GSK3β.

My data showed lower p-GSK3β Ser 9 in NPC^{NIH} (Figure 7) mice which coincided with our earlier p-AKT results. The higher GSK3β activity may suggest higher phosphorylation of tau and/or lower expression of glycogen synthase which modulates glycogen synthesis in NPC^{NIH} mice. One distinct common hallmark of NPC in both patients and NPC^{NIH} mice is hyperphosphorylated tau (Love, Bridges et al. 1995; Suzuki, Parker et al. 1995; Sawamura, Gong et al. 2001; Bu, Li et al. 2002; Distl, Treiber-Held et al. 2003). It is speculated that this symptom may be due to the increased GSK3β activity in NPC^{NIH} mice. On the other hand, it is uncertain if GSK3β is solely responsible as several other kinases such as MAP kinase and cdk5 (Cyclin-dependent kinase 5) were reported to induce hyperphosphorylated tau. Further analysis on the tau residues which have been phosphorylated will be necessary to identify potential kinases involved. A detail table of tau phosphorylation and corresponding kinases is available under http://cnr.iop.kcl.ac.uk/hangerlab/tautable.

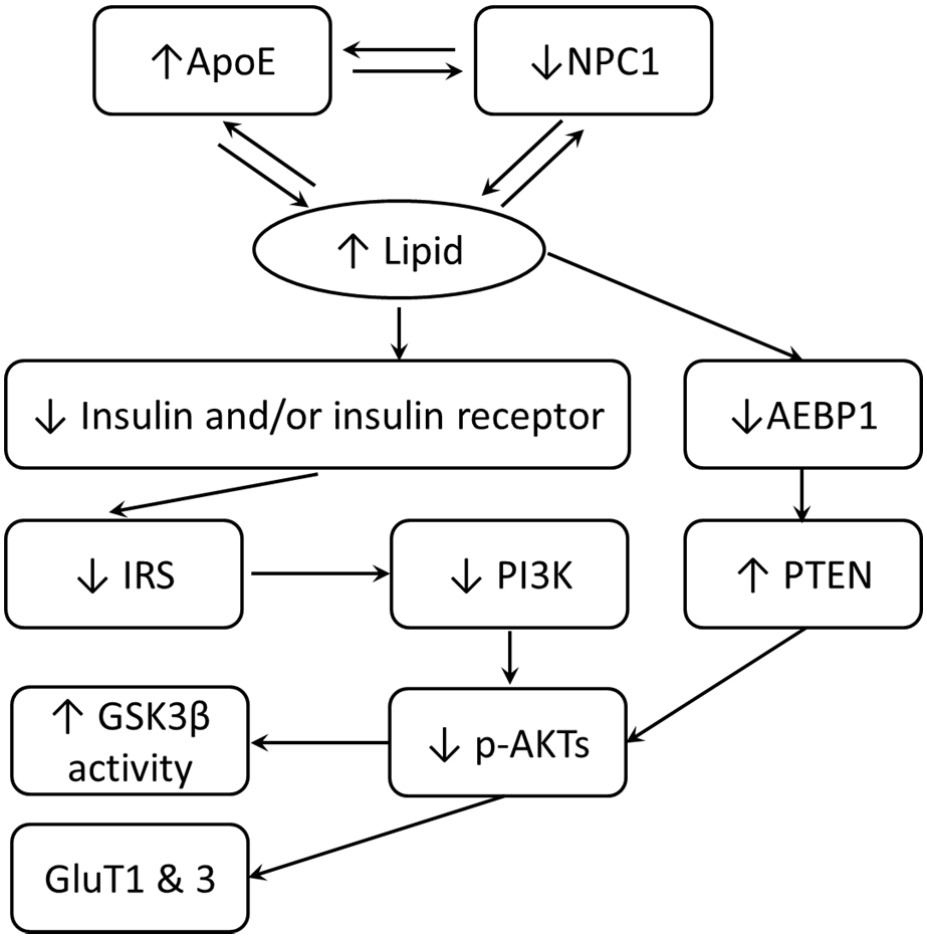

Figure 9 Schematic diagram of how ApoE may modulate GSK3β activity in the CNS of NPC^{NIH} mice

The lipid-rich environment in NPC^{NIH} mice inhibits the activity of insulin, IR and/or IRS. This significantly lowered the expression of both IRS and AEBP1. Further downstream, the phosphorylation of AKT diminished via two possible pathways, PI3K and PTEN. The activity of GSK3β is inhibited when phosphorylated. This led to higher GSK3β activity in the CNS of NPC^{NIH} mice.

The only publication that had investigated the PI3K/AKT signalling pathway in the CNS of NPC^{NIH} mice was by Bi et al in 2005 (Bi, Liu et al. 2005). Surprisingly, most of their data indicated an elevation in AKT activity and relevant molecules as opposed to the lab's findings. They postulated that higher AKT activity may lead to neurodegeneration involving TNFα (Tumor necrosis factor-alpha) and GSK3β. Distinctions between the results may be

due to the different time points and the methodologies samples were obtained. Results presented in Bi et al were derived from animal brains varying from 1-4 weeks while our samples were from 5-9 week old mice. It is arguable that subtle age differences have little influence in the observations. On the contrary it is noteworthy that most of the transgenic mice pathological features only surface from the 5^{th} week.

Phosphorylated AKT is involved in several other physiological functions apart from mediating GSK3β. It has been shown to alter GluT trafficking from intracellular vesicles to the plasma membrane via AS160. Impaired AS160 transgenic mice exhibited impaired glucose homeostasis and insulin sensitivity (Kane, Sano et al. 2002; Sano, Kane et al. 2003; Ng, Ramm et al. 2008; Chen, Wasserman et al. 2010). Similar levels of glucose were detected in the NPC^{NIH} and control mice, suggesting that the activity of GluT might not have been altered in these transgenic animals. We were unable to determine the bio-activity of GluT on the plasma membrane however little differences were observed in GluT protein level between the CNS of both diseased and wildtype mouse models (Figure 8). FoxO1 transcription factor which promotes the expression of gluconeogenic enzymes is also regulated by AKT. It is negatively regulated by the PI3K/AKT signalling pathway and has been shown to be compromised under insulin resistant conditions (Brunet, Bonni et al. 1999; Kops, de Ruiter et al. 1999). Together, AKT is largely recognized as the key protein actively involved in glucose metabolism through various avenues. In spite of these, significant studies are still required to decipher its role under a lipid-rich environment.

3.3.4 Aebp1 mediated AKT signalling

Other than the PI3K/AKT signalling pathway, I was also interested in other molecules that may be associated to ApoE and contribute to the downstream GSK3β activity. Aebp1 is an 82 kDa transcriptional repressor and is ubiquitously expressed, with exceptionally high expression in the adipose, macrophage, liver, lung, spleen and brain (Ro, Kim et al. 2001). Existing literature indicate that it holds key regulatory roles in adipogenesis, obesity (Ro, Zhang et al. 2007), energy homeostasis (Zhang, Reidy et al. 2005) and more recently atherosclerosis (Bogachev, Majdalawieh et al. 2011). Aebp1 has also been associated with cholesterol metabolism and inflammation through peroxisome proliferator-activated receptor gamma 1 (PPARγ1) and liver X receptor alpha (LXRα). Aebp1 overexpression in macrophages has been shown to reduce both PPARγ1 and LXRα (Majdalawieh, Zhang et al. 2006) in cholesterol homeostasis (Tontonoz, Nagy et al. 1998; Venkateswaran, Laffitte et al. 2000; Chawla, Boisvert et al. 2001).

My findings indicated diminishing expression of Aebp1 and up regulation of PTEN (Phosphatase and tensin homolog) (Figure 6) in NPC^{NIH} mice which are in line with the initial results from the PI3K/AKT signalling pathway. The combination of increased PTEN and reduced PI3K lead to significantly lowered AKT activity in NPC^{NIH} mice. These data further strengthen our previous result and suggest that PI3K/AKT pathway may not be the sole mediator of AKT phosphorylation in NPC^{NIH} mice.

The interaction between Aebp1 and PTEN was first established using yeast two hybrid (Gorbenko, Kuznetsov et al. 2004), subsequently experiments then showed that Aebp1 negatively regulates PTEN (Ro, Zhang et al. 2007). PTEN has also been identified as a negative regulator of insulin signalling and sensitivity as it down regulates the pro-survival serine/threonine protein kinase AKT. Tissue-specific PTEN knockout mice exhibited significant improvement in glucose tolerance and insulin sensitivity (Kurlawalla-Martinez, Stiles et al. 2005). It is possible that PTEN may be associated with the phosphorylation level of AKT and GSK3β activity. This assumption was partially proven in Ro's co-immunoprecipitation study whereby Aebp1 knockout mice displayed significantly lower p-AKT Thr 308(Ro, Zhang et al. 2007). In spite of these, these mice did not develop any symptoms that are associated with a defective glucose metabolism similar to our NPC^{NIH} mice. This phenomenon may be tissue specific as altered AKT activation was only reported in the white adipose tissues but not the skeletal muscles. My data may prove valuable as these observations have yet to be reported in the CNS of NPC patients/animals.

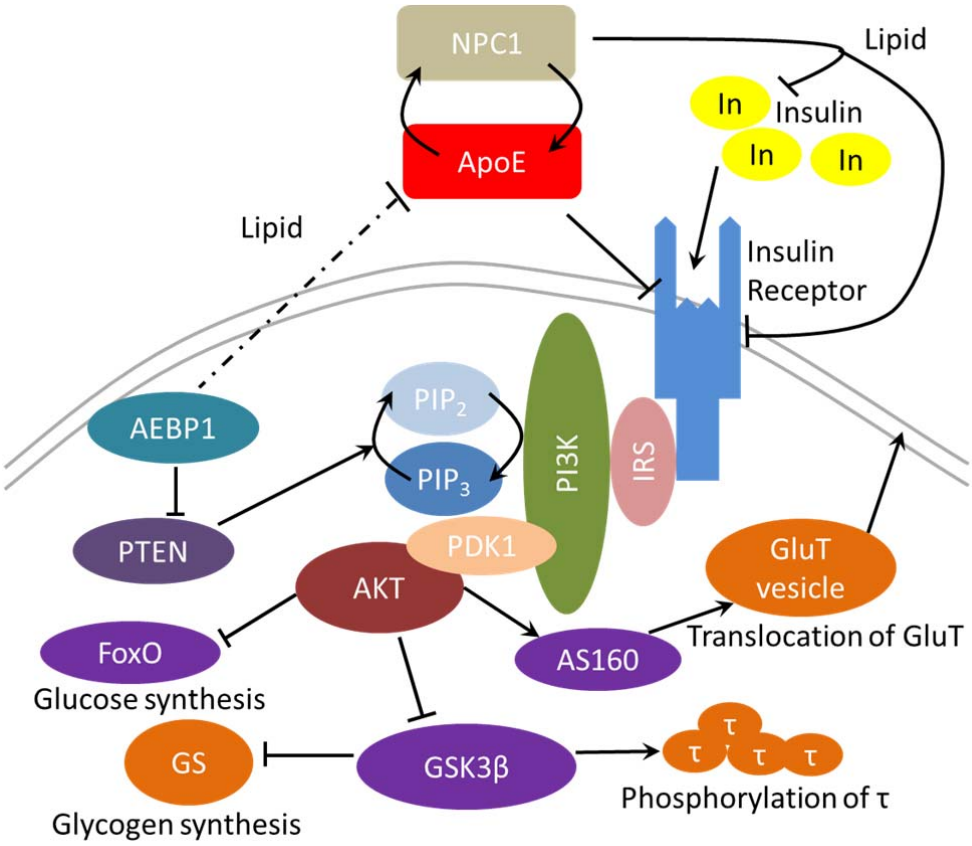

Figure 10 Schematic diagram proposing possible links across NPC1, ApoE and lipid metabolism in the CNS

In a high lipid environment, the PI3K/AKT signalling pathway was significantly reduced and results in elevated GSK3β activity. The expression of Aebp1 was also severely diminished as the body attempted to clear the lipid by up regulating key cholesterol mediators. Both effects synergistically contributed to the elevated activity of GSK3β via the PTEN/AKT signalling pathway.

3.4 Summary

In conclusion, the lab has explored the possible influence of ApoE-driven lipid regulation on PI3K/AKT signalling pathway. It is important to recognize that ApoE and cholesterol metabolism may carry key contributions to insulin and glucose regulation in the CNS. Although insulin signalling and lipid dysfunction have often been associated with metabolic diseases, insufficient data are available to establish a direct link between these systems. My data provided clues and further understanding to how differential apolipoprotein expression may have resulted in a lipid-overwhelmed CNS which in turn led to an attenuated PI3K/AKT signalling (Figure 10). I have also brought forward a possible molecular pathway and role of Aebp1 in the CNS (Figure 10).

CHAPTER 4

HUMAN APOLIPOPROTEIN E POLYMORPHISM AFFECTS BRAIN INSULIN SIGNALLING IN A MOUSE MODEL

4 Human apolipoprotein E polymorphism affects brain insulin signalling in a mouse model

4.1 Introduction

4.1.1 Human apolipoprotein E isoforms and diseases

Limited literature is available on human apolipoprotein E (huApoE) function in the brain. Although it has been associated with higher risk of hypercholesterolemia and dementia with ageing, the underlying mechanism remains elusive. As described in the first chapter, there are three major huApoE isoforms, E2, E3 and E4 (Mahley 1988. Majority of the population are huApoE3 carriers while huApoE4 is a strong genetic risk factor for atherosclerosis and AD {Hauser, 2011 #710; Zannis, Kardassis et al. 1993). Other than huApoE isoforms, the differential expression level of huApoE proteins in the CNS is also linked to neurological symptoms such as NPC and AD (Ramaswamy, Xu et al. 2005; Riddell, Zhou et al. 2008; Sullivan, Han et al. 2011).

4.1.2 Knowledge from NPC studies

In the earlier chapter, varying insulin profiles and mouse apolipoprotein E (muApoE) between NPC^{NIH} and wildtype mouse models was reported in our lab (Chua, Lim et al. 2010). Based on these observations, I decided to explore the activity of PI3K/AKT signalling pathway in NPC^{NIH} mouse model. Proteins involved in PI3K/AKT signalling pathway were significantly down regulated with age when most of the neurological symptoms surfaced. Based on these findings, I speculated possible correlation between cholesterol and PI3K/AKT signalling pathway involving ApoE expression.

HuApoE3 and E4 mouse models are ideal tools to investigate how ApoE isoforms may affect cholesterol metabolism and PI3K/AKT signalling pathway.

4.1.3 HuApoE targeted replacement (TR)mouse model

There are several human ApoE transgenic mice available and they are all created on ApoE knock-out background. These animals differ on the promoter driving the expression of huApoE. These include the glial fibrillary acidic protein (GFAP) promoter which is largely found within glial cells (Sun, Wu et al. 1998; Holtzman, Bales et al. 2000); the neuron-specific enolase (NSE) promoter which is largely found within neuronal cells (Raber, Wong et al. 1998; Buttini, Orth et al. 1999) and lastly, the endogenous mouse ApoE promoter which expresses the designated huApoE in the huApoE targeted replacement (TR) mouse models (Piedrahita, Zhang et al. 1992; Sullivan, Mezdour et al. 1998; Knouff, Hinsdale et al. 1999).

Studies have shown that both GFAP-huApoE and NSE-huApoE transgenic mice exhibit varying degrees of cognition defects (Raber, Wong et al. 1998; Raber, Wong et al. 2000; Hartman, Wozniak et al. 2001). On top of that, difference in gender susceptibility has also been reported (Raber, Wong et al. 2000; Hartman, Wozniak et al. 2001). These labs postulated that marked differences are contributed by the different expression profiles of huApoE due to the promoter. Henceforth, the usage of KI animals driven by the native mouse ApoE promoter is relevant to this study.

This mouse model of choice (B6.129P2-Apoe$^{tm3(APOE*3/4)Mae\ N8}$) has a defined C57BL6/J background and is homozygous for a human ApoE3 or ApoE4 gene targeted replacement of the endogenous mouse ApoE. It expresses huApoE driven by the muAPoE regulatory sequences. Abnormal serum lipid profiles and atherosclerotic symptoms only surfaced under high-fat diet for both huApoE TR mouse. Although the huApoE4 mouse model develops more severe atherosclerotic lesions and twice the level of cholesterol than in huApoE3 mouse model (Knouff, Hinsdale et al. 1999).

4.1.4 HuApoE expression profiles in ApoE3/4 carriers and B6.129P2-Apoe$^{tm3(APOE*3/4)Mae\ N8}$ mouse models

Current literature suggests that huApoE expression in these knock-in mouse models display regional differences although they are both driven by the same muApoE promoter. Sullivan and collaborators showed that the distribution of plasma lipoproteins and ApoE differed between huApoE3, E4 and wild type mice kept on a normal chow diet (Sullivan, Mezdour et al. 1997; Sullivan, Mace et al. 2004). On top of that, similar huApoE expression variation was observed in clinical brain samples between ApoE3 and ApoE4 allele carriers (Sullivan, Mace et al. 2004; Ramaswamy, Xu et al. 2005).

CSF of huApoE4 TR mice has been show to contain fewer ApoE molecules as compared to the huApoE3 TR mouse model (Riddell, Zhou et al. 2008). Similar observation was observed in individuals with ApoE4 alleles, whereby significantly lower ApoE was detected in comparison to those without an

ApoE4 allele (Larson, Ordovas et al. 2000; Poirier 2005). Vice versa, we also found contradicting reports of higher ApoE in brains and plasma of AD patients (Artiga, Bullido et al. 1998). Interestingly, under stress conditions, intense expression of ApoE was observed in injured neurons and in response to excitotoxic injury (Xu, Bernardo et al. 2006).

These observations are particularly interesting since both huApoE3 and huApoE4 are targeted replacement for muApoE. Both animals are expected to exhibit comparable level of huApoE but it appears that this is not the case. This suggests that the disparity in huApoE protein level may be regulated by both the expression and also the stability of the protein.

Higher huApoE4 degradation was observed in human astrocytoma cell lines and huApoE4 TR mouse brain as compared to huApoE3. This led the author to postulate that huApoE4 has poorer protein stability, thus a higher tendency to degrade (Riddell, Zhou et al. 2008). These findings further complicate my current study as isoform differences may affect the protein level of huApoE present in the CNS of both human and mouse.

4.1.5 Cholesterol profiles in ApoE3/4 carriers and B6.129P2-Apoe$^{tm3(APOE*3/4)Mae\ N8}$ mouse models

As discussed in the introduction, ApoE plays a pivotal role in the transportation of cholesterol in both the peripheral and central nervous system. These evidence led research groups to look into the cholesterol metabolism of huApoE TR mouse model. Marked decrease in the brain cholesterol of

huApoE4 TR as compared to WT mouse models (Hamanaka, Katoh-Fukui et al. 2000). No significant difference in the serum cholesterol level was observed between huApoE3 and E4 TR mouse models under normal diet (Mann, Thorngate et al. 2004).

Effective delivery of cholesterol has been observed in huApoE3 as compared to huApoE4 in primary astrocytes culture (Gong, Kobayashi et al. 2002). It is postulated that less effective transportation of lipids in ApoE4 carriers may have an effect on myelin repair in the CNS. This is particularly prominent later in life with increased age and myelin deterioration.

4.1.6 Hypercholesterolemia associated glucose and insulin profiles

Literature studies suggest huApoE isoform and/or expression variations may affect cholesterol and insulin profiles. Similarly, glucose hypometabolism have been observed in certain brain regions of ApoE4 allele carriers (Lee, Lee et al. 2003; Mosconi, Sorbi et al. 2004; Drzezga, Riemenschneider et al. 2005; Reiman, Chen et al. 2005; Langbaum, Chen et al. 2010)

Genetically obese (ob/ob) mice and rats under high fat diet induced obesity have significantly higher serum cholesterol and lower ApoE mRNA as compared to control diet animals. Furthermore, intracerebroventricular infusion of ApoE and injection of anti-ApoE to the serum have been shown to regulate food intake which in turn contributes to dietary cholesterol in these animals (Shen, Tso et al. 2008).

Cholesterol has been shown to induce insulin resistance in rat primary cortical cultures. Cholesterol treated cells exhibit an attenuated insulin signalling pathway. It renders insulin receptors less responsive to insulin (Taghibiglou, Bradley et al. 2009). These observations proposed strong links across huApoE isoform, cholesterol metabolism and insulin signalling.

4.1.7 Experimental considerations

The huApoE4 isoform is associated with higher risk of cholesterol dysfunction and neurological diseases, especially in older adults. Since ageing is an important factor, the animals were studied up to 72 weeks to investigate the effect of ageing on top of huApoE isoform differences. Both huApoE3 and huApoE4 TR mice were studied across three separate time points to monitor the progressive effects of ageing and varying huApoE isoforms.

Gender plays an important role in certain genetic risk disease. It has been reported that female gender is at a higher risk for neurological disease (Darreh-Shori, Brimijoin et al. 2006). On top of that, female NSE-ApoE4 mouse model has been shown to be more susceptible to cognitive deficits (Raber, Wong et al. 2000). Coincidentally, ApoE isoform and gender have been shown to influence glucose regulation in AD patients (Aisen, Berg et al. 2003). In view of these considerations, only female huApoE TR mice were used for this study.

Cholesterol, glucose and insulin assays were conducted on huApoE TR mouse brain lysates and plasma to determine potential physiological differences in

these animals. Circulating blood is able to transverse the blood brain barrier. It is important to include the serum/plasma analysis in this study as it is the sole transit between the peripheral and CNS.

4.1.8 Hypothesis

It is evident that differential ApoE protein level is associated with the CNS lipid profile. It is may also be affecting glucose and/or insulin metabolism through the PI3K/AKT signalling pathway. Earlier studies on NPC mouse models support this notion.

I hypothesized huApoE isoform differences together with ageing may affect glucose metabolism in the CNS. There is limited literature associating huApoE and glucose metabolism in the CNS. On top of that, I was unable to find any publication that has addressed glucose metabolism in huApoE TR mouse models over a time course. The academic impact of deciphering this molecular mechanism is significant and it may allow us to identify other potential risks of huApoE genetic variation.

4.2 Results

4.2.1 Total cholesterol in the brain and plasma of female huApoE TR mouse models

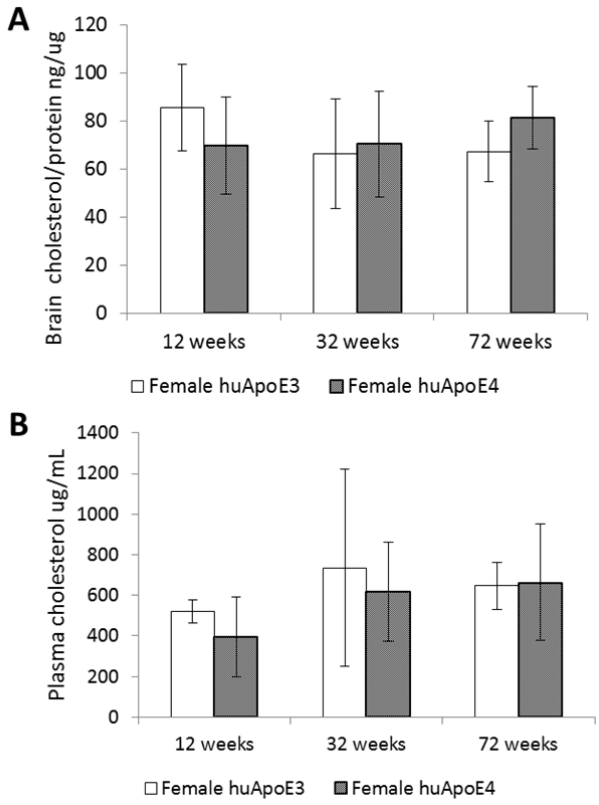

Figure 11 Total amount of cholesterol in plasma and brain lysates of huApoE3 and huApoE4 TR mice

Amplex red cholesterol assays of samples from (A) brain lysates, (B) plasma across all three time points (12th, 32nd and 72nd week) of female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice. The brain lysates was normalized to its total protein measured via BCA method. The plasma samples were normalized to its volume. Each value represents the mean ± SD for individual mouse brain sample (n = 5 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

No significant change was observed in the cholesterol content of both brain and plasma (Figure 11A and 11B) of huApoE3 and huApoE4 TR mice at 12, 32 and 72 week old mouse brain lysates and plasma.

4.2.2 HuApoE expression in the CNS of female huApoE TR mouse models

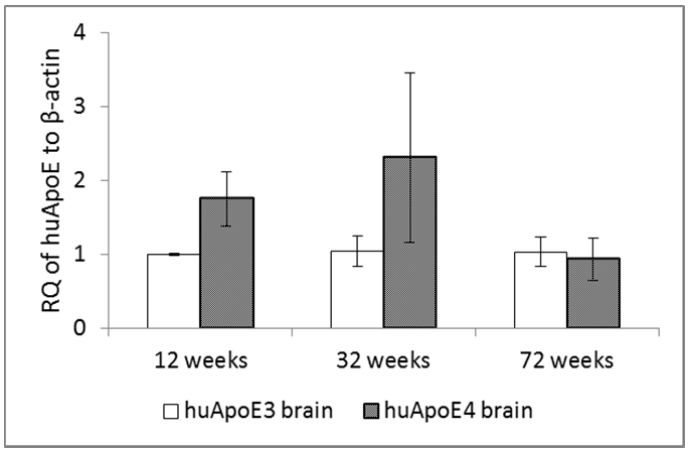

Figure 12 Relative quantification of huApoE mRNA in the brain of huApoE3 and huApoE4 TR mice

ΔΔCt analysis was performed with Ct values obtained with Taqman hydrolysis assays and normalized against β- actin for female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice. Each value represents the mean ± SEM for individual mouse brain sample ($n \geq 3$ at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

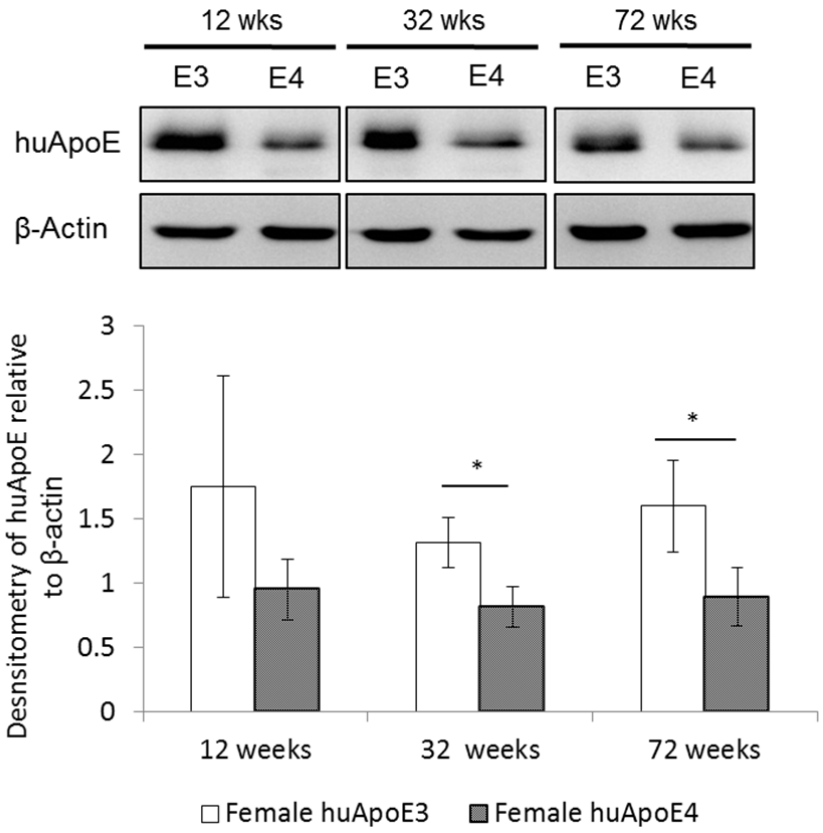

Figure 13 Brain huApoE protein expression level in the brain of huApoE3 and huApoE4 TR mice

Western blot analysis of human Apolipoprotein E (huApoE) and β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of huApoE relative to β-actin level in the brain lysates of female huApoE3 (white bar) and huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

No significant change was observed in huApoE mRNA level (Figure 12) across 12, 32 and 72 week old female mouse brain lysates. Further protein analysis via Western blot indicates that there is little change in the huApoE protein expression level of 12 week old female mouse brain lysates. However, significantly lower huApoE protein expression in huApoE4 TR mice at 32 week old (-38%) and 72 week old (-45%) female mouse models (Figure 13).

4.2.3 Glucose and insulin level in the brain and plasma of female huApoE TR mouse models

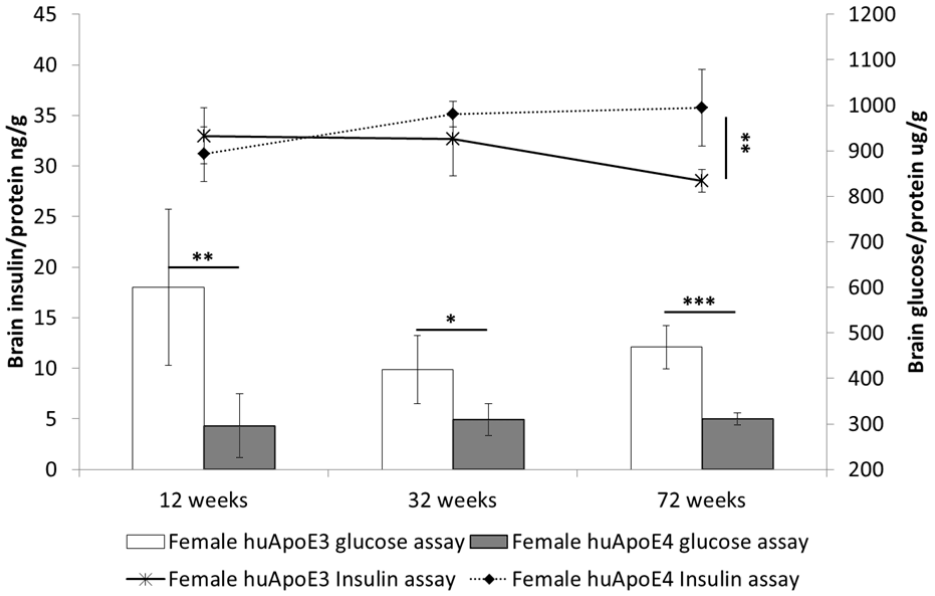

Figure 14 Total glucose and insulin in brain lysates of huApoE3 and huApoE4 TR mice

Glucose assays of samples from brain lysates across all three time points (12th, 32nd and 72nd week) of female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice. Insulin assays of samples from brain lysates across all three time points (12th, 32nd and 72nd week) of female huApoE3 (solid line) and female huApoE4 (dotted line) TR mice Glucose and insulin readings were normalized to its total protein measured via BCA method. Each value represents the mean ± SD for individual mouse brain sample (n = 5 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

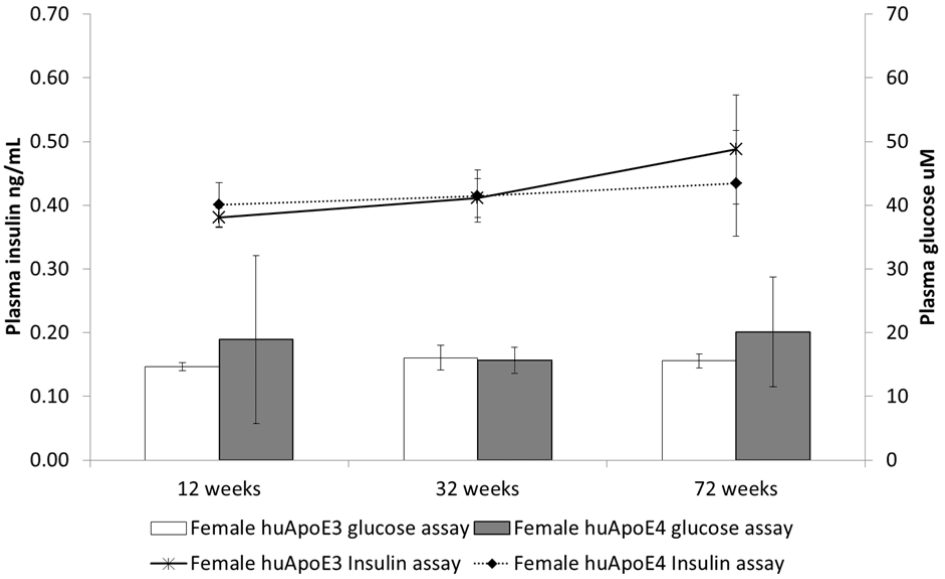

Figure 15 Total glucose and insulin in plasma samples of huApoE3 and huApoE4 TR mice

Glucose assays of plasma samples across all three time points (12th, 32nd and 72nd week) of female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice. Insulin assays of plasma samples across all three time points (12th, 32nd and 72nd week) of female huApoE3 (solid line) and female huApoE4 (dotted line) TR mice. Glucose and insulin readings were normalized to the volume. Each value represents the mean ± SD for individual mouse plasma sample (n = 5 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

Brain lysates showed significant loss of total glucose across all time points (Figure 14). -51%, -27% and -34% losses in huApoE4 as compared to huApoE3 TR at 12, 32 and 72 week old mouse brain lysates respectively. Significant increase of insulin (+25%) was only found in 72 week old huApoE4 TR mouse brain samples while there is no change in 12 and 32 week old brain samples (Figure 14).

Plasma samples from both huApoE3 and huApoE4 TR mice exhibit similar profiles (Figure 15). This is particularly interesting as the profiles of both glucose and insulin in the plasma differ from the profiles in the CNS.

4.2.4 PI3K/AKT protein profile in the CNS of huApoE3 and huApoE4 TR mice

No significant change was observed in protein expression of IRS1 in the brain lysate of both mouse models across all three time points (Figure 16A and 16B). Notable up-regulation of IRS2 was observed in 12 week old huApoE4 TR mice (+68%). However, the trend reversed in 72 week old huApoE4 TR mice, with significant loss of IRS2 (-46%) in the CNS (Figure 16A and 16C).

IRβ protein expression was relatively consistent between huApoE3 and huApoE4 TR mouse models at 12^{th}, 32^{nd} and 72^{nd} week time point (Figure 17A and 17B). Significantly higher expression of IGF1Rβ (Insulin growth factor 1 receptor-beta) was observed in huApoE4 TR mice at 12^{th} (+104%) week time point (Figure 17A and 17C). The activities of these proteins were quantitated by targeting p-IRβ and p-IGF1Rβ. Notable losses of p-IRβ and p-IGF1Rβ were observed via Western blot densitometry (Figure 17A and 17C) in the last two time points, 32^{nd} (-46%) and 72^{nd} (-53%) weeks.

Other than IRS proteins, PI3K proteins are also involved in the activation of AKT. Western blot densitometry showed 54% and 51% loss of PI3K p85 (Figure 18A and 18C) in huApoE4 (as compared to huApoE3 TR mouse) 32 and 72 week old female mice respectively. On the hand, I did not observe any significant change in the expression of PI3K p110 (Figure 18A and 18B) in the brain

Figure 19A and 19C showed comparable expression of AKT in both huApoE3 and huApoE4 TR mice at all three time points. Further analysis was conducted on both p-AKT S473 (Figure 19B) and T308 (Figure 19D) to study AKT activity in both mouse models. Significant loss of p-AKT S473 was observed in 72 week old huApoE4 (-39%) as compared to the huApoE3 TR mice. Similarly, significant loss of p-AKT T308 was observed in both 32 (-61%) and 72 (-49%) week old huApoE4 relative to huApoE3 TR mice.

Figure 20 showed notable loss of GluT4 (-48%) in female huApoE4 as compared to huApoE3 TR mice at the 72^{nd} week time point. Comparable expression of GluT4 was expressed in earlier time point of 12^{th} and 32^{nd} week time points.

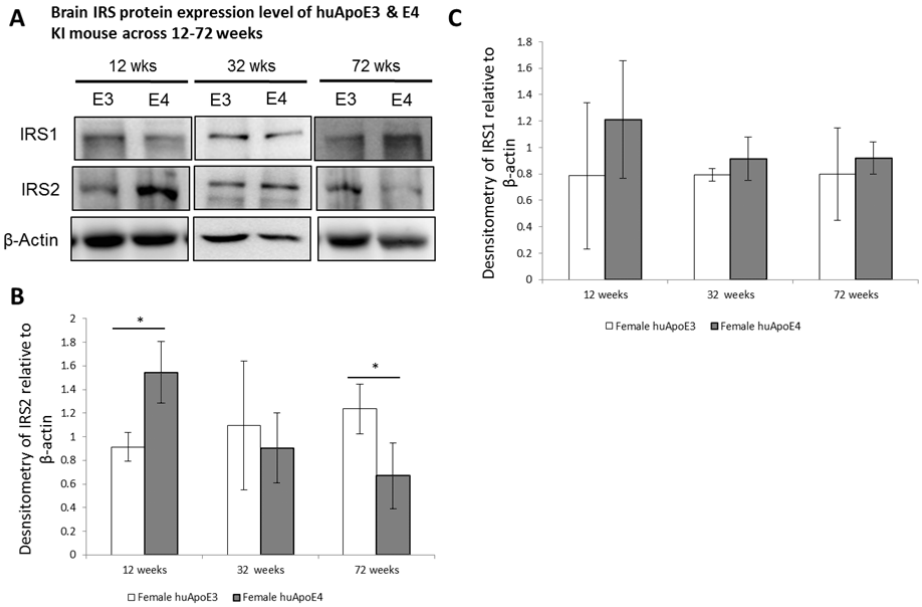

Figure 16 Brain insulin receptor substrate (IRS) protein expression level of huApoE3 & E4 TR mice

(A) Western blot analysis of insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) IRS1 and (C) IRS2 in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

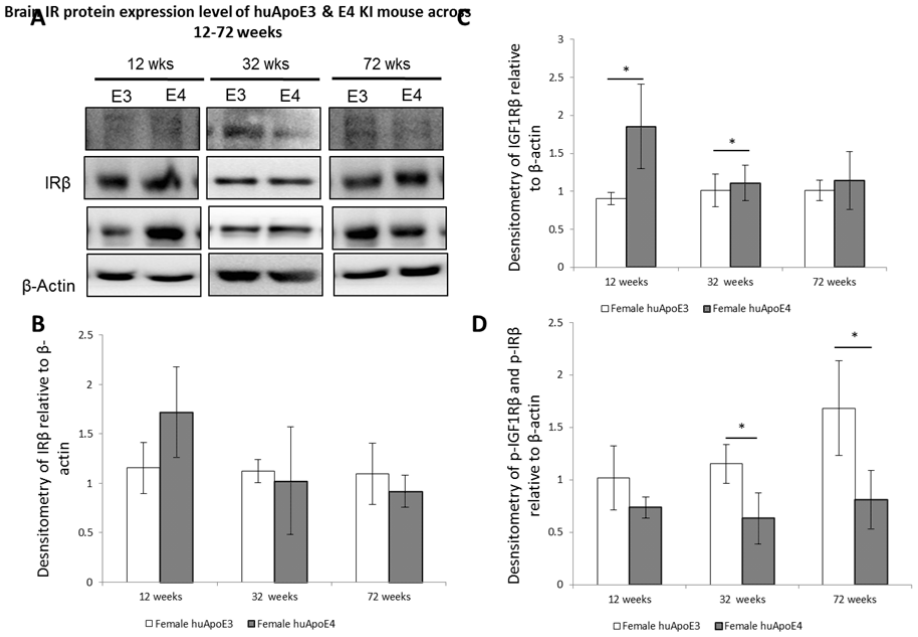

Figure 17 Brain insulin receptor (IR) protein expression level of huApoE3 & E4 TR mice

(A) Western blot analysis of phosphorylated insulin receptor β (p-IRβ), phosphorylated insulin growth factor receptor β (p-IGFRβ), insulin receptor β (IRβ) and insulin growth factor receptor β (IGF1Rβ). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) p-IRβ + p-IGFRβ , (C) IRβ and (D) IGF1Rβ relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

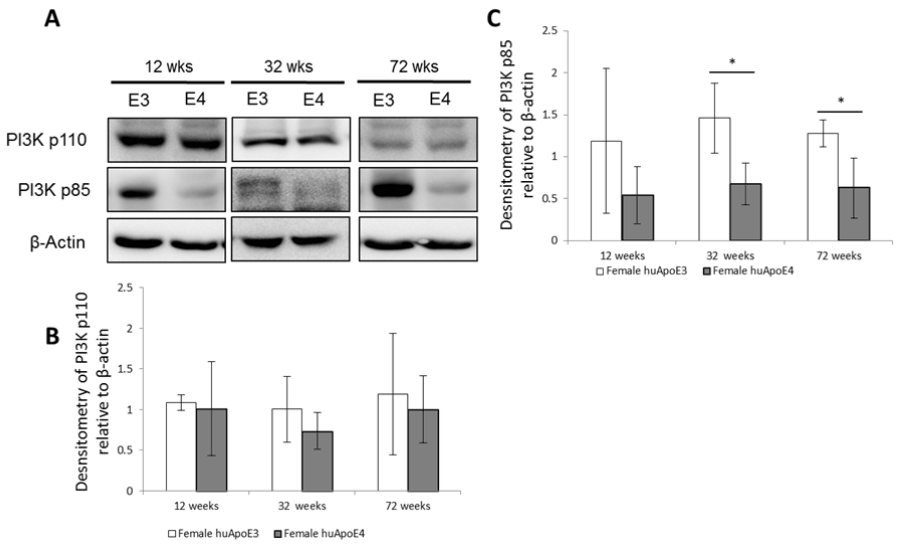

Figure 18 Brain phosphatidylinositol 3-kinases (PI3K) protein expression level of huApoE3 & E4 TR mice across 12-72 weeks.

(A) Western blot analysis of phosphatidylinositol 3-kinases p110 (PI3K p110) and phosphatidylinositol 3-kinases p85 (PI3K p85). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) PI3K p110, (C) PI3K p85 relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

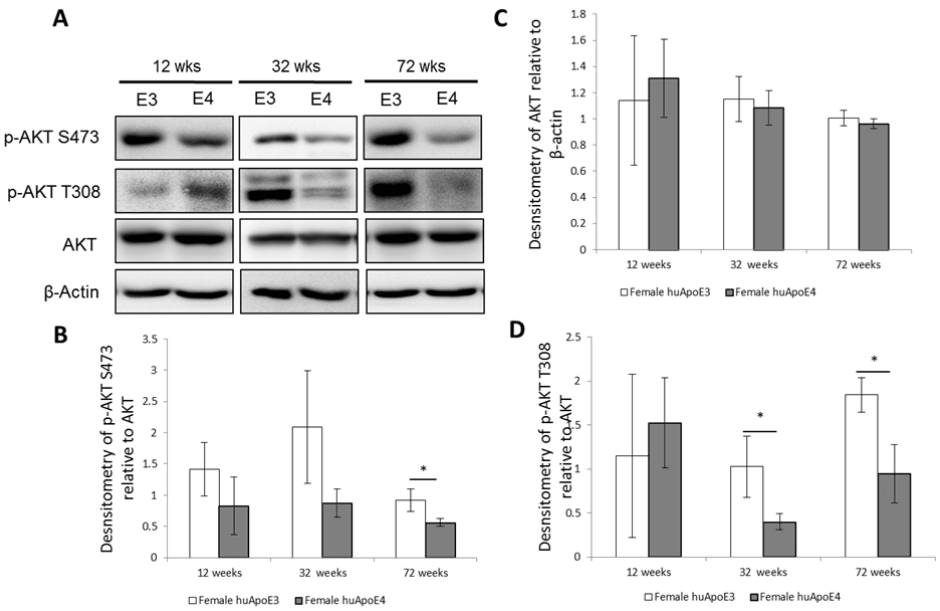

Figure 19 Brain AKT protein expression level of huApoE3 & E4 TR mice

(A) Western blot analysis of phosphorylated AKT Serine 473 (p-AKT S473), phosphorylated AKT Threonine 308 (p-AKT T308) and AKT. β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) p-AKT S473, (C) AKT and (D) p-AKT T308 relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

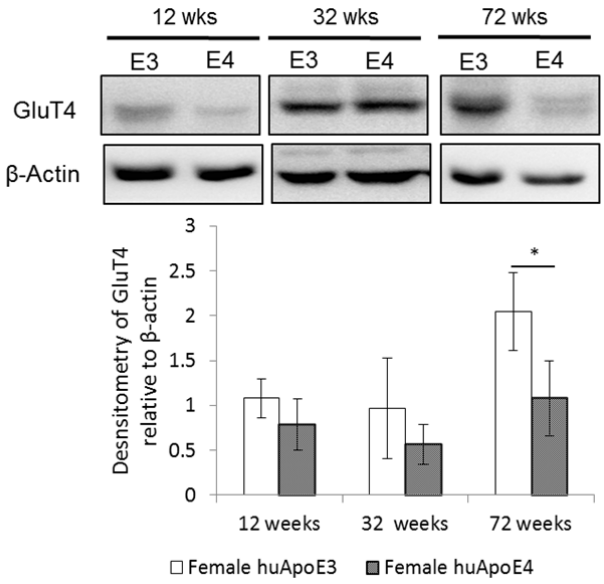

Figure 20 Brain glucose transporter 4 protein expression level of huApoE3 & E4 TR mice

Western blot analysis of glucose transporter 4 (GluT4). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of GluT4 relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse brain sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using

4.3 Discussion

4.3.1 Cholesterol, glucose and insulin profiles in the CNS of human apolipoprotein TR mouse models

There was no difference in the brain cholesterol level between both huApoE3 and E4 TR mice (Figure 11A). Earlier studies in NPC mouse model suggest that the level of ApoE protein may be associated with the brain cholesterol level. HuApoE expression in both mouse models differ significantly (Figure 12), however it does not seem to have any impact on brain cholesterol level. It is possible other than ApoE, other apolipoproteins such as ApoD (Franz, Reindl et al. 1999) may be regulating the cholesterol level. It is also noteworthy that extensive loss of cholesterol found in NPC mouse model was speculated to be the result myelin loss which has not been reported in huApoE4 TR mouse model.

I did not find any major difference in the plasma cholesterol of both huApoE3 and E4 TR mice (Figure 11B). This is in line with current literature as abnormal lipid metabolism and atherosclerotic symptoms were only observed when these animals underwent high fat diet.

Other than cholesterol metabolism, dysregulated glucose metabolism has also been associated with neurological diseases such as AD. Lower brain glucose metabolism together with ApoE4 have been associated with cognitive declination later in life (Reiman, Caselli et al. 1996; Drzezga, Riemenschneider et al. 2005; Reiman, Chen et al. 2005; Caselli, Dueck et al. 2009; Kalpouzos, Chetelat et al. 2009). PI3K/AKT signalling pathway which

plays an important role in glucose metabolism has been found to be altered (previous chapter) in NPC disease mouse model. In addition, insulin profile in NPC disease mouse model has also been shown to be affected (Ong, Lim et al. 2012). Owning to the above evidences, glucose and insulin assays were performed on brain samples of both huApoE TR mice.

Lower glucose levels were observed in huApoE4 TR mouse brain lysates at all three different points (12^{th}, 32^{nd} and 72^{nd} week). My data is in line with most reports whereby significant loss of glucose activity were observed in clinical and mouse models suffering from neurological impairment (Gerozissis 2008; Liu, Liu et al. 2011). These observations led me to speculate that glucose was not effectively transported into cells. Further insulin assays showed no difference in the insulin level of both 12 and 32 week old huApoE TR mice. 72 week old huApoE4 TR mice exhibited 25% higher brain insulin level. This might suggest that the brain has attempted to increase the amount of insulin in order to boost the glucose uptake in the cells. This is likely a feedback response due to prolong hypoglycaemic condition in the CNS. This is commonly observed in insulin resistant muscle cells whereby higher amount of insulin is required to allow the uptake of glucose into the cells. In summary, distinct glucose and insulin profiles were displayed in both animals and this may be due to huApoE isoform difference. This is also supported by reports of ApoE in associated with insulin in lipid metabolism studies (Descamps, Bilheimer et al. 1993; Ogbonna, Theriault et al. 1993).

In sharp contrast with the CNS, I did not pick up any difference in both the plasma glucose and insulin profiles of both huApoE TR mouse models. This indicates that plasma glucose and insulin may have little or no association with the CNS. It is likely that both the peripheral and CNS are mutually exclusive in huApoE TR mouse models.

4.3.2 huApoE protein level in the CNS

From the NPC studies, I learned that ApoE expression regulate the PI3K/AKT signalling pathway. Measurement of huApoE protein level was conducted via real time qPCR and Western blot analysis. The real time data showed consistent transcripts level in both huApoE TR mice across all three time points (12^{th}, 32^{nd} and 72^{nd} week). Western blot densitometry analysis, surprisingly, showed up to 45% lower huApoE protein in huApoE4 (compared to huApoE3) TR mouse brain lysates. The protein disparity is not the result of varying expression in both animals but due to varying protein stability. Firstly, both huApoE isoforms are regulated by the same muApoE regulatory elements; secondly, there was no difference in the brain huApoE transcripts of both huApoE TR mouse models. Lastly, huApoE4 has been reported to be less stable and prone to degradation (Huang, Liu et al. 2001; Riddell, Zhou et al. 2008) as compared to huApoE3.

It is also interesting to note that lower glucose content was detected in the cells as early as the 12^{th} week while the onset of huApoE degradation seem to be only visible at the 32^{nd} week onwards . This indicates that, other than huApoE protein level, huApoE isoform may be responsible for lower glucose uptake in

the brain cells as early as the 12th week. The molecular mechanism linking huApoE isoforms and glucose metabolism in the CNS remain unsolved, however comparable observations have been reported (Drzezga, Riemenschneider et al. 2005; Reiman, Chen et al. 2005; Ercoli, Siddarth et al. 2006; Rimajova, Lenzo et al. 2008; Langbaum, Chen et al. 2010; Reiman, Chen et al. 2010)

4.3.3 Loss of IRS2 in the CNS of huApoE4 TR mouse model

The cholesterol, glucose and insulin assays results prompted me to further investigate and identify possible molecular pathway/s which may lead to these observations. Parallel to my previous NPC studies, I decided to investigate the PI3K/AKT signalling pathway which plays a key role in glucose metabolism. IRS and IR-related proteins are involved in the upstream signalling of PI3K/AKT pathway. There was no change in the protein level of IRS1 despite huApoE isoform differences at all three different time points. However, I noticed significantly higher level of IRS2 in huApoE4 TR mice as early as the 12th week. Interestingly, by 72nd week, this trend reversed and it recorded up to 46% loss in IRS2 in huApoE4 TR TR mice.

The IRS2 KO mouse model has been widely used as for diabetic and/or glucose metabolism studies. Dysregulation of IRS2 in different cell types have been shown to affect the regulation of glucose transport (Sadagurski, Weingarten et al. 2005). In the CNS, IRS2 has been suggested to be involved in brain growth, neuronal proliferation (Schubert, Brazil et al. 2003), synaptic

plasticity (Costello, Claret et al. 2012) and longevity (Taguchi, Wartschow et al. 2007).

As discussed in the introduction, ApoE and ApoD are largely involved in the transportation of lipid in the CNS. Significant lower level of ApoE in the brain of huApoE4 TR mice may have affected the brain development at a young age. In order to compensate for this loss, up regulation of IGF1Rβ (to be discussed later) was observed. This in turn led to the notable increase in IRS2 as early as the 12th week. The significant loss of IRS2 in older mice (72 weeks old) was recorded. This coincides with clinical data whereby marked loss of IRS2 was also observed in elderly subjects with cognitive impairment. These changes also correlate with the severity of neurodegeneration (Freude, Schilbach et al. 2009).

4.3.4 PI3K/AKT signalling in the CNS of huApoE TR mouse models

Protein analysis of IRβ showed little difference between both huApoE TR mouse models. However, I recorded up to 104% increment of IGF1Rβ in 12 week old huApoE4 as compared to huApoE3 mouse. These data support earlier findings whereby IGF1Rβ may have resulted in notable higher IRS2 protein in 12 week old huApoE TR mice. Further analysis of p-IRβ and p-IGF1Rβ showed significant loss of these proteins in 32 and 72 week old female huApoE4 TR mice. The link between insulin receptor activity and IRS2 expression has been reported (White 2002; Brady 2004). Comparable observations were made in this study.

Tyrosine phosphorylation of IRS1 and IRS2 lead to the binding of SH2 domains containing proteins such as the PI3K p85 subunit which in turn activates the PI3K p110 subunit (Farese, Sajan et al. 2005). This allows the conversion of PIP2 to PIP3 leading to downstream activation of AKT. My results showed loss of p-IRβ and IGF1Rβ proteins, followed by diminishing IRS2 and PI3K p85 proteins in huApoE4 TR female mice, in particular older mice (72 week old). PI3K is widely documented for its roles in periphery tissues such as liver, muscle and adipocytes, however there is also a growing interest on its functionality in the CNS. Local administration of PI3K inhibitor in the CNS has been shown to block insulin signalling pathway (Obici, Feng et al. 2002; Obici, Zhang et al. 2002). These further enforce the importance of PI3K and the loss of PI3K p85 may affect other downstream proteins of this signalling pathway.

The loss of IRS2 was observed in 72 week old ApoE4 TR mice while loss of PI3K p85 subunit was observed in 32 and 72 week old ApoE4 TR mice. It is likely that other than IRS2, the expression of PI3K may be modulated by some other pathways. Existing data is unable to identify concrete evidence highlighting the effects of altered PI3K p85 subunit protein level to PI3K activity, and several reports shared similar challenge (Andreelli, Laville et al. 1999; Rieusset, Chambrier et al. 2001; Rieusset, Roques et al. 2001; Barbour, Mizanoor Rahman et al. 2005). Regardless, PI3K is recognized as a key player in insulin signalling. The loss of PI3K p85 will lead to the suppression of AKT activity. This coincides with current observations; significant loss of p-AKT

Threonine 308 and Serine 473 in older huApoE4 as compared to huApoE3 TR mice.

Lower PI3K/AKT signalling has been shown to impair neuroprotection (Dudek, Datta et al. 1997; Hong, Kwon et al. 2001; Hirai, Hayashi et al. 2004; Manning and Cantley 2007). The genetic effects of huApoE4 in the CNS of mice only surfaced at later time point. This indicates that the overall attenuated activity of PI3K/AKT signalling observed in huApoE4 animals is associated with the effects of ageing.

4.3.5 Lower GluT4 expression complements the observations in PI3K/AKT signalling pathway

The expression of GluT4 is significantly lower in the CNS of huApoE4 TR mouse at older time point (72 week old). This is likely attributed by the lower PI3K/AKT signalling. Previous animal and cell culture studies have also showed that PI3K/AKT signalling pathway regulates GluT4 activity (Cong, Chen et al. 1997; Hernandez, Teruel et al. 2001; Huang, Huang et al. 2009). Comparable observations were made in T2DM patients whereby the transcripts for PI3K are much lower in the skeletal muscle and adipose tissues (Ducluzeau, Perretti et al. 2001).

In summary, the CNS of huApoE3 and huApoE4 TR mouse models exhibit different PI3K/AKT signalling profiles with increasing age. This is coupled with significant loss of GluT4 protein expression and abnormally higher insulin in older huApoE4 TR mice. It is possible that the attenuated

PI3K/AKT signalling led to the loss of GluT4 protein expression. As part of the body's effort to compensate for the loss of GluT4 and together with falling glucose concentration in the CNS, it drove the insulin level higher.

4.4 Summary

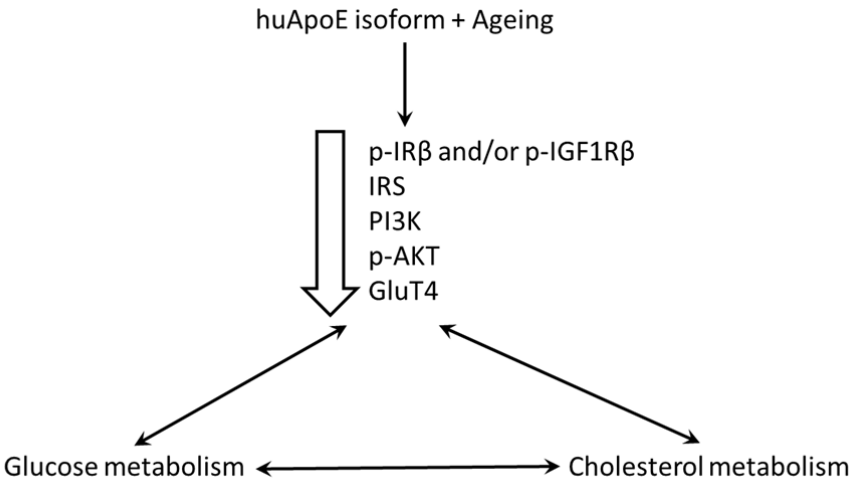

Figure 21 Overall representation of my findings addressing the effects of huApoE isoform and ageing on PI3K/AKT signalling in the CNS

Varying glucose and insulin profiles were observed in the CNS. Further analysis showed attenuated PI3K/AKT signalling in the CNS. The two major variables in this study are huApoE isoform and ageing.

Distinct glucose and insulin profiles were observed in the CNS of both huApoE targeted replacement mouse models. In fact, most of these differences were prominent in older mice. Physiological impact of ageing, huApoE genotype and protein level were three of the key areas I have addressed in this study.

The huApoE3 and E4 TR mouse models are excellent tool for us to study the effect of huApoE genotype on the physiology of the animal. ApoE isoform has been reported to be associated with hypometabolism, particularly in the CNS and the effects were evident in elderly population (Langbaum, Chen et al. 2010; Tao, Liu et al. 2011; Chen, Ayutyanont et al. 2012). Limited information is available addressing the link between huApoE and PI3K/AKT signalling except for a recent microarray study (Simpson, Ince et al. 2011). These results together with existing literature provide considerable amount of information linking these three factors.

CHAPTER 5

HUMAN APOLIPOPROTEIN E POLYMORPHISM AFFECTS INSULIN SIGNALLING IN THE LIVER OF HUAPOE TR MOUSE MODELS

5 Human apolipoprotein E polymorphism affects insulin signalling in the liver of huApoE TR mouse models

5.1 Introduction

5.1.1 Apolipoprotein E isoforms affects plasma cholesterol

ApoE plays a central role in the regulation of periphery lipoproteins. It functions as a ligand for LDL-, VLDL- and remnant receptors. It is largely synthesized by hepatocytes and macrophages in the peripheral system. More importantly, ApoE is responsible for the clearance of lipoproteins through the reverse cholesterol transport system by the liver. This process reduces plasma cholesterol associated with atherosclerosis susceptibility (Mahley 1988; Mahley and Rall 2000). HuApoE3 and E4 bind to LDLR with different affinity. This gives rise to varying efficiency in the clearance of plasma cholesterol. Other than cholesterol, clinical study has also showed significantly lower plasma ApoE in huApoE4 carrier (Larson, Ordovas et al. 2000).

HuApoE4 carriers are associated with increase plasma cholesterol concentration and a higher risk of atherosclerosis. In particular, huApoE4 carriers have exceptionally high LDL-cholesterol level (Gylling, Kontula et al. 1995; Mahley and Rall 2000; Han, Heath et al. 2002; Tascilar, Dursun et al. 2009).

ApoE-deficient mice have been reported to develop severe dyslipidemia and atherosclerotic lesions (Nakashima, Plump et al. 1994). Mouse models expressing huApoE3 and E4 have normal cholesterol profiles and do not

develop atherosclerosis (Sullivan, Mezdour et al. 1997; Knouff, Hinsdale et al. 1999). Under western high-fat diet, both mouse models suffer from hypercholesterolemia and huApoE4 TR mouse model displays more severe symptoms. In line with this observation, a report has also shown that ApoE4 carriers are more sensitive to diet and exhibit wider variation in cholesterol level (Kallio, Salmenpera et al. 1998).

5.1.2 Apolipoprotein E and glucose metabolism

ApoE polymorphism has been suggested to modify the effect of insulin on coronary artery disease, body mass, TG, total and LDL cholesterol levels (Laakso, Kesaniemi et al. 1991; Despres, Verdon et al. 1993; Orchard, Eichner et al. 1994; Valdez, Howard et al. 1995). Diabetes has also been reported to be associated with 2-4 fold higher risk of coronary artery disease (Soedamah-Muthu, Chaturvedi et al. 2004). It is speculated that glucose dysfunction may be linked to ApoE and/or cholesterol metabolism in the peripheral system. A recent study on HuApoE TR mouse models drew similar conclusion. HuApoE4 TR mice gained less weight but showed signs of impaired glucose tolerance as compared to huApoE3 TR mice (Arbones-Mainar, Johnson et al. 2010).

On the other hand, some literature indicates no correlation between ApoE polymorphism and insulin resistance. However, the author did highlight that there are some limitations to his study (Meigs, Ordovas et al. 2000). In summary, the molecular mechanisms associating insulin levels or insulin

resistance and ApoE isoform remains elusive (Shriver, Boerwinkle et al. 1991; Meigs, Ordovas et al. 2000).

5.1.3 Knowledge from previous studies

In the last two chapters, I learned that differential ApoE expression and isoform have an impact on the CNS. Specifically, my observations showed distinct changes in the PI3K/AKT signalling pathway in the brain. These effects appear to be limited to the CNS as the cholesterol, insulin and glucose results between the plasma and the CNS exhibited different profiles. However, insulin dosage to the peripheral system of human subjects with different huApoE genotype has been shown to have differential cognitive effect (Craft, Asthana et al. 1999; Craft, Asthana et al. 2003). It is suggested that huApoE genotype may influence the patients' sensitivity to insulin. Other than isoform differences, I found significantly lower huApoE4 as compared to huApoE3 in the CNS of these mouse models. Similar trend has been reported in human plasma ApoE concentration (Larson, Ordovas et al. 2000). The expression level of ApoE in the liver of these huApoE TR mouse models has not been investigated.

5.1.4 Experimental considerations

Other than huApoE genotype variation, this study will study the expression level of huApoE in the liver since it is the major organ synthesizing huApoE. I have also incorporated ageing as a variable for this study. These animals were kept up to 72 weeks to investigate the effect of ageing. Only female animals were used for this study. Cholesterol, glucose and insulin assays were

conducted in huApoE TR mouse liver lysates to determine potential physiological differences. Earlier data showed distinct assay profiles in both the CNS and plasma thus indicating that both the peripheral and CNS function independently.

5.1.5 Hypothesis

Published data suggest that differential huApoE protein levels and/or huApoE genotype is/are associated cholesterol and possibly glucose metabolism. I am interested to find out if PI3K/AKT signalling in the peripheral system which plays a key role in the regulation of glucose and insulin exhibits different profiles as observed in the CNS of huApoE TR mouse models.

It hypothesized that huApoE isoform differences together with ageing may affect glucose metabolism in the liver. In line with my earlier CNS data, it is speculated the PI3K/AKT signalling pathway may be affected. No publication has addressed glucose metabolism and/or PI3K/AKT signalling in these mouse models over a time course. The academic impact of deciphering this molecular mechanism may be useful and complementary to earlier CNS data.

5.2 Results

5.2.1 Total cholesterol in the liver of female huApoE3 and huApoE4 TR mouse models

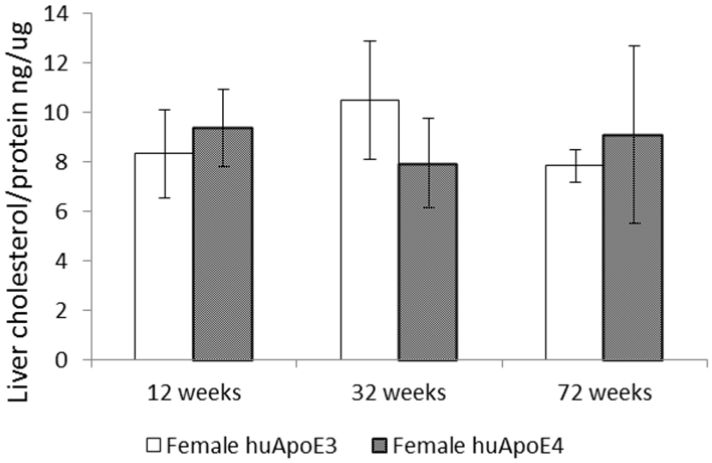

Figure 22 Total amount of cholesterol of liver lysates in female huApoE3 and huApoE4 TR mice

Amplex red cholesterol assays of samples from liver lysate across all three time points (12th, 32nd and 72nd week) of female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice. The liver lysate was normalized to its total protein measured via BCA method. Each value represents the mean ± SD for individual mouse liver samples (n = 5 at each time point for each mouse line). Whereby, * p value $\leq$ 0.05, ** p value $\leq$ 0.01, *** p value $\leq$ 0.001 using Student's t-test.

No significant change was observed in the cholesterol content of the liver tissue lysates (Figure 22) of huApoE3 and huApoE4 TR mice at 12th, 32nd and 72nd week time points

5.2.2 HuApoE expression of female huApoE3 and huApoE4 TR mice

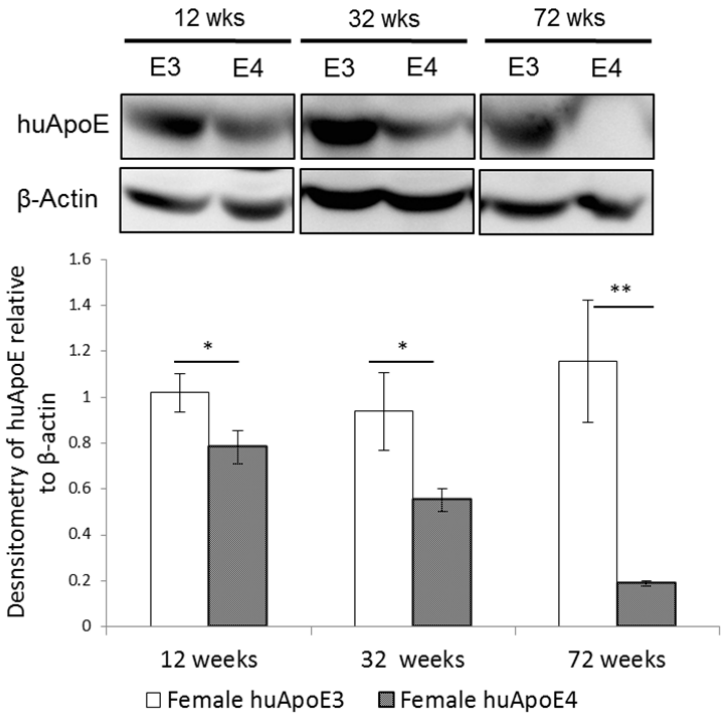

Figure 23 Liver huApoE protein expression level in huApoE3 and huApoE4 TR female mice

Western blot analysis of human Apolipoprotein E (huApoE) and β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of huApoE relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

Protein analysis via Western blot on liver samples of these two mouse models indicate significantly lower huApoE protein expression in huApoE4 TR at 12 week old (-24%), 32 week old (-42%) and 72 week old (-84%) female mice (Figure 23). These results suggest lower amount of huApoE in huApoE4 TR as compared to huApoE3 TR mice. This phenomenon was observed in liver samples as early as the 12th week.

5.2.3 Glucose and insulin level in the liver of female huApoE3 and huApoE4 TR mice

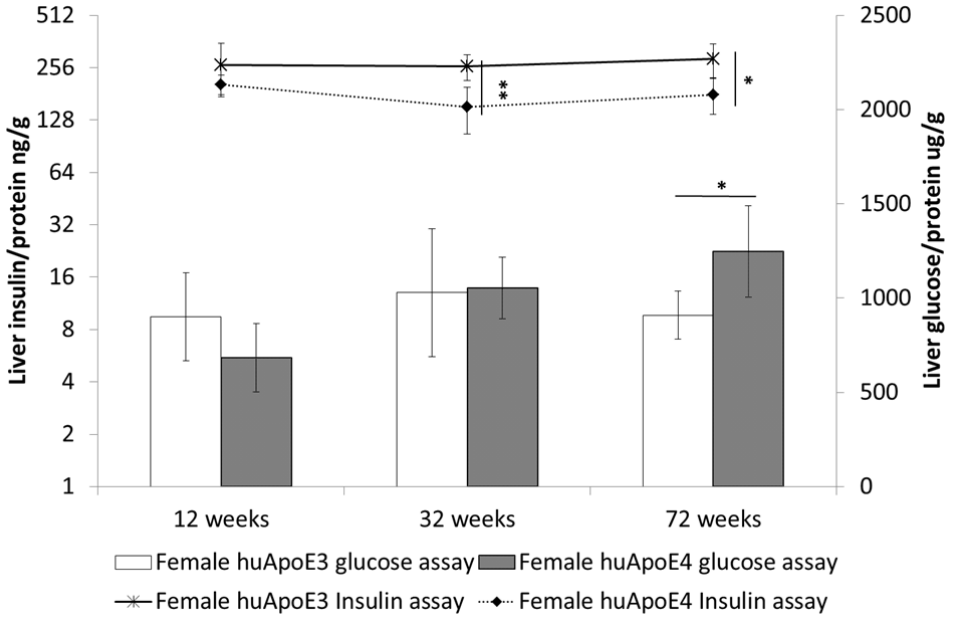

Figure 24 Total glucose and insulin in liver lysates of female huApoE3 and huApoE4 TR mice

Glucose assays of samples from liver lysates across all three time points (12th, 32nd and 72nd week) of female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice. Insulin assays of samples from liver lysates across all three time points (12th, 32nd and 72nd week) of female huApoE3 (solid line) and female huApoE4 (dotted line) TR mice. Glucose and insulin readings were normalized to its total protein measured via BCA method. Each value represents the mean ± SD for individual mouse liver sample ($n \leq 3$ at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

Relative similar total glucose was detected in the 12 and 32 week old liver lysates. However notable increase in total glucose (+37%) of huApoE4 liver lysate was observed in the 72nd week time point (Figure 24). Insulin assays showed little change at the 12th week time point. Insulin was significantly lower in huApoE4 liver lysate as compared to huApoE3 at both 32nd (-42%) and 72nd (-38%) week time points (Figure 24).

5.2.4 PI3K-AKT protein profile in the liver of huApoE3 and huApoE4 TR mice

Western blot densitometry studies were conducted in liver tissue lysates. There was little difference in the expression of IRS1 at earlier time points, 12th and 32nd week. At 72nd week, significant loss of IRS1 expression (-95%) (Figure 25A and 25C) was observed in huApoE4 as compared to huApoE3 TR mice. For IRS2, I observed relatively uniform expression in 12 – 72 week old liver lysates of both mouse models (Figure 25A and 25B).

IGF1Rβ was evenly expressed in huApoE3 and huApoE4 TR female mouse liver lysates at all three time points (Figure 26A and 26B). At the 12th and 32nd week, there was no change in the expression of IRβ in both mouse models but at the 72nd week, I found significant increase in the expression of IRβ (+48%) in huApoE4 TR mice (Figure 26A and 26C). p-IRβ and p-IGF1Rβ showed no expressional difference in 12 and 32 week old animals. Significant drop in p-IRβ and p-IGF1Rβ (-93%) expression was observed in 72 week old huApoE4 TR mice as compared to huApoE3 TR mice (Figure 26A and 26D).

The liver lysates analysis of PI3K p85 and p110 showed little or no change in the expression of these proteins in 12 and 32 week old huApoE TR female mice. Notable loss of both PI3K p85 (-42%) and p110 (-71%) proteins were observed in older female huApoE TR mice (72 week old) (Figure 27). Subsequently, I had also studied the expression of AKT proteins in female huApoE TR mouse liver samples. Comparable expression of AKT was

observed in both huApoE3 and huApoE4 TR female mice at all three time points (Figure 28A and 28C). There was no change in p-AKT S473 in 12 week old mice while significant loss of p-AKT S473 was observed at 32 (-58%) and 72 (-44%) week old huApoE4 as compared to huApoE3 female mice (Figure 28A and 28B). Similar trend was observed for p-AKT T308 but the onset of this loss was only observed at 72 week old huApoE4 (-54%) mice. 12 and 32 week old mice have relatively similar expression of p-AKT T308 (Figure 28A and 28D). Lastly, there was little or no change in GluT4 at 12 and 32 week old huApoE TR mice. However at the 72nd week time point, there is 39% loss of GluT4 in huApoE4 as compared to huApoE3 TR female mouse liver lysates (Figure 29).

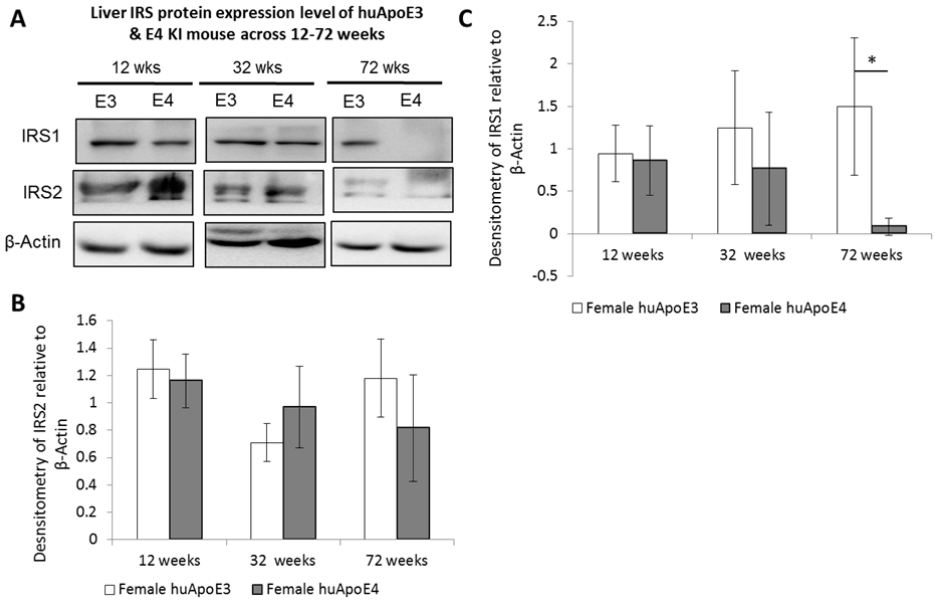

Figure 25 Liver insulin receptor substrate (IRS) proteins expression level of huApoE3 & E4 TR mice

(A) Western blot analysis of insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) IRS1 and (C) IRS2 in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver samples (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

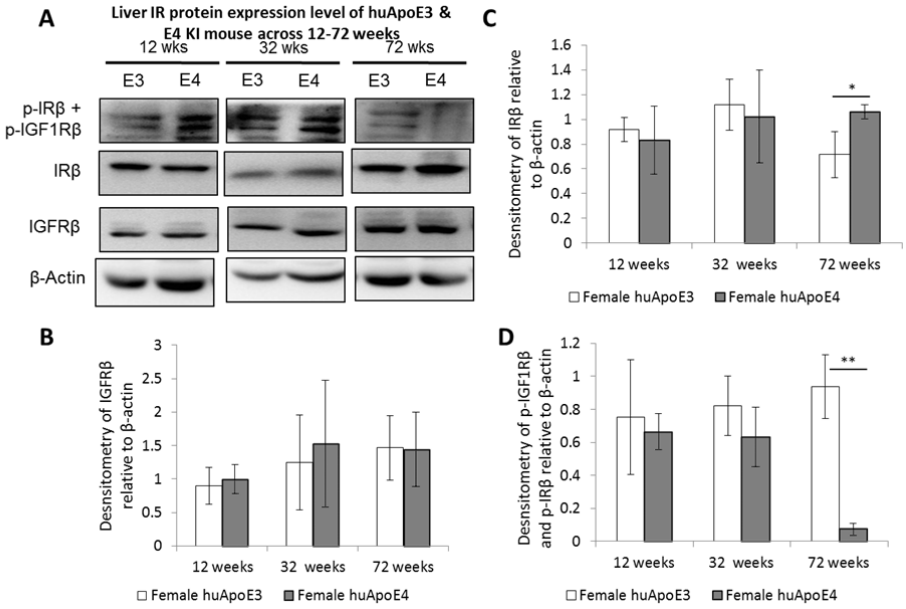

Figure 26 Liver insulin receptor (IR) proteins expression level of huApoE3 & E4 TR mice

(A) Western blot analysis of phosphorylated insulin receptor β (p-IRβ), phosphorylated insulin growth factor receptor β (p-IGFRβ), insulin receptor β (IRβ) and insulin growth factor receptor β (IGF1Rβ). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) p-IRβ + p-IGFRβ , (C) IRβ and (D) IGF1Rβ relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

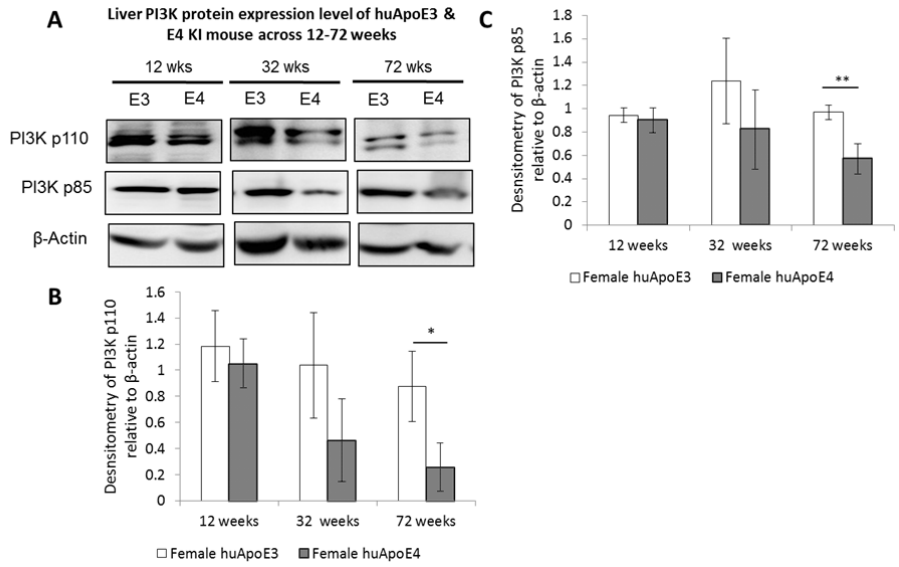

Figure 27 Liver phosphatidylinositol 3-kinases (PI3K) proteins expression level of huApoE3 & E4 TR mice

(A) Western blot analysis of phosphatidylinositol 3-kinases p110 (PI3K p110) and phosphatidylinositol 3-kinases p85 (PI3K p85). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) PI3K p110, (C) PI3K p85 relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver samples (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

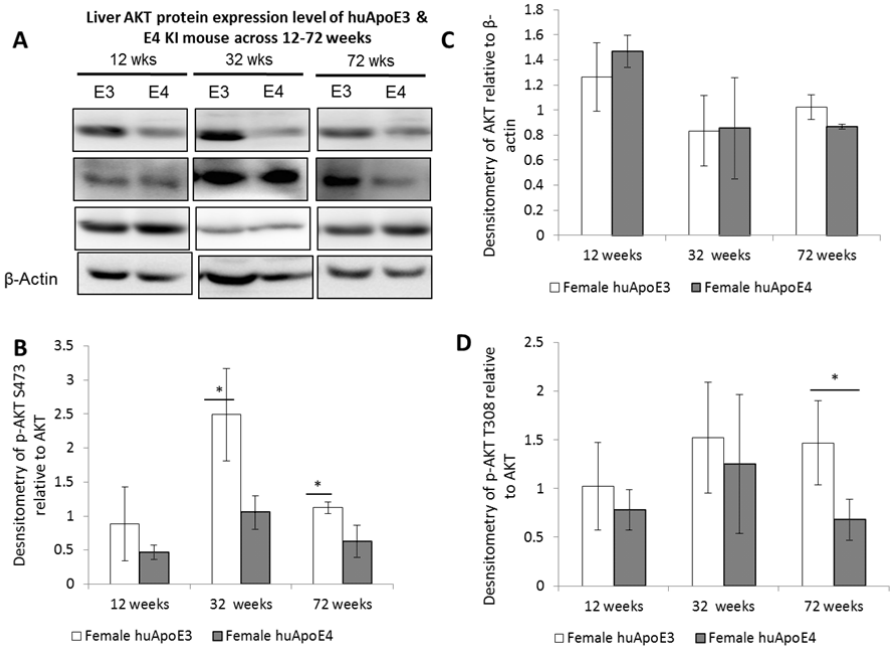

Figure 28 Liver AKT proteins expression level of huApoE3 & E4 TR mice

(A) Western blot analysis of phosphorylated AKT Serine 473 (p-AKT S473), phosphorylated AKT Threonine 308 (p-AKT T308) and AKT. β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of (B) p-AKT S473, (C) AKT and (D) p-AKT T308 relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver samples (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

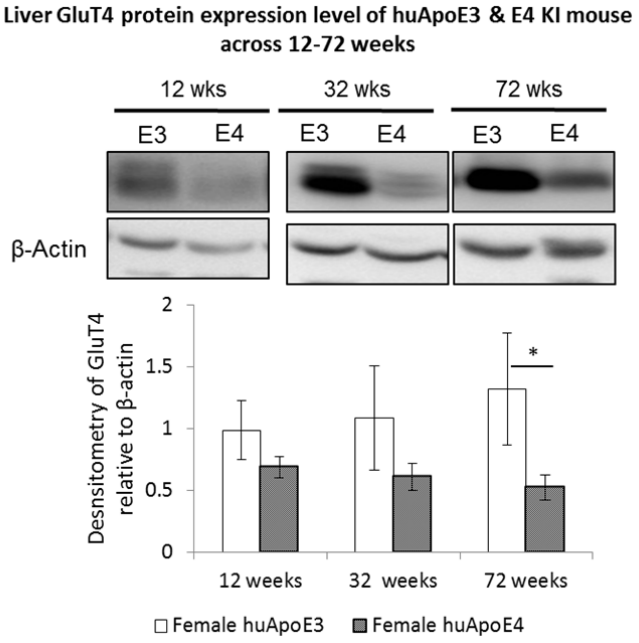

Figure 29 Liver glucose transporter 4 protein expression level of huApoE3 & E4 TR mice

Western blot analysis of glucose transporter 4 (GluT4). β-actin was immnunoblotted to ensure similar gel loading of the starting material in each sample. Densitometry analysis of GluT4 relative to β-actin level in female huApoE3 (white bar) and female huApoE4 (grey bar) TR mice were performed using the NIH ImageJ software. Each value represents the mean ± SEM for individual mouse liver sample (n = 3 at each time point for each mouse line). Whereby, * p value ≤ 0.05, ** p value ≤ 0.01, *** p value ≤ 0.001 using Student's t-test.

5.3 Discussion

5.3.1 Cholesterol and glucose metabolism in the liver

My data showed that the regulation of cholesterol across the blood plasma, liver and brain lysates is not affected by the genotype and age differences. Mouse models expressing huApoE3 and E4 have been shown to have similar cholesterol profiles and do not develop atherosclerosis (Sullivan, Mezdour et al. 1997; Knouff, Hinsdale et al. 1999). My results showed similar observations and ageing did not affect the cholesterol level. However, under western high-fat diet, both mouse models suffer from hypercholesterolemia. The same study also showed that ApoE4 TR animals are more sensitive to diet and exhibit wider variation in cholesterol level (Kallio, Salmenpera et al. 1998). This is in line with clinical data whereby HuApoE4 carriers are associated with increase plasma cholesterol concentration and a higher risk of atherosclerosis (Gylling, Kontula et al. 1995; Mahley and Rall 2000; Han, Heath et al. 2002; Tascilar, Dursun et al. 2009).

Earlier assay studies of glucose and insulin on brain lysates and blood plasma showed distinct profiles in both the peripheral and CNS. In this study, notable glucose and insulin changes were also observed in the liver of 72 week old huApoE TR mice. Although the circulating blood system was not affected, the regulation of glucose and insulin in the liver exhibited changes associated with ApoE genetic variation, particularly in older animals.

ApoE polymorphism has been reported to affect the effects of insulin on coronary artery disease, body mass, TG, total and LDL cholesterol levels

(Laakso, Kesaniemi et al. 1991; Despres, Verdon et al. 1993; Orchard, Eichner et al. 1994; Valdez, Howard et al. 1995). A recent study on huApoE TR mouse models drew similar conclusion. It was found that huApoE4 TR mice gained less weight but showed signs of impaired glucose tolerance as compared to huApoE3 mice (Arbones-Mainar, Johnson et al. 2010).

Data from this study showed higher level of glucose and lower amount of insulin in the liver of huApoE4 TR mice as compared to huApoE3. These results suggested that huApoE genotype and/or protein level may influence the regulation of glucose and insulin in the peripheral system of older animals, regardless of cholesterol level. In order to verify this statement, I have explored key the PI3K/AKT signalling pathway which may contribute to this phenomenon.

5.3.2 HuApoE isoforms and PI3K-AKT signalling pathway

Other than cholesterol and glucose assays, clinical studies also showed significantly lower plasma ApoE in huApoE4 carrier (Larson, Ordovas et al. 2000). This is particularly interestingly as both the liver and brain tissues in huApoE TR mice displayed lower huApoE protein level too. Glucose and insulin concentration have been associated with varying huApoE level (Dashti, Williams et al. 1989; Kawashima, Chen et al. 2009; Arbones-Mainar, Johnson et al. 2010).

Likewise to the CNS, significantly lower liver huApoE expression was observed in huApoE4 TR mouse models across all time points. This supported my earlier conclusion and existing reports (Huang, Liu et al. 2001; Riddell, Zhou et al. 2008) that huApoE4 may be more prone to degradation than huApoE3 thus leading to the differences.

Preliminary Western blot analysis showed severe loss of IRS1 in huApoE4 TR liver lysates as compared to huApoE3 at the 72nd week time point while IRS2 showed little change across all time points. Serine/ Threonine phosphorylation of IRS1 has been reported to trigger IRS1 degradation (Pederson, Kramer et al. 2001). Up to 93% loss of p-IRβ and p-IGF1Rβ were also observed in the liver of 72 week old huApoE4 TR mice. The above two findings demonstrated that huApoE isoforms may regulate IRS proteins activation and possibly associated with ageing.

Decreased insulin binding followed by reduced receptor tyrosine kinase activity and increased IRS1 protein degradation have been showed in liver, muscle and adipocytes of TD2M mouse models (Kerouz, Horsch et al. 1997; Paz, Hemi et al. 1997; Li, DeFea et al. 1999; Pederson, Kramer et al. 2001). IRS1 is the main docking protein for downstream signalling to PI3K/AKT signalling pathway.

Western blot results showed that lower PI3K p85 and p100 proteins which are likely due to reduced tyrosine activated IRS1 in 72 week old huApoE4 as compared to huApoE3 TR mice. The importance of PI3K and its role in the peripheral system such as the liver (Farese, Sajan et al. 2005) has been highlighted in the previous discussion. These findings also showed significantly lower AKT Serine 473 and Threonine 308 phosphorylation in older mice. The entire series of data demonstrated strong correlation between PI3K/AKT signalling pathway and the effects of huApoE isoforms with ageing. An attenuated PI3K/AKT signalling due to reduced IRβ activity and IRS1 degradation are consistently recorded at later time point (72^{nd} week). Western blot mapping PI3K/AKT signalling pathway is possibly associated with the preliminary glucose and insulin assays. In addition, ApoE has been reported to be involved in insulin and glucose metabolism (Dashti, Williams et al. 1989; Kawashima, Chen et al. 2009; Arbones-Mainar, Johnson et al. 2010).

Western blot analysis also exhibited loss of GluT4 across all time points in huApoE4 as compared to huApoE3 TR liver lysates. The down regulation of the PI3K/AKT signalling pathway was only prominent in older huApoE4 TR

mice. In contrast, the onset for the loss of GluT4 was consistently found across all time points. It is possible that loss of GluT4 may be due to several factors other than PI3K/AKT signalling pathway (Dohm, Elton et al. 1991; Ducluzeau, Perretti et al. 2001; Kampmann, Christensen et al. 2011). One of these pathways includes the AMPK signalling pathway which regulates glucose utilization via the expression and translocation of GluT4 to the plasma membrane. This process is independent of insulin (Hayashi, Hirshman et al. 1998; Russell, Bergeron et al. 1999; Witczak, Sharoff et al. 2008).

5.4 Summary

In summary, the hepatic system of huApoE3 and huApoE4 TR mouse models exhibit distinctive glucose, insulin and PI3K/AKT signalling profiles, particularly in older mice. In addition, significantly attenuated PI3K/AKT signalling pathway was mapped out in the huApoE4 TR mouse hepatic system. Unfortunately, it remains a challenge to differentiate if these results were associated with varying huApoE isoform or protein level. Notable loss of GluT4 protein expression in huApoE4 TR mice was also observed. It is possible that PI3K/AKT signalling pathway is involved in the regulation of glucose and insulin in the hepatic system.

CHAPTER 6

CONCLUDING REMARKS

6 Concluding remarks

The biology I have focused on revolves around ApoE and its impact on the regulation of lipid and glucose, especially in the CNS. Dysregulation of lipid and glucose have been associated with neurological, hypercholesterolemia and other metabolic disease such as T2DM. Longitudinal studies on NPC and huApoE mouse models had also allowed me to address the importance of ageing. This is especially in huApoE TR mouse model whereby dysregulation of glucose metabolism is largely prevalent especially in older mice.

The initial NPC mouse model study concluded that varying muApoE expression level and lipid metabolism may have disrupted the PI3K/AKT signalling pathway. I was also able to identify a possible role for Aebp1 in the CNS via PI3K/AKT signalling pathway. In addition to these results support the trio relationship across PI3K/AKT, glucose (insulin) and lipid (ApoE) metabolism in the CNS.

I have extended my study of ApoE and lipid metabolism into huApoE TR mouse models. Preliminary results produce virtually no change in the cholesterol profiles between both isoforms across all time points. It was surprising that ageing did not affect the cholesterol profiles. Conversely, I did noticed varying glucose and insulin profiles between the two huApoE TR mouse models in both the peripheral and CNS. Comparable to the NPC study, severely attenuated PI3K/AKT signalling pathway was recorded in both the peripheral and CNS of huApoE TR mice.

One of the major limitations of huApoE TR animal studies is differentiating if these observations are consequences of varying huApoE isoforms or protein levels. The data from NPC mouse model leans towards the hypthesis that varying ApoE level may be the causative factor. Regardless, these data support the underlying hypothesis that PI3K/AKT signalling pathway and the regulation of glucose might be linked to huApoE and ageing.

Conclusively, this thesis addressed the importance of ApoE genotype and its association with potential glucose metabolic pathways, possibly neurological and metabolic diseases. The effects of isoform differences become more evident with the effects of ageing.

CHAPTER 7

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7 Bibliography

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