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The effects of distinct fatty acids on central leptin sensitivity, hypothalamic inflammation, and central-regulated hepatic metabolism

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The effects of Distinct Fatty Acids on Central Leptin Sensitivity, Hypothalamic Inflammation, and Central-regulated Hepatic Metabolism

A thesis submitted in fulfilment of the
requirements for the award of the degree

DOCTOR OF PHILOSOPHY

From

University of Wollongong

By

LICAI CHENG

2015

CERTICATION

I, Licai Cheng, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is entire my own work unless referenced or acknowledged. This manuscript has not been submitted for qualification at any other academic institution.

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Licai Cheng

June 30, 2015

STATEMENTS

In accordance with the University of Wollongong thesis committee 'Guidelines for Preparation and Submission of HDR Thesis (September 2012), this PhD thesis is presented in 'Journal Article Style'. It is comprised of two original studies published and two original studies submitted in peer reviewed journals of which I am the first author. I hereby declare that I am the primary designer of these studies, have carried out experimental procedures, data analysis and manuscript preparation.

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June 30, 2015

I consent to the presentation of this PhD in 'Journal Article Style' and I acknowledge the above statement pertaining to student contribution to be correct.

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STATEMENT OF CONTRIBUTION OF OTHERS

I, Licai Cheng, declare that this thesis, submitted in fulfilment the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is entirely my own work unless otherwise referenced or acknowledged. Co-authors (Xu-Feng Huang, Yinghua Yu, and Alexander Szabo) of the journal articles included in the thesis are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts. Co-author Qingsheng Zhang is the research fellow in our school, who has provide comments on revision of my manuscripts. Co-author Hongqin Wang contributed to technical training and data analysis. Co-author Ms. Danielle Camer is my team member for editorial revision of the manuscript. Co-author Yizhen Wu is my team member for data analysis.

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LIST OF ABBREVIATIONS

DIO	Diet-induced obesity
LepR	Leptin receptor
STZ	Streptozotocin
ICV	Intracerebroventricular
G6Pase	Glucose 6-phosphatase
PEPCK	Phosphoenolpyruvate carboxykinase
GLUTs	Glucose transporters
GK	Glucokinase
FAS	Fatty acid synthase
ACC	Acetyl-CoA carboxylase
SCD1	Stearoyl-CoA desaturase 1
CPT1	Carnitine palmitoyltransferase 1
PPARα	Peroxisome proliferator-activated receptor α
SREBP-1c	Sterol regulatory elementbinding protein 1c
ACOX	Peroxisomal acyl-coenzyme A oxidase 1
HMG-CoA reductase	3-hydroxy-3-methylglutaryl-CoA reductase
ApoA1	Apolipoprotein A1
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
TH	Tyrosine hydroxylase
TG	Triglyceride
ARC	Arcuate nucleus
PVN	Paraventricular nucleus
VMH	Ventromedial hypothalamus
JAK2	Janus activated kinase 2
STAT3	Signal transducer activator of transcription 3
PI3K	Phosphoinositide 3- kinase
SOCS3	Suppressor of cytokine signalling 3
Akt/PKB	Protein kinase B
FOXO1	Forkhead box protein O1

NPY	Neuropeptide Y
POMC	Proopiomelanocortin
AgRP	Agouti-related peptide
CART	Cocaine- and amphetamine-related transcript
TNF-α	Tumor necrosis factor α
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
MyD88	Myeloid differentiation factor-88
TRAF6	Tumor necrosis factor receptor-associated factor-6
IKK-β	IκB kinase β
NF-κB	Nuclear factor -kappa B
JNK	c-Jun N-terminal kinase
TLR4	Toll-like receptor 4
RT-PCR	Real-time polymerase chain reaction
PA	Palmitic acid
ARA	Arachidonic acid
DHA	Docosahexaenoic acid
ALA	α-linolenic acid
SFA	Saturated fatty acids
PUFA	Polyunsaturated fatty acids

ABSTRACT

Consumption of a fat-rich diet is implicated in the development of central leptin resistance and obesity in modern societies. Epidemiological evidence suggests that saturated fatty acids (SFA) and n-6 polyunsaturated fatty acids (n-6 PUFA), highly consumed in Western diets, induce potent inflammation and impair leptin signalling in the hypothalamus, leading to the dysregulation of central leptin on body energy homeostasis and peripheral metabolism. However, n-3 PUFA and n-3 PUFA derivatives have well-known anti-inflammatory properties, and exert anti-obesity effects by improving central leptin sensitivity and its metabolic action in peripheral tissues. However, the role and mechanism of distinct fatty acids, especially directly act on central nervous system, regulate central leptin sensitivity, hypothalamic leptin signalling pathways, and hepatic energy homeostasis remain largely undiscovered.

The present thesis aims to determine the effect of intracerebroventricular (icv) injection of distinct fatty acids on central leptin sensitivity in C57BL/6J male mice. Body energy homeostasis, hypothalamic leptin signalling, and centrally regulated hepatic glucose and lipid metabolism in response to distinct fatty acids will be characterised. The contribution of hypothalamic inflammatory effects induced by different fatty acids will also be investigated. The fatty acids to be examined are SFA palmitic acid (PA), n-6 PUFA arachidonic acid (ARA), n-3 PUFA docosahexaenoic acid (DHA), and n-3 PUFA derivative α-ethyl DHA ethyl ester.

We demonstrate that the icv administration of PA and ARA induces central leptin resistance, evidenced by the inhibition of central leptin's suppression on food intake and body weight gain. In addition to central leptin resistance, the hypothalamic leptin JAK2-STAT3 and PI3K-Akt signalling pathways were impaired, with the down-regulation of

leptin signalling mediators pSTAT3, pJAK2, pAkt, and pFOXO1. Furthermore, the central administration of PA and ARA blunted the leptin-induced decrease of hepatic gluconeogenesis, glucose transportation, lipogenesis, cholesterol synthesis, and increase in hepatic β-oxidation. PA and ARA induced potent hypothalamic pro-inflammatory effects and increased pro-inflammatory cytokines and inflammatory mediators, as well as increased leptin signalling negative regulator SOCS3. On the other hand, central injection of DHA and DHA derivative exerted an anorexigenic effect by reducing energy intake and body weight gain in high-fat diet (HFD) mice. Both DHA and DHA derivative improved leptin JAK2-STAT3 and PI3K-Akt signalling in the hypothalamus, and consequently restored central leptin-mediated hepatic glucose and lipid metabolism. In addition, we also demonstrate that PA and ARA can inhibit, while DHA can improve central leptin action in mediating hypothalamic sympathetic activity, which may associated with the impaired or promoted hepatic energy metabolism.

In summary, elevated central PA and ARA concentrations induce leptin resistance and pro-inflammatory response in the central nervous system, which is associated with the dysregulation of the central leptin effect on energy homeostasis and hepatic metabolism. DHA and DHA derivative reverse HFD-induced adiposity, and decrease hypothalamic inflammation, which contributes to an increased central leptin sensitivity and improved regulation of hepatic metabolism. Thus, the administration of distinct fatty acids may provide realistic and alternative therapeutic strategies for the treatment of obesity and associated metabolic disturbances.

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Chapter One

1.1 Introduction

The prevalence of obesity is a major worldwide health problem, has led to increasingly a number of life threatening diseases as well as enormous associated personal, social and economic costs. There is an urgent need for improved therapeutics and a better understanding of the physiological process that balances energy intake and energy expenditure. Leptin resistance is a key feature of obesity and related metabolic disorders such as type II diabetes and metabolic syndrome. The consequence of leptin resistance is an inability to regulate energy intake and expenditure properly by utilising leptin for negative energy balance regulation. Improving central leptin sensitivity will break the vicious cycle of the dysregulation of energy balance evident in obesity.

Diets contain different types of fats. Saturated fatty acids (SFA), n-3 polyunsaturated fatty acids (n-3 PUFA), and n-6 PUFA have been shown to differentially modulate overall energy metabolism by affecting central leptin sensitivity. For instance, both dietary and central administrations of SFA have been shown to induce central leptin resistance, accompanied by defective leptin signal transducer activator of transcription 3 (STAT3) and phosphoinositide 3-kinase (PI3K) signalling in the hypothalamus (Kleinridders et al., 2009, Bates et al., 2003, Munzberg et al., 2004). n-6 PUFA can increase the risk of leptin resistance, obesity, and diabetes in humans and rodents (Phillips et al., 2010, Nuernberg et al., 2011). On the other hand, n-3 PUFA and n-3 PUFA derivatives have been shown to exert some beneficial effects on leptin resistance and obesity (Pimentel et al., 2012, Rossmeisl et al., 2009, Cintra et al., 2012). However,

the direct effects of SFA and n-3 PUFA on leptin signalling in specific regions of hypothalamus and neuronal-mediated hepatic metabolism are not thoroughly elucidated. And especially the effects of n-6 PUFA and n-3 PUFA derivatives on central leptin sensitivity, hypothalamic leptin signalling and neuronal mediated hepatic metabolism are largely unknown.

Evidence indicates that central leptin plays a primary role in the regulation of glucose and lipid metabolism in the liver and other tissues (Denroche et al., 2012). Leptin administration enhances insulin-mediated suppression on hepatic glucose production, hepatic gluconeogenesis, and glucose transportation in rodents (Rossetti et al., 1997, Burcelin et al., 1999, German et al., 2011, Hidaka et al., 2002). Moreover, mounting evidence indicates that leptin has a beneficial effect on hepatic lipid metabolism in regulating lipogenesis, fatty acid β-oxidation, and cholesterol metabolism (Prieur et al., 2008, Gallardo et al., 2007). In addition, it has been suggested that the central leptin action on hepatic metabolism can be regulated by the alteration of central leptin sensitivity. For instance, HFD rich in SFA induces decreased central leptin sensitivity and defective leptin signalling in the hypothalamus, which leads to the dysregulation of peripheral metabolism, including hepatic steatosis, hyperglycaemia, and lipidaemia etc. (Warne et al., 2011, Milanski et al., 2009). Leptin signalling pathways, janus activated kinase 2 (JAK2)-STAT3 and pI3K-protein kinase B (Akt) signalling in the hypothalamus have been shown to be involved in hepatic glucose and lipid metabolism (Buettner et al., 2006, Buettner et al., 2008, Morton et al., 2005). For instance, attenuated hypothalamic leptin PI3K signalling is reported to contribute to adiposity and hepatosis in diet-induced obesity (DIO) (Warne et al., 2011). Therefore, determining the effects of distinct fatty acids on central leptin sensitivity will help us to understand the

mechanism underlying central leptin resistance, obesity, and associated metabolic disturbances.

The mechanism of central leptin resistance and DIO has not yet been elucidated completely. Hypothalamic inflammation has been shown to be an important contributor to leptin resistance and DIO (Carvalheira et al., 2003, Posey et al., 2009). Recently, the signalling pathways, IKK-β/nuclear factor-kappa B (NF-κB), toll-like receptor 4 (TLR4), and JNK signalling in the hypothalamus, have been suggested to be implicated in the inflammation underlying DIO (Tsukumo et al., 2007, Shi et al., 2006a). Moreover, distinct fatty acids have different inflammatory properties. SFA and n-6 PUFA, highly present in the typical Western diet, have been demonstrated to stimulate potent inflammation in the hypothalamus by activating TLR4/NF-κB signalling (Kleinridders et al., 2009). n-3 PUFA and n-3 PUFA derivatives have been shown to exert anti-inflammatory effects in the hypothalamus by reducing the expression of inflammatory cytokines and other inflammatory mediators (Cintra et al., 2012). In addition, it has been shown that the hypothalamic pro-inflammatory effects induced by SFA lead to central leptin resistance (Kleinridders et al., 2009), while the anti-inflammatory effects induced by n-3 PUFA are able to revert the central leptin resistance and related metabolic disorders (Cintra et al., 2012). Therefore, determination of the hypothalamic inflammation effects induced by distinct fatty acids may contribute to the improvement and prevention of central leptin resistance and obesity.

Therefore, through the present thesis, we will determine the type of fatty acid that reduces central leptin sensitivity, as well as the type of fatty acid that best improves central leptin sensitivity. I aim to explore the molecular mechanism of specific fatty acids in regulating central leptin action on energy homeostasis, hypothalamic signalling,

and hepatic glucose and lipid metabolism. We determine to explore the contribution of hypothalamic TLR4/NF-κB and JNK/NF-κB inflammatory signalling to central leptin resistance and DIO. The knowledge obtained from these studies may lead to practical dietary interventions with the proper use of fatty acids for the control of obesity and type II diabetes.

1.2 Literature Review

1.2.1 Obesity Prevalence

Obesity is a medical condition in which abnormal or excessive adipose mass accumulation resulting from a chronic imbalance between energy intake and expenditure (Weiser et al., 1997, Surwit et al., 1988). The incidence of obesity is increasing at an alarming rate, and obesity is now considered to be a worldwide epidemic. Nearly 35% of the adult population in most developed countries are clinically obese (WHO Statistics, 2013). The global epidemic of obesity has led to increasingly serious medical problems. Firstly, the rapid increase of obesity has increased the incidence of leptin resistance, insulin resistance, and type II diabetes, which correlates with the increased risk for numerous adverse health consequences (Visscher and Seidell, 2001). For instance, about 80% of the individuals with type II diabetes are classified as overweight or obese, and 30% of obese children under the age of 12 display insulin resistance (Canete et al., 2007). DIO also leads to multiple metabolic dysregulations, such as hypertension, metabolic syndrome, hyperglycaemia, hypertriglyceridaemia, and dyslipidaemia (Kopelman, 2000). Moreover, obesity is an important risk factor for cardiovascular disease and cancer. It has been demonstrated that obesity may increase the risk of myocardial infarction by up to 55% (Yusuf et al., 2005). In addition to the

serious medical consequences, the health problems related to obesity impose substantial economic burdens on individuals, families, and communities.

Several factors contribute to the worldwide epidemic of obesity and type II diabetes. Important etiologic factors include the increased consumption of diets rich in saturated fat, the prevalence of the Western diet (rich in n-6 PUFA), and the overall decreased intake of n-3 PUFA (Spiegelman and Flier, 2001). Combined with a sedentary lifestyle, the outcome is excess energy storage in the form of fat deposits in the body, which exacerbates the development of obesity and associated metabolic disorders. However, there is no effective current therapy to combat the obesity epidemic. Through understanding the effects of specific fatty acids on leptin resistance and obesity, and determining the causes and pathogenesis of obesity, it may be possible to design therapeutic targets to prevent and treat obesity and its associated metabolic disorders.

1.2.2 Leptin and Central Leptin Resistance

1.2.2.1 Leptin

Leptin is a 16 kDa adipocyte-derived hormone consisting of 167 amino acid residues. Leptin communicates the repletion of body energy stores to central nervous system, which suppresses food intake and increases energy expenditure by controlling behaviour and metabolic responses (Fruhbeck, 2006, Bjorbaek and Kahn, 2004b). However, leptin deficiency owing to the mutation of leptin or leptin receptor (LepR) results in increased food intake and reduced energy expenditure and immune function, eventually leading to obesity (Myers et al., 2010, Bjorbaek, 2009). Generally, serum leptin levels are closely related to body weight and total body fat. The leptin levels and leptin gene expression tend to be higher in HFD obese models (Considine et al., 1996).

Leptin exerts its biological action through binding to and activating the LepR, of which multiple isoforms exist. In mice, the long isoform (LepRb) consists of 1,162 amino acids and is the only isoform with a clearly demonstrated signalling capacity (White et al., 1997). The absence of LepRb in *db/db* mice results in obesity, impaired growth, infertility and diabetes mellitus (White et al., 1997). LepR is highly expressed in the central nervous system, particularly in the hypothalamus, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), ventromedial nucleus (VMH), and lateral hypothalamus (LH). Leptin acts via the LepR to stimulate the expression of pro-opiomelanocortin (POMC) located in the ARC. POMC generates a range of smaller biologically active peptides, which mediates an anorectic response. Leptin also inhibits orexigenic pathways mediated by neurons expressing the melanocortin antagonist Agouti-related peptide (AgRP) and neuropeptide Y (NPY).

The present studies will examine the effects of distinct fatty acids on central leptin sensitivity in the specific mediobasal hypothalamus (MBH) and PVN. The reason is that they are critical sites for leptin to regulate food intake, energy balance, and peripheral metabolism homeostasis (Williams et al., 2009). MBH is the primary centre of integration of nutrient-related signals (e.g. glucose, fatty acids, and hormones) critical to the regulation of energy homeostasis. As part of the MBH, the ARC serves as the leptin signalling centre for energy homeostasis regulation (Ring and Zeltser, 2010). ARC neurons, containing cocaine- and amphetamine-related transcript (CART) and POMC derivative α-melanocyte stimulating hormone (α-MSH), project to the PVN to generate the anorectic effect. The PVN has been shown to be a powerful mediator on central leptin to regulate energy homeostasis (Michaud et al., 1998).

1.2.2.2 Leptin signalling

Leptin activates several signalling pathways in the central nervous system. The JAK2-STAT3 pathway has been proven to be a major pathway of leptin signalling (Ghilardi et al., 1996, Ghilardi and Skoda, 1997, Rosenblum et al., 1996). In this leptin signalling cascade, leptin binds to the functional form of the LepR, and results in the activation of JAK2, a cytoplasmic tyrosine kinase. Activated JAK2, in turn, mediates phosphorylation at the specific receptor tyrosine residue, which then serves as a docking site for STAT3. Therefore, STAT3 becomes phosphorylated. The phosphorylated STAT3 becomes dimerized and translocates to the nucleus where they bind and regulate related gene transcription (Darnell, 1997). The activation of the STAT3 pathway will induce POMC, which is subsequently processed into α-MSH which inhibits appetite and increases body expenditure. Additionally, leptin JAK2-STAT3 signalling is negatively regulated by the suppressor of cytokine signalling 3 (SOCS3) (Naka et al., 1997). Leptin specifically induces SOCS3 mRNA levels in the hypothalamus (Baskin et al., 2000, Bjorbaek et al., 1998) and activates SOCS3 expression in NPY and POMC neurons (Elias et al., 1999). SOCS3 has been demonstrated to attenuate leptin signalling by inhibiting JAK2 signal transduction (Sasaki et al., 1999), Akt activation (Ernst et al., 2009), and insulin receptor substrate (IRS) phosphorylation (Ueki et al., 2004) (Fig. 1).

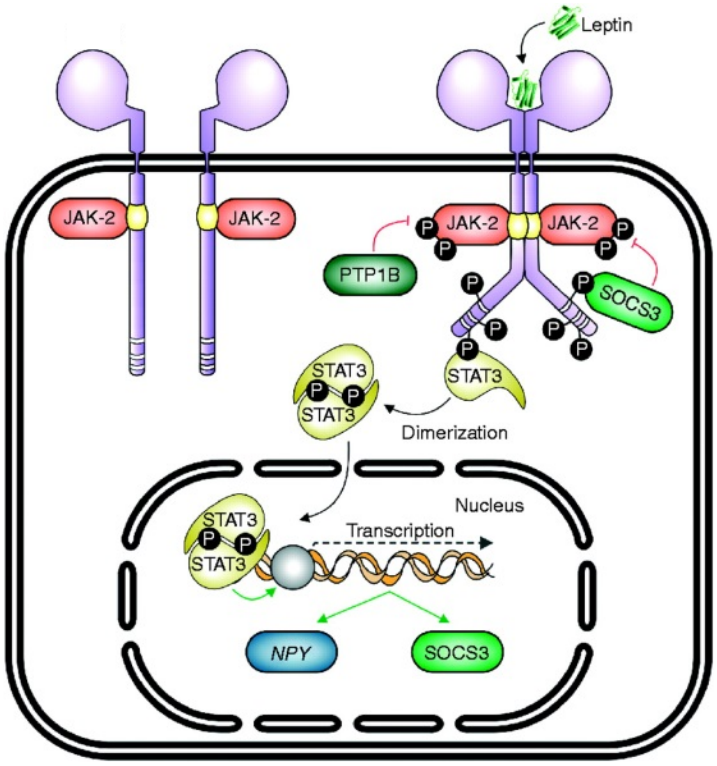

Figure 1. Mechanism of the JAK2-STAT3 signalling pathway

In the leptin-STAT3 signalling cascade, leptin binds to lepR, results in the activation of JAK2. Activated JAK2 mediates phosphorylation at the specific receptor tyrosine residue, which then serves as a docking site for STAT3. STAT3 becomes phosphorylated. Phosphorylated STAT3 becomes dimerized and translocates to the cell nucleus. Within the nucleus, STAT3 can bind and regulate related gene transcription. SOCS3 can suppress the action of leptin by binding to JAK2 and tyrosine residues. JAK-2: janus activated kinase 2, STAT3: signal transducer activator of transcription 3, SOCS3: suppressor of cytokine signalling 3, PTP1B: protein-tyrosine phosphatase 1B, NPY: neuropeptide Y. Figure adapted from Marroquı´ L. *et al* (Marroqui et al., 2012).

Furthermore, leptin PI3K signalling in the hypothalamus plays a crucial role in the development of central leptin resistance and obesity, and contributes to whole-body energy homeostasis (Koch et al., 2010). Leptin and insulin both have the potential to activate PI3K signalling in neurons and other cell types. In the hypothalamus, leptin

binds to the LepR and activates JAK2 via phosphorylation, leading to the phosphorylation of IRS proteins, which in turn activates PI3K and downstream molecules Akt. The phosphorylation of Akt finally induces the phosphorylation of forkhead box protein O1 (FOXO1) in the nucleus and inactivates FOXO1-mediated transcription (Wauman and Tavernier, 2011) (Fig. 2). Leptin inhibits both the activity and expression of hypothalamic FOXO1 through the PI3K pathway. Mechanistically, FOXO1 regulates food intake and energy expenditure by stimulating the expression of orexigenic NPY and AgRP (Ropelle et al., 2009) and inhibiting the expression of POMC. The deletion of FOXO1 in POMC neurons results in decreased food intake and body weight in mice (Plum et al., 2009). In addition, the deletion of IRS2 in the brain causes obesity in mice (Lin et al., 2004). Pharmacological inhibition of the PI3K in the hypothalamus prevents leptin-induced anorexia in mice (Niswender et al., 2001). Above evidence demonstrated the important role of leptin PI3K pathway in regulating energy homeostasis in DIO.

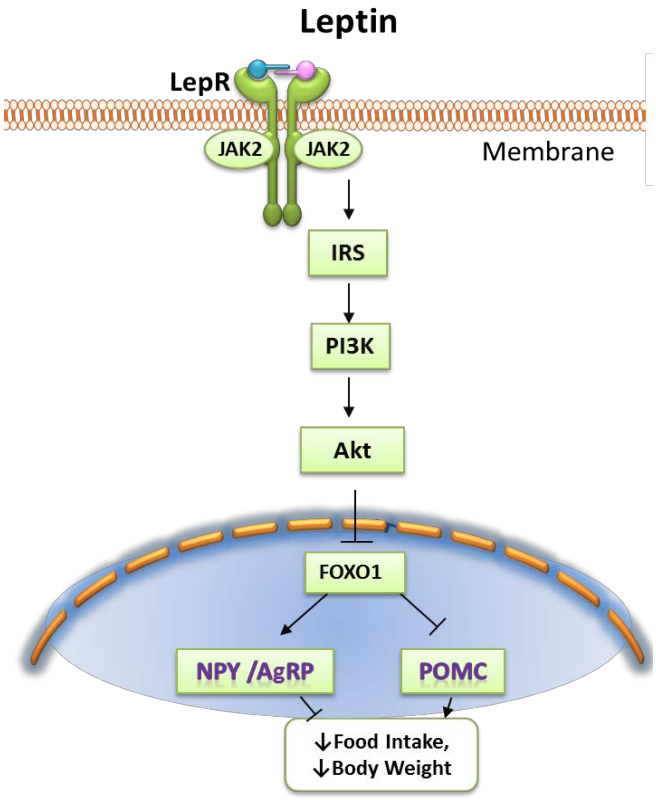

Figure 2. Mechanism of the PI3K-Akt signalling pathway

Both the leptin and insulin receptors play a role in the initial step of IRS 1/2 phosphorylation, which binds to the subunit of PI3K. PI3K phosphorylates PIP2 and following mediators, which activates and phosphorylates protein kinase B (Akt), and finally induces the phosphorylation of FOXO1 in the nucleus, inactivating FOXO1-mediated transcription. JAK2: janus activated kinase 2, IRS: insulin receptor substrate, PI3K: phosphoinositide 3-kinase, FOXO1: forkhead box protein O1, NPY: neuropeptide Y, AgRP: agouti-related peptide, POMC: proopiomelanocortin, PIP2: phosphatidylinositol 4,5-bisphosphate.

1.2.2.3 Central leptin resistance and associated mechanism

Physiologically, leptin suppresses appetite, increases thermogenesis, and induces weight loss through a classical negative feedback mechanism. However, in times of excess

energy storage, impaired responses or ‘resistance’ to afferent input of hypothalamus from leptin would be predicted to favour weight gain, fat accumulation, and leptin resistance. Therefore, leptin resistance is characterised by the failure of elevated circulating leptin to suppress appetite and weight gain, which in turn exacerbates obesity (Enriori et al., 2006). Furthermore, the leptin resistance in the central nervous system has attracted much attention. In the C57BL/6J mice model, both peripheral and central leptin resistance have been shown to be induced during the development of DIO (Lin et al., 2000). Since central leptin resistance is considered to be the primary risk factor for the pathogenesis of overweight and obesity, understanding the mechanisms involved in the development of leptin resistance is crucial for the development of clinical treatment.

Many mechanisms have been proposed to explain leptin resistance, including impairment in leptin transportation, LepR signalling, and leptin target neurons. Previous studies strongly suggest that impaired leptin signalling in the central nervous system plays an important role in the development of central leptin resistance and DIO (Munzberg et al., 2004). Firstly, the hypothalamic STAT3 signalling has been demonstrated to be involved in DIO. For instance, central leptin resistance and defective hypothalamic STAT3 signalling have been observed in DIO rodents (El-Haschimi et al., 2000, Kleinridders et al., 2009). Other consistent reports show that defective STAT3 signalling is also observed before the DIO or exposure to a HFD in obesity-prone rats (Levin et al., 2004). In particular, the defective STAT3 signalling in the specific site or sites of the hypothalamus in DIO has been investigated. Munzberg *et al*. first reported that central leptin resistance with diminished leptin-mediated STAT3 phosphorylation was induced in the ARC in HFD mice (Munzberg et al., 2004). Another study examined

pregnancy-related leptin resistance and reported that the impaired leptin-induced STAT3 phosphorylation exists in the ARC and VMH regions in rats (Ladyman and Grattan, 2004). These previous studies suggest that defective leptin STAT3 signalling in the hypothalamus may be responsible for the pathogenesis of central leptin resistance and obesity. In addition, the critical role of SOCS3 as a negative regulator of leptin signalling in the central nervous system has been demonstrated by previous studies in DIO (Zhang et al., 2008a). Increased expression of SOCS3 in the hypothalamus has been observed in DIO rodents, accompanied by leptin resistance, insulin resistance, and hepatic steatosis (Enriori et al., 2007, Munzberg et al., 2004). On the other side, mice lacking SOCS3 in neurons have increased phosphorylation of STAT3 in VMH, improved anorexigenic effect of leptin and glucose homeostasis, and are protected from the development of DIO (Zhang et al., 2008a). Evidently, this experimental evidence indicates that SOCS3 in neurons negatively regulates leptin signalling and plays an important role in mediating central leptin sensitivity, energy homeostasis, and glucose metabolism.

The impairment of the hypothalamic leptin PI3K signalling pathway has been shown to play a critical role in central leptin resistance and peripheral glucose and lipid metabolism during DIO (Metlakunta et al., 2008, Zhao et al., 2002, Warne et al., 2011). Metlakunta *et al*. reported that the PI3K pathway of leptin signalling was impaired in the hypothalamus (MBH) by HFD exposure for 4 weeks (Metlakunta et al., 2008). Further, the chronic activation of the hypothalamic PI3K pathway increased leptin sensitivity and decrease adiposity, while the pharmacological inhibition of PI3K activity blocked the anorectic effect of leptin (Zhao et al., 2002, Hill et al., 2008). Furthermore, it has been suggested that the PI3K signalling in the hypothalamus is associated with the

regulation of central leptin on hepatic glucose and lipid metabolism in DIO (Warne et al., 2011). For instance, Warne *et al*. reported that the impairment of PI3K signalling in hypothalamic neurons causes severe hepatic steatosis (Warne et al., 2011). Altogether, these lines of evidence clearly establish an important role of PI3K signalling in energy homeostasis and peripheral metabolism in transducing leptin action in the hypothalamus. However, the effects of distinct fatty acids on specific leptin STAT3 and PI3K signalling pathways in the hypothalamus are still unclear.

1.2.3 The Regulation of Central Leptin on Hepatic Energy Metabolism

1.2.3.1 Central leptin regulation on hepatic glucose metabolism

Leptin plays an important role in the regulation of glucose homeostasis (Fruhbeck and Salvador, 2000). It has been proven that the hypothalamus is implicated as a key centre for the glucose-lowering action of leptin and peripheral glucose homeostasis mediation (Schwartz et al., 1996). Previous vivo studies indicate that leptin acts directly on specific peripheral cells, such as hepatocytes, islet cells, and adipocytes, to regulate glucose homeostasis (Emilsson et al., 1997, Muller et al., 1997). Several vitro studies have shown that the systemic administration of leptin not only enhances glucose turnover in normal rodents (Rossetti et al., 1997), but also ameliorates impaired glucose metabolism in leptin deficient *ob*/*ob* mice (Pelleymounter et al., 1995), insulin-resistant mice (Shimomura et al., 1999), and insulin-deficient streptozotocin (STZ)-induced diabetic rats (Chinookoswong et al., 1999).

The precise mechanism by which central leptin modulates hepatic glucose metabolism has not been fully elucidated. It is possible that leptin mediates glucose metabolism by regulating the key genes that encode glucose metabolism in the liver (Denroche et al.,

2012). In normal Sprague-Dawley rats, an intracerebroventricular (icv) injection of leptin (1.5 ug/6 h) stimulated hepatic gluconeogenesis by increasing Glucose 6-phosphatase (G6Pase) and Phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression (Gutierrez-Juarez et al., 2004). However, central leptin has been shown to suppress hepatic gluconeogenesis in obese rodents (German et al., 2011, Hidaka et al., 2002). For instance, in STZ-Diabetic rats, an icv infusion of leptin (3 ug/day) for 6 days normalised the hepatic glucose metabolic dysregulation, and inhibited hepatic gluconeogenesis by reducing the G6Pase mRNA levels (Hidaka et al., 2002). In addition, previous studies indicate that hepatic glycolysis is suppressed by leptin with down-regulated glucokinase (GK) activity in obese rodents (Tang and Chen, 2010). However, a few in vitro studies show that it is stimulated by leptin (Hidaka et al., 2002).

In addition, the regulation of central leptin on glucose uptake may be one of the possible mechanisms underlying the regulation of heptaic homeostasis by central leptin. Glucose uptake is primarily mediated by glucose transpotters (GLUTs). In the liver, GLUT2 and GLUT4 are the primary GLUTs responsible for transporting glucose across the hepatic plasma membrane into hepatocytes (Oka et al., 1990). The transcription of the GLUT2 gene in the liver has been shown to be up-regulated during hyperglycaemic states and in type II diabetes (Oka et al., 1990). Leptin has an inhibitory effect on hepatic glucose transportation. For instance, an icv infusion of leptin down-regulates GLUT2 mRNA expression in the liver of STZ-Diabetic rats, which contributes to the restoration of glucose metabolism (Hidaka et al., 2002).

Another mechanism of the regulation of leptin on hepatic glucose metabolism may be associated with altered leptin sensitivity and signalling in the central nervous system. Previous experimental evidence suggests that hypothalamic leptin PI3K signalling is an

important determinant neuronal mechanism of glucose metabolism (Morton et al., 2005, Niswender et al., 2001). In the context of DIO, impaired PI3K signalling in the hypothalamus contributes to impaired hepatic glucose metabolism (Metlakunta et al., 2008). Consistently, Warne *et al*. also demonstrates that the attenuation of PI3K signalling in LepRb neurons promotes hepatic steatosis (Warne et al., 2011). Furthermore, another mice study shows that a disruption leads to an impaired mRNA translation of insulin and GLUT2 (Chen et al., 2009). On the other hand, both antisense 'knockdown' of hypothalamic insulin receptors and the infusion of a PI3K inhibitor cause peripheral insulin resistance and hepatic metabolic disturbances in rats (Obici et al., 2002, Gelling et al., 2006). These data collectively show that that leptin PI3K signalling in the hypothalamus is implicated in the regulation of hepatic glucose metabolism during DIO. In addition, other studies demonstrate that hypothalamic leptin STAT3 signalling may be also involved in the regulation of glucose homeostasis (Myers, 2004). One study has shown that the disruption of the LepR/STAT3 signalling in mice not only results in hyperphagia, neuroendocrine dysfunction, and obesity, but also exacerbates insulin resistance and glucose intolerance compared to *db/db* animals (Bates et al., 2005). Consistently, it has also been shown that the central infusion of a STAT3 peptide inhibitor prevented the suppressive effect of central leptin on gluconeogenesis (Buettner et al., 2006). Therefore, previous studies indicate that the activation of hypothalamic STAT3 signalling is required for hepatic glucose metabolism.

1.2.3.2 Central leptin regulation on hepatic lipid metabolism

Several studies support a direct effect of central leptin on hepatic lipid metabolism (Silver et al., 1999, Farooqi et al., 2002). Leptin exerts its effect on lipid metabolism

predominantly through the hypothalamus by regulating the transcription of key genes that involved in metabolism (Fei et al., 1997, Gallardo et al., 2007). Leptin administration inhibits lipogenesis, while promotes lipolysis in rodents (Bryson et al., 1999). For instance, in wild-type mice, the central infusion of leptin for 7 days decreased triglyceride (TG) levels and hepatic lipogenic enzymes, including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD1), and sterol regulatory element binding protein 1c (SREBP-1c), (Gallardo et al., 2007). In *ob*/*ob* mice, an icv infusion of leptin (1 ug) for 3 days inhibited hepatic lipogenesis by down-regulating ACC, FAS, and SCD1 mRNA levels (Prieur et al., 2008). In contrast, in leptin-deficient *ob*/*ob* mice, *de novo* lipogenesis markers (e.g. ACC, FAS, and SCD1) in the liver were improved, accompanied by increased fasting glucose, insulin, and hepatic TG content (Perfield et al., 2013). These results demonstrate the possible role of leptin regulation on lipogenesis in the hepatic metabolism underlying DIO.

Furthermore, the regulation of leptin on hepatic fatty acid oxidation has been demonstrated. Central leptin has been suggested to increase hepatic fatty acid oxidation both in normal and obese rodents, resulting in lower tissue TG accumulation (Havel, 2004, Gallardo et al., 2007). For instance, adenovirus-induced hyperleptinaemia has been demonstrated to up-regulate peroxisome proliferator-activated receptor α (PPARα), a master mediator of enhanced hepatic β-oxidation in wild-type mice (Lee et al., 2002). Another report from Prieur *et al.* indicated that central leptin acts directly on the liver to increase lipid oxidative metabolism by up-regulating acyl-CoA oxidase (AOX), carnitine palmitoyltransferase 1 (CPT1), and acetyl-CoA acetyltransferase 1 (ACAT1) mRNA levels in *ob*/*ob* mice (Prieur et al., 2008).

In addition, emerging data now indicates that central leptin is an important regulator in the control of hepatic cholesterol metabolism. It has been demonstrated that leptin inhibits cholesterol synthesis and improves cholesterol transportation (Prieur et al., 2008). For instance, a low dose icv injection of leptin was effective to decrease the *de novo* synthesis of cholesterol by decreasing the activities of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) in normal Sprague-Dawley rats (Vanpatten et al., 2004). In obese mice, icv injection of leptin down-regulated the gene expression of HMG-CoA reductase and up-regulated the gene expression of apolipoprotein A1 (ApoA1) in the liver (Prieur et al., 2008). ApoA1 is the main component of high-density lipoprotein (HDL). As a result, it can be seen that leptin central administration is sufficient to decrease plasma TG and free fatty acid levels by approximately 50% and suppress the low-density lipoprotein (LDL)/HDL1-cholesterol profile.

1.2.3.3 Central leptin regulation on hepatic glucose and lipid metabolism via tyrosine hydroxylase (TH)

Experimental evidence indicates that central leptin regulates peripheral glucose and lipid metabolism by affecting sympathetic nervous system activity (Stanley et al., 2010, Warne et al., 2011). For instance, chronic central administration of leptin down-regulates hepatic lipogenic genes expression by activating the sympathetic nervous system (Warne et al., 2011). The attenuation of leptin-mediated PI3K signalling in the hypothalamus in turn causes severe hepatic steatosis and decreased hepatic sympathetic tone (Warne et al., 2011). In addition, leptin is known to activate the sympathetic nervous system through increasing catecholamine output in rodents and humans (Buettner et al., 2008, Rosenbaum et al., 2005, Satoh et al., 1999). TH is a rate-limiting

enzyme in the synthesis of catecholamine. TH-positive neurons in the hypothalamus directly project to brainstem autonomic regions (Geerling et al., 2010). From there, the catecholamine-synthesising neurons in the brainstem send efferent signals to the spinal cord and exert autonomic control on many organs, including the liver, to regulate glucose and lipid metabolism. In addition, in the context of obesity, the TH expression in the hypothalamus is decreased, which accounts for the down-regulation of sympathetic outflow (Li et al., 2009, Shi et al., 2013). Therefore, TH activation in the hypothalamus may be a significant indicator of sympathetic nervous system activity connecting the central action of leptin to hepatic glucose and lipid metabolism.

1.2.4 The Effects of Distinct Fatty Acids on Leptin Sensitivity

SFA, n-6 PUFA, n-3 PUFA, and n-3 PUFA derivatives have been shown to differentially modulate overall energy metabolism, leptin and insulin sensitivity (Cintra et al., 2012, Munzberg et al., 2004). The biochemical and molecular mechanisms underlying central leptin sensitivity changes induced by distinct fatty acids remain unclear. Understanding and exploring the effects of fatty acids on central leptin sensitivity and the pathogenesis of obesity may enable us to design therapeutic targets for the prevention and treatment of obesity and associated complications.

A number of studies have demonstrated that the levels of specific fatty acids in plasma are reflective of fatty acids consumed in the diet (Ma et al., 1995, Raatz et al., 2001). The intake of typical western diet has an influence on the fatty acids found in circulation. For instance, the high intake of safflower oil (high n-6 PUFA content) led to significant increases in 20:3 (n-6) levels but decreases in 18:3 (n-3) and 20:5 (n-3) levels (Sinclair et al., 1994). The high consumption of fish, fish oil, and docosahexaenoic oil (high in n-3 PUFA) increased n-3 PUFA and decreased n-6 PUFA

compositions of plasma fractions in human (Vidgren et al., 1997). Previous studies with arachidonic acid (n-6 PUFA), docosahexaenoate (n-3 PUFA) , palmitate (SFA), and the essential fatty acids linoleic acid and linolenic acid, each labelled with 14C or 3H, showed that fatty acids administered orally or intravenously were rapidly taken up by the brain and quickly incorporated into brain lipids (Washizaki et al., 1994) (Innis, 2007) (Wainwright et al., 1992). Previous studies show that there is a significant positive relationship between the levels of n-3 PUFA (especially DPA and DHA) and n-6 PUFA (especially ARA) in plasma and their levels in cerebrospinal fluid in humans (Guest et al., 2013). The icv injection method used for fatty acid administration in our project would be expected to mimic the central effects of fatty acids. The dosage used in our project was based upon a previous literature report in rodents (Kleinridders et al., 2009)."

1.2.4.1 Palmitic acid and leptin sensitivity

SFA are derived from dairy products, fatty meats, palm oil, coconut oil, and some processed foods. Palmitic acid (PA, C16:0) is the most common SFA in the human diets (Gunstone et al., 2007). SFA are important candidates for diet-induced central leptin and insulin resistance (Galgani et al., 2008, Koch et al., 2014, Kleinridders et al., 2009). For instance, maintaining a high-SFA diet tends to reduce insulin and leptin sensitivity, and even cause leptin resistance in the central nervous system (Benoit et al., 2009, Milanski et al., 2009). In addition to central leptin resistance, a diet rich in SFA has been observed to impair leptin-evoked STAT3 and PI3K signalling in the hypothalamus (Metlakunta et al., 2008). Previous studies suggest that the molecular mechanism of high SFA consumption induced central leptin and insulin resistance is implicated in hypothalamic TLR4 signalling (Milanski et al., 2009). In the hypothalamus, dietary

SFA trigger a pro-inflammatory response, which leads to an increased inflammatory gene transcription and hypothalamic dysfunction predominantly by inducing TLR4 activation (Moraes et al., 2009, Milanski et al., 2009). Further, Milanski *et al.* further demonstrated that icv injection of stearic and arachidic acid (SFA) activated pro-inflammatory gene expression in the hypothalamus in a TLR4-dependent manner (Milanski et al., 2009). The central administration of palmitate (SFA) has been shown to promote the interaction between TLR4 and myeloid differentiation factor-88 (MyD88, an essential signal adaptor for most TLRs), induce downstream inflammation signalling, and consequently activate an inflammatory response (Kleinridders et al., 2009). Furthermore, recent studies have demonstrated that the central inflammation induced by SFA may contribute to central leptin resistance and defective leptin-activated STAT3 signalling in the hypothalamus (Zhang et al., 2008b). Therefore, SFA play an important role in hypothalamic inflammation and the development of central leptin resistance and obesity during HFD.

PA is a primary SFA, and can be found in meat, cheese, butter, and dairy products, accounting for approximately 65% of SFA and 32% of total fatty acids in human serum (Yu et al., 2012). Recently, PA has drawn particular attention for its effect on leptin signalling (Shi et al., 2006a). It has been shown that both dietary and central infusion of PA cause leptin resistance in the central nervous system (Benoit et al., 2009, Posey et al., 2009, Milanski et al., 2009). In particularly, Kleinridders *et al*. reported that an single icv injection of palmitate (66 pmol) acutely induced central leptin resisitance and inhibited central leptin anorexigenic action in regulating food intake and body weight gain in male C57/BL6 mice (Kleinridders et al., 2009). However, the precise role and mechanism of PA on central leptin sensitivity, leptin signalling in specific regions of the

hypothalamus, and central-mediated hepatic glucose and lipid metabolism is still unclear.

1.2.4.2 Arachidonic acid and leptin sensitivity

Major sources of n-6 PUFA are vegetable oils such as corn, safflower, soybean, and typical Western diet. ARA is the most biologically relevant n-6 PUFA. With an increased consumption of n-6 PUFA and an decreased n-3 PUFA intake, the n-6 PUFA to n-3 PUFA ratio can be as high as 25:1 (Simopoulos, 2010). The high n-6 PUFA to n-3 PUFA ratio increases the risk of serious of chronic disease, including type II diabetes and cardiovascular diseases (Das, 2006). Previous evidence about the effects of n-6 PUFA on leptin sensitivity is scarce. Some studies have reported that a HFD rich in n-6 PUFA induced insulin and leptin resistance in mice (Nuernberg et al., 2011), rats (Storlien et al., 1991, Jucker et al., 1999), and humans (Heine et al., 1989). In addition, the effects of n-6 PUFA on leptin sensitivity in central nervous system have been explored by Pimentel *et al*. He reported that a high-soy oil diet impaired hypothalamic insulin signalling by inhibiting insulin receptor and IRS-2 activation, and decreasing Akt serine phosphorylation (Pimentel et al., 2012, Pimentel et al., 2013). However, some studies show different results, and indicate that n-6 PUFA increase leptin and insulin sensitivity in rodents and obese humans (Storlien et al., 1997, Asp et al., 2011, Summers et al., 2002, Perez-Matute et al., 2003). Therefore, more evidence is needed to determine the effects of n-6 PUFA on central leptin sensitivity and the development of obesity.

Arachidonic acid (ARA, 20:4 *n*-6), an predominant n-6 PUFA, is a major component of mammalian cell membranes, and accounts for up to 25% of all phospholipid fatty acids (Calder, 2006). ARA is synthesised in the liver from linoleic acid (LA, 18:2, n-6), and is

transported to other cell types via serum albumin or lipoproteins (Simopoulos, 1999). A major function of ARA is as a precursor to the eicosanoid family of hormones that modulate immune and inflammatory responses in the body (Habenicht et al., 1990). Excessive production of eicosanoids from ARA gives rise to pathophysiological signalling and increases inflammation (Fritsche, 2008), which may contribute to a number of disease states, including obesity (Garaulet et al., 2001), diabetes (Pelikanova et al., 2001), metabolic syndrome (Williams et al., 2007), and heart disease (Kark et al., 2003). Specifically, ARA has been shown to decrease insulin and leptin sensitivity, decrease glucose tolerance, and promote adiposity (Ailhaud et al., 2006, Asp et al., 2011). However, until now, the impact of direct central administration of ARA on leptin signalling in the hypothalamus and central regulated hepatic glucose and lipid metabolism has not been investigated.

Cumulative evidence indicates that ARA induces a potent pro-inflammatory response in the central nervous system. ARA can alter the gene expression and production of inflammatory mediators, such as increasing pro-inflammatory cytokines and decreasing anti-inflammatory cytokines (Calder, 2011). For instance, a diet enriched with soy oil (n-6 PUFA) for 2 months led to increased levels of tumor necrosis factor receptor-associated factor-6 (TRAF6) and NF-κB p65 in the hypothalamus in rats, indicating the stimulation of the inflammatory NF-κB pathway in the central nervous system (Pimentel et al., 2013). Furthermore, ARA is processed by the cyclooxygenase-2 (COX-2) or lipoxygenase metabolic pathways into eicosanoids (prostaglandins, thromboxanes, leukotrienes) (Di Marzo, 1995). ARA-derived eicosanoids have pro-inflammatory effects (Hanaka et al., 2009). For instance, refeeding with soy diet increased c-Fos immunoreactivity in the DMH (+271%) and LH (+303%) of the hypothalamus

(Watanabe et al., 2009). In addition, ARA has been proved to increase NF-κB activation, promote IKK associate with NF-κB and IκB complex, and lead to the pro-inflammatory response (Zhu et al., 2008, Siriwardhana et al., 2012). Therefore, previous studies suggest that hypothalamic inflammation induced by ARA may be associated with the development of central leptin resistance and obesity.

1.2.4.3 Docosahexaenoic acid and leptin sensitivity

n-3 PUFA, present in milk and fatty fish (e.g. salmon, tuna, and mackerel). The principal n-3 PUFA include α-linolenic acid (ALA, C18:3, n-3), DHA, and eicosapentaenoic acid (EPA, 20:5 n-3). n-3 PUFA are known to have numerous beneficial effects on body health. It is well documented that n-3 PUFA exert a protective function against obesity by increasing leptin sensitivity both in peripheral tissues and in central nervous system (Reseland et al., 2001, Ukropec et al., 2003, Cintra et al., 2012). For instance, dietary n-3 PUFA have been shown to reduce the expression of leptin mRNA and the level of plasma leptin in peripheral tissues in vivo and in vitro (Reseland et al., 2001, Ukropec et al., 2003, Mori et al., 2004). In recent years, more evidence has shown that n-3 PUFA increase the leptin sensitivity in the central nervous system in regulating energy homeostasis and improving hypothalamic leptin signalling (Cintra et al., 2012). For instance, in a mouse model of DIO, both dietary substitution of flax seed oil (rich in C18:3) and central injection of ALA reversed hypothalamic leptin resistance by increasing the activation of pJAK2, pSTAT3, pAkt, and pFOXO1 in the hypothalamus (Cintra et al., 2012). Consistently, another study showed that the consumption of a diet enriched with fish oil prevented hyperleptinaemia by stimulating hypothalamic Akt serine phosphorylation (Pimentel et al., 2012). Furthermore, n-3 PUFA has been reported to prevent some metabolic disturbances induced by HFD by

preventing the glucose tolerance and leptin and insulin resistance (Daniele et al., 1997, Rajas et al., 2002), decreasing TG, cholesterol, and VLDL (Kasbi Chadli et al., 2012), as well as increasing HDL levels (Rivellese and Lilli, 2003, Carpentier et al., 2006).

n-3 PUFA have well-known anti-inflammatory effects in both peripheral tissues and central nervous system. n-3 PUFA serve as precursors for eicosanoids. However, unlike n-6 PUFA, the eicosanoids derived from n-3 PUFA (e.g. EPA and DHA) are anti-inflammatory (Schmitz and Ecker, 2008) (Fig. 3). n-3 PUFA can improve the inflammatory profile by altering the inflammatory mediators, such as pro-inflammatory cytokines, anti-inflammatory cytokines in vitro and in vivo (Novak et al., 2003, Zhao et al., 2004, Caughey et al., 1996, Kelley et al., 1999). Recently, the anti-inflammatory effects of n-3 PUFA in central nervous system have also been provided. For instance, a diet rich in fish oil for 2 months reduced the levels of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and TRAF6, and increased the levels of anti-inflammatory factor interleukin 10 (IL-10) in the hypothalamus in male rats (Pimentel et al., 2013). Consistently, the direct central administration of n-3 PUFA has a similar effect, as shown by a report indicated that icv treatment of n-3 PUFA ALA for 7 days reduced hypothalamic inflammation by decreasing the expression of inflammatory markers, TNF-α, IL-6, and pJNK, and increasing the expression of the anti-inflammatory cytokine IL-10 (Cintra et al., 2012). In addition, GPR120, as an unsaturated fatty acid receptor, has been suggested to be involved in the anti-inflammation in the hypothalamus induced by n-3 PUFA underlying DIO. A central injection of n-3 PUFA has been demonstrated to reverse central leptin resistance by exerting an anti-inflammatory effect by activation GPR120 in rodents (Cintra et al., 2012).

Docosahexaenoic acid (DHA, 22:6 n-3) is an n-3 PUFA primarily found in marine fish. DHA participates in regulation of a great number of functions in an organism. Firstly, DHA is an important structural component of the cell membranes in the brain and retina, and help to maintain normal brain function (Schmitz and Ecker, 2008). DHA is taken up by the brain in preference to other fatty acids, and the turnover of DHA in the brain is very fast (Horrocks and Yeo, 1999). Therefore, DHA plays a very important role in human growth and intellectual development during fetal development, early infancy, and old age (Ruxton et al., 2005). Furthermore, DHA is beneficial for preventing and treating several diseases, such as hypertension, diabetes mellitus, arthritis, cardiovascular disease, and some cancers. In addition to the preventive effect on these diseases, DHA also shows beneficial effects on the central regulation of hepatic glucose and lipid homeostasis (Clarke, 2000, Han et al., 2008, Carpentier et al., 2006). Additionally, DHA exhibits anti-inflammatory properties by competitively inhibiting the ARA cascades, most importantly via the COX pathway, thus reducing the pro-inflammatory eicosanoids derived from ARA (Lo et al., 1999, Weldon et al., 2007) (Fig. 3). However, the effect of direct central injection of DHA on central leptin sensitivity, hypothalamic inflammation, and central-mediated hepatic energy metabolism has not been demonstrated.

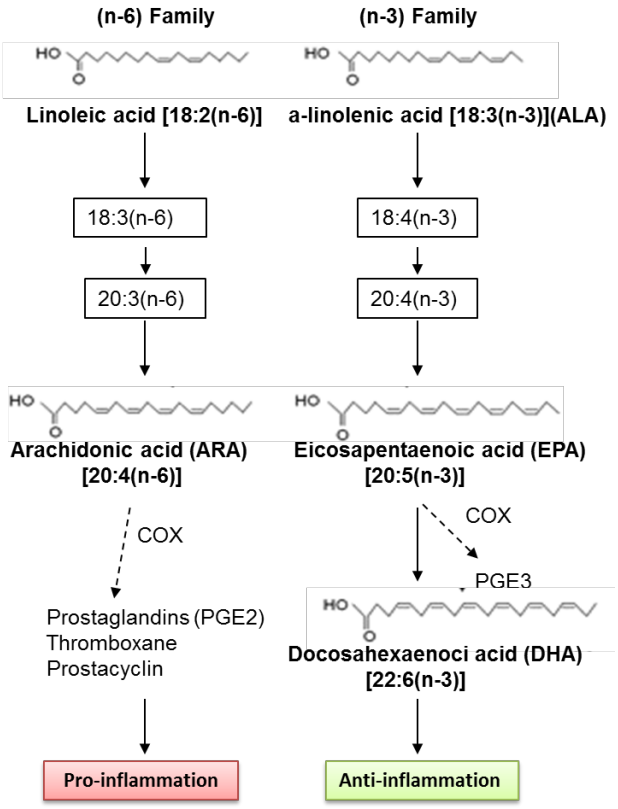

Figure 3. Metabolisms of n-3 PUFA and n-6 PUFA

ARA and DHA are the parent (n-6) and (n-3) long-chain PUFA. ARA is converted from LA. And EPA and DHA are converted from ALA. ARA-derived eicosanoids are pro-inflammatory, while the mediators formed from EPA and DHA are anti-inflammatory. COX: cyclooxygenase. Figure adapted from Kalupahana *et al*. (Kalupahana et al., 2011)

1.2.4.4 α-ethyl DHA ethyl ester and leptin sensitivity

α-ethyl DHA ethyl ester, as a promising DHA derivative, exhibits a similar range of beneficial effects on obesity and associated metabolic disorders as natural n-3 PUFA. It has been shown that α-ethyl DHA ethyl ester can prevent and even partially reverse the development of obesity and associated metabolic disturbances, including glucose intolerance, fat accumulation, and dyslipidaemia in C57BL/6J HFD mice (Rossmeisl et al., 2009). However, there are few reports on the effect of DHA derivative on insulin

and leptin sensitivity. Only one study showed that DHA derivative markedly decreased food intake, feeding efficiency, and plasma leptin levels, reflecting the improved effect of DHA derivative on leptin sensitivity (Rossmeisl et al., 2009).

Consistent with the effects of n-3 PUFA, DHA derivatives show beneficial effects on the regulation of glucose and lipid metabolism. In addition to preventing glucose intolerance, DHA derivative (α-ethyl DHA ethyl ester) has been shown to not only to lower plasma TG, non-esterified fatty acids, and cholesterol levels, but also to strongly promote hepatic fatty acid oxidation by increasing the mRNA expression of PPARα, AOX-1, and CPT1α in HFD rodents (Rossmeisl et al., 2009). Since SCD1 gene expression in skeletal muscle was shown to be down-regulated by α-ethyl DHA ethyl ester, this could imply a suppressive effect of DHA derivative on lipogenesis (Rossmeisl et al., 2009).

Systematic metabolomic studies reveal that n-3 PUFA derivatives exert potent anti-inflammatory effects (Morin et al., 2011). For example, CRBM-0244, a DHA monoglyceride derivative, has anti-inflammatory properties and prevents airway hyper-responsiveness in lung tissue (Morin et al., 2011). This study showed that DHA derivative prevented the degradation of Iκ-Bα and the subsequent nuclear translocation of the NF-κB p65 subunit. However, until now, no studies have reported the effects of DHA derivative on central leptin sensitivity, hypothalamic inflammation, and neuronal-regulated hepatic metabolism.

1.2.5 Hypothalamic Inflammation Mechanism involved in HFD-induced Obesity

1.2.5.1 Hypothalamic IKK-β/NF-κB signalling

A large body of evidence reveals that hypothalamic inflammation during HFD is associated with the development of central leptin resistance and obesity (Yu et al., 2013, Zhang et al., 2008b). IKK-β/NF-κB signalling is a key intracellular pro-inflammatory pathway involved in this process. Various studies have demonstrated that IKK-β/NF-κB signalling in the hypothalamus is activated in HFD-fed rodents (Zhang et al., 2008b). For instance, HFD consumption for 1 week induced a low-grade hypothalamic inflammation, evidenced by the increase of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (De Souza et al., 2005). The increased cytokines activate the NF-κB inflammatory signalling pathway by phosphorylating and degrading IκBα, and allowing NF-κB proteins to translocate to the nucleus and regulate the transcription of numerous genes, and induce a serious of inflammatory responses (Hayden and Ghosh, 2008). Recently, hypothalamic inflammation has even been observed after 1 day HFD feeding (Thaler et al., 2012) and acute fatty acids (SFA and OA) central injection (Posey et al., 2009, Zhang et al., 2008b). Furthermore, constitutive activation of NF-κB inflammatory signalling in the hypothalamus induces central leptin resistance and impairs leptin signalling in rodents. For instance, a study performed by Zhang *et al*. revealed that overnutrition activated the IKK-β/NF-κB inflammatory pathway in the hypothalamus, and was accompanied by blunted hypothalamic leptin and insulin signalling (Zhang et al., 2008b). On the other hand, in the same report, a genetic or pharmacological blockade of hypothalamic inflammatory signals (e.g. IKK-β) was shown to improve leptin sensitivity and pSTAT3 activation in the hypothalamus and protect against obesity. Therefore, hypothalamic inflammation has been suggested to be a predominant contributor to central leptin resistance and obesity.

In addition, up and down-stream hypothalamic inflammatory mediators affect leptin signalling by regulating the leptin signalling negative regulators, such as SOCS3. HFD-activated IKK-β/NF-κB signalling, especially the activation of pro-inflammatory cytokine expression in the hypothalamus has been shown to be associated with the promotion of SOCS3 expression (Wang et al., 2012, Zhang et al., 2008b). More evidence shows that the overexpression of both hypothalamic IKK-β and IL-6 signalling elevates SOCS3 mRNA expression through the IKK-β/NF-κB pathway. The overexpression of SOCS3 induced by IL-6 signalling induces the degradation of IRS proteins, which plays a specific role in the regulation of energy balance and the development of central leptin sensitivity and obesity (Tups, 2009, Zhang et al., 2008b, Morton and Schwartz, 2011). These findings reveal that the up-regulation of hypothalamic SOCS3 plays a critical role in mediating hypothalamic inflammation and leptin resistance. Above all, strategies to reduce the aberrant activation of inflammatory signalling in the hypothalamus are of great interest to improve the central leptin and insulin action and prevent obesity and related diseases.

1.2.5.2 Hypothalamic TLR4 signalling

TLR4 is the receptor for LPS and plays a critical role in innate immunity. The activation of TLR4 signalling leads to a pro-inflammatory response by regulating the induction of cytokines and the expression of other immune-related genes. In the hypothalamus, TLR4 is predominantly expressed by microglia. SFA have been reported to induce inflammation via TLR4 signalling (Huang et al., 2012). In the signalling cascades, free SFA or LPS bind and activate TLR4, which binds to MyD88 and after some intermediate steps leads to the recruitment of TRAF6. Its interactions with several proteins lead to phosphorylation of the inhibitory factor Iκ*B*. This releases NF-κB,

whose subunit undergoes phosphorylation and translocates to the nucleus, where it binds to its target genes to produce pro-inflammatory cytokines and a series of inflammatory response (Verstak et al., 2009, Spiegelman and Flier, 2001). It has been suggested that hypothalamic TLR4 signalling is the molecular mechanism which link the consumption of HFD to leptin and insulin resistance and obesity (Tsukumo et al., 2007, Shi et al., 2006a). Hypothalamic TLR4 expression and activity are increased in HFD-feeding (Wang et al., 2012, Ropelle et al., 2010). The interaction between TLR4 and MyD88 in the hypothalamus has also been demonstrated to be promoted by the central administration of SFA (palmitate), which stimulates TLR4 couple with intracellular inflammatory signalling cascades JNK and IKK-β/NF-κB, and induce hypothalamic inflammation (Kleinridders et al., 2009). On the other hand, the peripheral and central administration of TLR4 inhibitor has been demonstrated to diminish the high SFA-induced hypothalamic inflammation, and decrease food intake and body weight gain in rats (Milanski et al., 2009). Similarly, the deletion of neuronal TLR adaptor molecule MyD88 protects against leptin resistance and obesity induced by HFD (Kleinridders et al., 2009). In addition, the same study shows that an icv injection of palmitate inhibits leptin-induced pSTAT3 in the hypothalamus, which is dependent on MyD88 signalling (Kleinridders et al., 2009). Taken together, these studies indicate that hypothalamic TLR4 signalling plays a critical role in the development of central leptin resistance and DIO.

1.2.5.3 Hypothalamic JNK signalling

The JNK inflammatory pathway appears to play a crucial role in the development of leptin resistance and obesity. JNK is activated by multiple inflammatory and environmental stimuli, and is thought to exert its pro-inflammatory effect by stabilising

mRNAs encoding pro-inflammatory cytokines and other inflammatory mediators (Velloso and Schwartz, 2011). The activity of JNK in the hypothalamus is up-regulated during chronic HFD in rodents (Prada et al., 2005, Belgardt et al., 2010). Consistently, the direct exposure of SFA arachidic acid in the central nervous system led to hypothalamic JNK activation (Milanski et al., 2009). The up-regulation of hypothalamic JNK activity induced by HFD has been suggested to be associated with the increase of NF-κB activation, TLR4 activity, pro-inflammatory cytokines, and SOCS3 gene expression (De Souza et al., 2005, Milanski et al., 2009). On the other hand, whole body and brain-specific JNK deletion and the icv infusion of a JNK inhibitor have been shown to protect against HFD-induced obesity (Belgardt et al., 2010, Hirosumi et al., 2002). Therefore, the JNK signalling may represent a candidate pathway for HFD-mediated leptin resistance and obesity. However, some studies reveal that JNK signalling is not responsible for mediating leptin resistance upon HFD feeding. Evidence shows that the inhibition of JNK in the brain, either by genetic deletion or pharmacological inhibition, fails to rescue the impairment of leptin anorexigenic action and STAT3 signalling induced by HFD (De Souza et al., 2005, Kleinridders et al., 2009). More evidence is needed to elucidate the contribution of hypothalamic JNK signalling to the development of central leptin resistance and obesity induced by dietary fatty acids.

1.3 Aims and Hypothesis

1.3.1 Aim

General Aim

To examine the effects of distinct fatty acids (PA, ARA, DHA, and α-ethyl DHA ethyl ester) on central leptin sensitivity, hypothalamic leptin signalling, hypothalamic inflammation, and central-regulated hepatic glucose and lipid metabolism, in order to elucidate the molecular mechanisms of central leptin resistance, obesity and associated metabolic disturbances.

Specific Aims

(1) Study 1 aims to determine the effect of central administration of PA on central leptin sensitivity, hypothalamic leptin JAK2-STAT3 and PI3K-Akt signalling, hypothalamic inflammation, and neuronal-mediated hepatic glucose and lipid metabolism.

(2) Study 2 aims to determine the effect of central administration of ARA on central leptin sensitivity, hypothalamic leptin JAK2-STAT3 and PI3K-Akt signalling, hypothalamic inflammation, and neuronal-mediated hepatic glucose and lipid metabolism.

(3) Study 3 aims to determine the effect of central administration of DHA and α-ethyl DHA ethyl ester on central leptin sensitivity, hypothalamic leptin JAK2-STAT3 and PI3K-Akt signalling, and hypothalamic inflammation.

(4) Study 4 aims to determine the effect of central administration of DHA and α-ethyl DHA ethyl ester on neuronal-mediated hepatic glucose and lipid metabolism.

1.3.2 Hypothesis

I hypothesis that the icv administration of PA and ARA will lead to central leptin resistance, evidenced by the inhibition of central leptin's anorexigenic effect, as well as impaired leptin JAK2-STAT3 and PI3K-Akt signalling in the hypothalamus. Decreased central leptin sensitivity may affect the central leptin regulation of hepatic metabolism,

and lead to the attenuation of hepatic glucose and lipid metabolism. PA and ARA will trigger a pro-inflammatory response in the hypothalamus and activate inflammatory cytokines and mediators, which contributes to the central leptin resistance and hepatic metabolic disturbances. The activation of hypothalamic TH in response to central leptin will be down-regulated by icv injection of PA and ARA.

I also hypothesis speculate that the central injection of DHA and α-ethyl DHA ethyl ester will have a significant anorexigenic effect in reducing energy intake and body weight gain in HFD mice. I expect that DHA and DHA derivative will restore the leptin JAK2-STAT3 and PI3K-Akt signalling pathway in the hypothalamus by up-regulating the key signalling mediators. As a result of increased central leptin sensitivity, the regulation of leptin on hepatic glucose and lipid metabolism will also be ameliorated. The central injection of DHA and DHA derivative corrects the HFD-induced hypothalamic inflammation by reducing the hypothalamic pro-inflammatory cytokines and inflammatory mediators. The effect of central leptin in regulating hypothalamic TH will be improved by DHA and DHA derivative, thus connecting the central action of leptin to hepatic energy metabolism.

1.4 Experimental Design and Methods

1.4.1 Ethics Statement

This study was approved by the Animal Ethics Committee, University of Wollongong (Application Approval #: AE12/03), and complied with the 'Australian Code of Practice for the Care and Use of Animals for Scientific Purposes' (Australian Government National Health and Medical Research Council, 2004). This is in accordance with the

International Guiding Principles for Biomedical Research Involving Animals. All efforts have been made to minimise animal stress and prevent suffering.

1.4.2 Animals, Diet and Drug Treatment

Male C57BL/6J mice were obtained from the Animal Resource Centre (Perth, WA, Australia) and housed in environmentally controlled conditions (temperature 22°C, 12 hour light/dark cycle). Mice were maintained on a normal lab chow diet (LC, Vella Stock feeds, Doonside, NSW, Australia). After 1 week of acclimatization, mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An icv cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) as described in our previous study (Wu et al., 2014). The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track in the brain sections of each animal (Fig. 4).

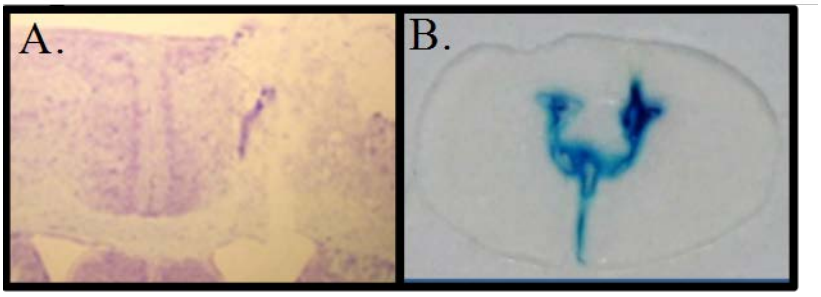

Figure 4. The location confirmation of cannula implantation

The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track in the brain sections of each animal (A). A pre-experimental confirmation of the correct location of the injection was carried out using a Methylene Blue injection (B).

1.4.3 Experimental Design

The experiments in this thesis have been divided into two parts. The first experiment (for Study 1 and Study 2) investigated the effect of icv injection of PA and ARA on central leptin sensitivity, hypothalamic inflammation, and central-regulated hepatic energy metabolism in normal mice. The second experiment (for Study 3 and Study 4) investigated the effect of icv injection of DHA and α-ethyl DHA ethyl ester on central leptin sensitivity, hypothalamic inflammation, and central-regulated hepatic energy metabolism in HFD mice.

1.4.3.1 Experiment proposal 1 (Study 1 and Study 2)

All of the 72 mice (10 weeks old, body weight: 22.74 ± 3.22g) were randomized into one of six groups: vehicle + saline (SS), vehicle + leptin (SL), PA + saline (PS), PA + leptin (PL), ARA + saline (AS), ARA + leptin (AL) (n=12/group). The mice were allowed to recover for 1 week after the cannula implantation. The mice received icv injection of PA, ARA, or vehicle (25 pmol/time/mouse, twice a day, 50 pmol/day) for 2.5 days (Abizaid and Horvath, 2008). Then, a central leptin sensitivity test was performed as described (Yu et al., 2013). After recovery for 3 days (day 5-7), the mice were treated with icv injection of PA and ARA for another 2.5 days (day 8-10) as described previously. On day 10, the mice were treated with an icv injection of either leptin (0.5 μg in 2 μl) or vehicle (2 μl saline) 1 hour after the last PA/ARA injection. The efficacy of central leptin in controlling blood glucose was determined by carrying out a glucose tolerance test 30 minutes after the icv leptin/saline administration (Roman et al., 2010). After 3 days of recovery (day11-13), another round of PA, ARA, or vehicle injection was conducted (day14-16), and subsequently the leptin/saline injection was administrated. 1 hour after the icv injection of leptin/saline, all of the mice were sacrificed (Fig. 5). As described previously (Ross et al., 2010), PA (P5585, Sigma-

Aldrich, Australia) and ARA (A9673, Sigma-Aldrich, Australia) were dissolved in 96% ethanol, dried using nitrogen gas, dissolved in 40% hydroxypropyl-b-cyclodextrin (HPB) (H107, Sigma-Aldrich), and then stored at -20 °C.

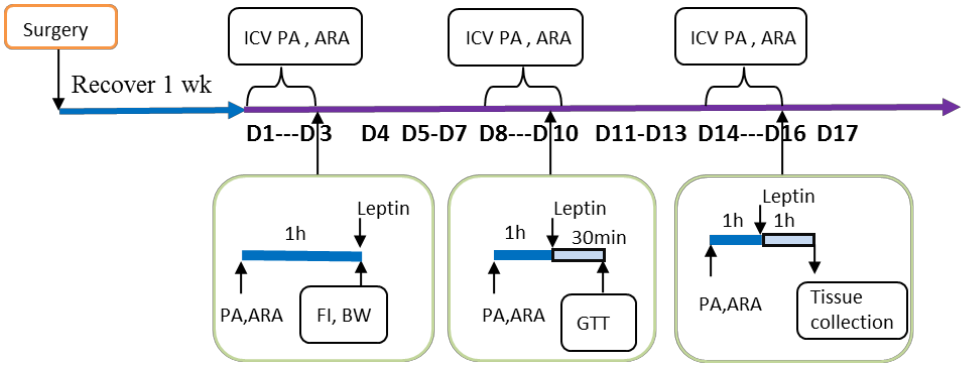

Figure 5. Flow chart of experiment for Study 1 and Study 2

wk(s): week(s); D1-3: day 1-3; icv: intracerebroventricular; h: hour(s); min: minute(s); FI: food intake; BW: body weight; GTT: glucose tolerance test; PA: palmitic acid; ARA: arachidonic acid.

1.4.3.2 Experiment proposal 2 (Study 3 and Study 4)

All of the 80 mice (10 weeks old, body weight: 25.35 ± 1.83 g) were randomized into one of eight groups: Lab chow + saline (LS), lab chow + leptin (LL), high-fat diet + saline (HS), high-fat diet + leptin (HL), high-fat diet +DHA + saline (HDS), high-fat diet + DHA + leptin (HDL), high-fat diet + DHA derivative + saline (HDdS), high-fat diet + DHA derivative + leptin (HDdL) (n=10/group). 1 week after the cannula implantation, the mice in the lab chow group were fed lab chow (5%, 3.21 kcal/g), and the mice in the HFD group were fed a high-fat diet (60%, 4.58 kcal/g). The mice in the HFD group were treated with icv injection of DHA, DHA derivative, or vehicle (1.5 nmol/time/mouse, twice a day, 3 nmol/day) at the same time for two days (Schwinkendorf et al., 2011). At the end of day 2, the mice in each group received either

an icv injection of leptin (0.5 µg in 2 µl saline) or vehicle (2 µl saline). Energy intake and body weight were measured at the beginning and at the end of HFD (48 hours) (Fig. 6). As described previously (Ross et al., 2010), DHA (D2534, Sigma-Aldrich) and α-ethyl DHA ethyl ester were dissolved in 96% ethanol, dried using nitrogen gas, dissolved in 40% HPB, and stored at -20 °C.

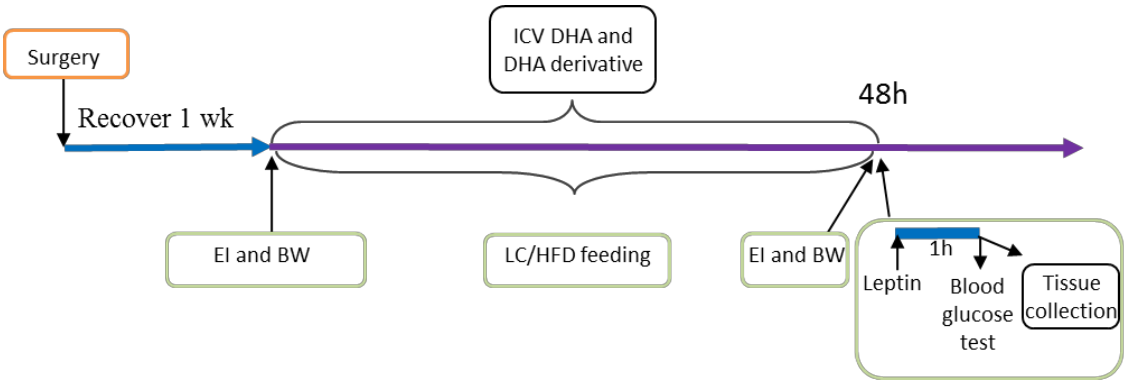

Figure 6. Flow chart of experiment for Study 3 and Study 4

wk(s): week(s); icv: intracerebroventricular; h: hour(s); EI: energy intake; BW: body weight; LC: lab chow; HFD: high fat diet; DHA: docosahexaenoic acid; DHA derivative: α-ethyl DHA ethyl ester.

1.4.4 Leptin Sensitivity Test

In Study 1 and Study 2, after fasting overnight, the mice were treated with an icv injection of either leptin (0.5 µg in 2 µl) or vehicle (2 µl saline) 1 hour after the last PA/ARA injection. The food intake for 1, 4, 16, 24 hours, and body weight for 24 hours and 48 hours were measured.

1.4.5 Glucose Tolerance Test

In Study 1 and Study 2, after the second interval of fatty acids and leptin injection, glucose tolerance tests were performed 30 minutes after leptin/saline injection. Blood

glucose was measured at 0, 30, 60, and 120 minutes after glucose administration (0.5 g/kg glucose, intraperitoneally) using a glucometer (Alameda, CA).

1.4.6 Blood Glucose Level Test

In Study 3 and Study 4, the blood glucose levels were examined 1 hour after the injection of leptin or saline using a glucometer.

1.4.7 Tissue Collection

After fasting overnight, the mice were treated with an icv injection of leptin or saline. 1 hour after the leptin/saline injection, the mice were sacrificed by CO_2 asphyxiation. The brain and liver were immediately collected, snap frozen in liquid nitrogen, and stored at -80 °C for further processing and analysis. In a cryostat at a temperature of -18°C, 500 µm frozen brain sections were cut from Bregma -0.58 mm to -2.72 mm according to a standard mouse brain atlas (Paxinos and Franklin, 2002). The MBH and PVN were dissected using a Stoelting Brain Punch (#57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) from frozen coronal sections based on previously described coordinates (Paxinos and Franklin, 2002, Yu et al., 2013).

1.4.8 Quantitative real-time PCR (qRT-PCR)

Total RNA from the hypothalamus and the liver was extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. Purity and concentration were determined with a Nanodrop 1000 spectrophotometer (Thermo Scientific). RNA was used to synthesize the first-strand complementary DNA using a high-capacity cDNA reverse transcription kit (AB Applied Biosystems, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed in a 20 µl final reaction volume using a SYBR green I master on a

Lightcycler 480 RT-PCR System (F. Hoffmann-La Roche Ltd, Switzerland). Amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The mRNA expression levels were normalized to GAPDH, which served as the internal control. Expression levels for each gene were calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct$ sample - ΔCt reference) as described previously (Livak and Schmittgen, 2001). The primers used are listed in Table 1. The mRNA expression of the hypothalamic cytokines (TNF-α, IL-1β, IL-6) in Study 3 and Study 4, and the mRNA expression of hepatic glucose and lipid metabolism in Study 1 - 4 were examined by qRT-PCR.

Table 1. The primers used in experiment

GENE	Forward primer	Reverse primer	NCBI reference
G6Pase	CTGTGAGACCGGACCAGGA	GACCATAACATAGTATACACCTGCTGC	NM_008061.3
PEPCK	CAGGATCGAAAGCAAGACAGT	AAGTCCTCTTCCGACATCCAG	NM_011044.2
GLUT2	ACCCTGTTCCTAACCGGG	TGAACCAAGGGATTGGACC	NM_031197.2
GK	GTGGTGCTTTTGAGACCCGTT	TTCAATGAAGGTGATTTCGCA	NM_010292.4
FAS	AGGGGTCGACCTGGTCCTCA	GCCATGCCCAGAGGGTGGTT	NM_007988.3
SCD1	CTTCTTGCGATACACTCTGG	TGAATGTTCTTGTCGTAGGG	NM_009127.4
HMG-CoA reductase	CACCTCTCCGTGGGTTAAAA	GAAGAAGTAGGCCCCCAATC	NM_008255.2
APoAI	GTGGCTCTGGTCTTCCTGAC	ACGGTTGAACCCAGAGTGTC	NM_009692.3
ACAT1	CCGAGACAACTACCCAAGGA	CACACACAGGACCAGGACAC	NM_009230.3
ACOX	ATGAATCCCGATCTGCGCAAGGAGC	AAAGGCATGTAACCCGTAGCACTCC	NM_015729.2
ACCa	GAAGTCAGAGCCACGGCACA	GGCAATCTCAGTTCAAGCCAGTC	NM_133360.2
PPARa	GCCTGTCTGTCGGGATGT	GGCTTCGTGGATTCTCTTG	NM_011144.6
CPT1a	TTGGGCCGGTTGCTGAT	GTCTCAGGGCTAGAGAACTTGGAA	NM_013495.2
SREBP-1c	ACCCTGGTGAGTGGAGGGACCATCTTGG	CTTTGCTTCAGTGCCCACCACCAGGTCTTT	NM_011480.3
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG	NM_008084.2
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	NM_013693.3
IL-1β	TACAAGGAGAACCAAGCAACGACA	GATCCACACTCTCCAGCTGCA	NM_008361.3
IL-6	GTGGCTAAGGACCAAGACCA	GGTTTGCCGAGTAGATCTCA	NM_031168.1

1.4.9 Western Blotting

Western Blotting was performed on protein extracts from frozen tissue as described in our previous study (Wu et al., 2014). The hypothalamus was quickly dissected and homogenized in solubilisation buffer on ice at 4 °C. The protein concentration of the supernatants was determined by BCA assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The whole tissue extracts were denatured by boiling (5 min) in Laemmli sample buffer containing 100 mM DTT. Precision plus protein^{TM} dual color marker (BioRad, #161-0374) was used as the molecular weight standard. Electrotransfer of proteins from the gel to nitrocellulose membranes was performed in a semi-dry transfer apparatus (Bio-Rad Laboratories). Nonspecific protein binding to the membrane was reduced by pre-incubation for 1 h at 22°C in blocking buffer. The nitrocellulose membranes were incubated overnight at 4°C with primary antibody (detailed in table 2). The blots were subsequently incubated with peroxidase-conjugated secondary antibodies for 1 hour. Specific bands were detected by chemiluminescence, and visualization/ capture was performed by exposure of the membranes to Amersham Hyperfilm ECL (GE Healthcare Life Sciences, USA). The expression of specific proteins was determined using the following antibodies: TNF-α, IL-1β, IL-6, pIκBα, pJAK2, JAK2, pJNK, and Akt from Santa Cruz Biotechnology (*Santa Cruz, CA*, USA), and pSTAT3, STAT3, SOCS3, pAkt, pFOXO1, FOXO1, TLR4, and pIkk from Cell Signalling Technology (Beverly, MA, USA), and TH (Anti-Tyrosine Hydroxylase from EMD Millipore (USA). Bands corresponding to the proteins of interest were scanned and band density analysed using the automatic imaging analysis system, Quantity One (Bio-Rad). All quantitative analyses were normalized to β-actin, based on our previous studies (du Bois et al., 2012). Due to the small amount of tissue in the MBH and PVN of the hypothalamus, we used a previously-described modified multi-strip western blot (Yu et al., 2013). Multi-

strip Western blotting is a modified immunoblotting procedure, which is based on simultaneous transfer of proteins from multiple gel-strips onto the same membrane, and is compatible with any conventional gel electrophoresis system. In contrast to the traditional "one protein detection per electrophoresis cycle", this procedure allows simultaneous monitoring of up to nine different proteins. All of the hypothalamic inflammation markers in Study 1 and Study 2, some of the inflammatory markers in Study 3 and Study 4 (pJNK, TLR4), and the leptin signalling mediators, and TH protein expression in the MBH and PVN in study 1 - 4 were examined by Western Blotting.

Table 2. The antibodies used in experiment

Peptide	Molecular weight	Name of Antibody	Manufacturer, catalog #, and/or name of provider	Species raised in; monoclonal or polyclonal	Dilution used
TNF-α	17KD	TNF-α (H-156)	Santa Cruz Biotechnology, sc-8301	rabbit, monoclonal	1:200
IL-1β	31KD	IL-1β (H-153)	Santa Cruz Biotechnology, sc-7884	rabbit, monoclonal	1:200
IL-6	21KD	IL-6 (H-183)	Santa Cruz Biotechnology, sc-7920	rabbit, monoclonal	1:200
TLR4	110KD	TLR4	Cell Signaling Technology, #2219S	rabbit, monoclonal	1:1000
pJNK	46, 54KD	pJNK (G-7)	Santa Cruz Biotechnology, sc-6254	mouse, monoclonal	1:1000
pJAK2	125KD	pJAK2 (Tyr1007/1008)	Santa Cruz Biotechnology, sc-21870	goat, polyclonal	1:1000
JAK2	128KD	JAK2 (C-10)	Santa Cruz Biotechnology, sc-390539	mouse, monoclonal	1:1000
pSTAT3	79KD	pSTAT3 (Y705)(D3A7)	Cell Signaling Technology, #9145S	rabbit, monoclonal	1:2000
STAT3	79,86KD	STAT3 (79D7)	Cell Signaling Technology, #4904S	rabbit, monoclonal	1:1000
SOCS3	26KD	SOCS3 (L210)	Cell Signaling Technology, #2932S	rabbit, polyclonal	1:1000
pAkt	60KD	pAkt (S473)	Cell Signaling Technology, #9271S	rabbit, polyclonal	1:1000
Akt	60KD	Akt (N-19)	Santa Cruz Biotechnology, sc-1619	goat, polyclonal	1:1000
pFOXO1	82KD	pFOXO1 (S256)	Cell Signaling Technology, #9461S	rabbit, polyclonal	1:1000
FOXO1	78-82KD	FOXO1 (L27)	Cell Signaling Technology, #9454S	rabbit, polyclonal	1:1000
pIKK	85, 87KD	pIKKa/b (S176/180, 16A6)	Cell Signaling Technology, #2697S	rabbit, polyclonal	1:1000
pIκBα	41KD	pIκBα (B-9)	Santa Cruz Biotechnology, sc-8404	mouse, monoclonal	1:1000

1.4.10 Statistical Analysis

Data were analysed using the statistical package SPSS 19.0 (SPSS, Chicago, IL, USA). The two-tailed student's t-test was used to compare hypothalamic cytokines expression between the vehicle groups and treatment groups (PA, ARA) in Study 1 and Study 2. One-way, two way analysis of variance (ANOVA) and the post hoc Tukey–Kramer honestly significant difference (HSD) test were used to analyse the food intake, energy

intake, body weight gain, the protein expression of hypothalamic leptin signalling molecules, the mRNA expression of hypothalamic inflammatory cytokines (Study 3 and Study 4), and mRNA expression of enzymes involved in hepatic glucose and lipid metabolism (Study 1 - 4). $p<0.05$ was regarded as statistically significant. Values are expressed as mean ± SEM.

Chapter Two

Palmitic acid induces central leptin resistance and impairs hepatic glucose and lipid metabolism in male mice (Study 1)

Reprinted from ***Journal of Nutritional Biochemistry***, **Cheng, L.**, Yu, Y., , Szabo A., Wu Y., Wang H., Camer D., Huang, X.-F. (2015). Palmitic acid induces central leptin resistance and impairs hepatic glucose and lipid metabolism in male mice. 2015 May 26 (5): 541-548.

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Palmitic acid induces central leptin resistance and impairs hepatic glucose and lipid metabolism in male mice☆

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Abstract

The consumption of diets rich in saturated fat largely contributes to the development of obesity in modern societies. A diet high in saturated fats can induce inflammation and impair leptin signaling in the hypothalamus. However, the role of saturated fatty acids on hypothalamic leptin signaling, and hepatic glucose and lipid metabolism remains largely undiscovered. In this study, we investigated the effects of intracerebroventricular (icv) administration of a saturated fatty acid, palmitic acid (PA, C16:0), on central leptin sensitivity, hypothalamic leptin signaling, inflammatory molecules and hepatic energy metabolism in C57BL/6 J male mice. We found that the icv administration of PA led to central leptin resistance, evidenced by the inhibition of central leptin's suppression of food intake. Central leptin resistance was concomitant with impaired hypothalamic leptin signaling (JAK2-STAT3, PKB/Akt-FOXO1) and a pro-inflammatory response (TNF-α, IL1-β, IL-6 and pIκBa) in the mediobasal hypothalamus and paraventricular hypothalamic nuclei. Furthermore, the pre-administration of icv PA blunted the effect of leptin-induced decreases in mRNA expression related to gluconeogenesis (G6Pase and PEPCK), glucose transportation (GLUT2) and lipogenesis (FAS and SCD1) in the liver of mice. Therefore, elevated central PA concentrations can induce pro-inflammatory responses and leptin resistance, which are associated with disorders of energy homeostasis in the liver as a result of diet-induced obesity.

Keywords: Palmitic acid; Leptin resistance; Hypothalamus; Inflammation; Glucose metabolism; Lipid metabolism

Chapter Three

Arachidonic acid impairs hypothalamic leptin signaling and hepatic energy homeostasis in mice (Study 2)

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Arachidonic acid impairs hypothalamic leptin signaling and hepatic energy homeostasis in mice

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ABSTRACT

Epidemiological evidence suggests that the consumption of a diet high in n-6 polyunsaturated fatty acids (PUFA) is associated with the development of leptin resistance and obesity. We aim to examine the central effect of n-6 PUFA, arachidonic acid (ARA) on leptin sensitivity and leptin-regulated hepatic glucose and lipid metabolism. We found that intracerebroventricular injection of ARA (25 nmol/day) for 2.5 days reversed the effect of central leptin on hypothalamic JAK2, pSTAT3, pAkt, and pFOXO1 protein levels, which was concomitant with a pro-inflammatory response in the hypothalamus. ARA also attenuated the effect of central leptin on hepatic glucose and lipid metabolism by reversing the mRNA expression of the genes involved in gluconeogenesis (G6Pase, PEPCK), glucose transportation (GLUT2), lipogenesis (FAS, SCD1), and cholesterol synthesis (HMG-CoA reductase). These results indicate that an increased exposure to central n-6 PUFA induces central cellular leptin resistance with concomitant defective JAK2-STAT3 and PI3K-Akt signaling.

Chapter Four

DHA and α-ethyl DHA ethyl ester improve central leptin sensitivity with anti-inflammatory response in high fat diet mice (Study3)

Conditional accepted by ***Endocrinology***, **Cheng, L.**, Yu, Y., Zhang Q., Wang H., Huang, X.-F. DHA and α-ethyl DHA ethyl ester improve central leptin sensitivity with anti-inflammatory response in high fat diet mice. 12/2015.

DHA and α-ethyl DHA ethyl ester improve central leptin sensitivity with anti-inflammatory response in high fat diet mice

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Abbreviated Title: DHA and DHA derivative improve central leptin sensitivity

Key words: DHA, α-ethyl DHA ethyl ester, leptin sensitivity, inflammation, hypothalamus

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Abstract:

Purpose: Anti-obesity effects and improved leptin sensitivity from n-3 polyunsaturated fatty acids have been reported in diet-induced obese animals. However, the acute central effect of DHA and DHA derivative on leptin signalling within specific regions of hypothalamus is still unclear. This study sought to determine the impact of DHA and DHA derivative (α-ethyl DHA ethyl ester) on energy homeostasis, hypothalamic leptin signalling, and hypothalamic inflammation.

Methods: Intracerebroventricular (icv) administration of DHA or DHA derivative (3 nmol/day) was performed for 2 days in C57BL/6 mice fed a high fat diet (HFD, 60%, 4.58 kcal/g).

Results: The central injection of DHA and DHA derivative not only reduced energy intake and body weight gain, but also corrected the HFD-induced hypothalamic inflammation. This was demonstrated by the reduced hypothalamic expression of pro-inflammatory molecules TNF-α, IL-1β, and IL-6, the inflammatory mediator pJNK, and the leptin signalling inhibitor SOCS3. In addition, both DHA and DHA derivative improved the leptin JAK2-STAT3 and PI3K-Akt signalling pathways in the hypothalamus by up-regulating the important mediators pJAK2, pSTAT3, and pAkt.

Conclusions: These results suggest that DHA and DHA derivative act directly on the hypothalamus to prevent HFD-induced inflammation and improve central leptin sensitivity, thus representing a promising potential therapeutic target for treating obesity and associated metabolic disturbances.

Introduction:

Hypothalamic leptin signalling plays a critical role in the development of obesity and its related metabolic disorders, such as type II diabetes (Bjorbaek and Kahn, 2004a, Posey et al., 2009). Improving central leptin sensitivity breaks the vicious cycle of energy balance dysregulation as evidenced in obesity. Leptin acts via several pathways, including the Janus kinase 2 (JAK2)-signal transducers and activators of transcription 3 (STAT3) and the phosphatidylinositol 3-kinase (PI3K)-Akt signalling in the central nervous system (Buettner et al., 2006). In diet induced obese rodents, leptin-induced pJAK2, pSTAT3, and pAkt activation in the hypothalamus are attenuated (Levin et al., 2004, Munzberg et al., 2004). This is accompanied by increased suppressor of cytokine signalling 3 (SOCS3), a negative regulator of the leptin signalling, which may be the cause of leptin resistance (Munzberg et al., 2004). In addition, it has been evident that the leptin PI3K signalling pathway is impaired during the development of diet induced obesity (DIO) in FVB/N mice (Metlakunta et al., 2008). Therefore, it seems that the defective regulation of above leptin pathways in the hypothalamus play a role in the pathogenesis of leptin resistance and obesity.

The sensitivity of various fatty acids to hypothalamic leptin plays an important role in the complex network of leptin signals controlling energy metabolism. Saturated fatty acids (SFA) have been shown to decrease leptin sensitivity and inhibit the anorexigenic effect of leptin in rodents (Kleinridders et al., 2009, El-Haschimi et al., 2000). n-3 polyunsaturated fatty acids (PUFA) and n-3 PUFA derivatives have been shown to exert some beneficial effects in against obesity and diabetes (Cintra et al., 2012, Pimentel et al., 2012, Rossmeisl et al., 2009). For instance, previous studies have found that n-3

PUFA improve hypothalamic leptin signalling by activating the phosphorylation of some downstream molecules of leptin signalling , including JAK2, STAT3 and Akt (Cintra et al., 2012). However, the role and mechanism of docosahexaenoic acid (DHA, 22:6 n-3) and DHA derivative (α-ethyl DHA ethyl ester) in modulating the hypothalamic leptin signalling underlying DIO remain largely undiscovered.

The molecular mechanisms by which high fat diet (HFD)-induced abnormal leptin activity and defective hypothalamic leptin signalling are involved in the hypothalamic inflammation (Carvalheira et al., 2003, Posey et al., 2009). SFA have been reported to induce hypothalamic inflammation by activating signal transduction through toll-like receptor 4 (TLR4) - nuclear factor-kB (NF-κB) signalling (Tsukumo et al., 2007, Shi et al., 2006b). The activation of TLR4 signalling leads to the coordinated induction of cytokines and other inflammatory mediators, such as tumor necrosis factor receptor-associated factor-6 (TRAF6), serine kinase c-Jun N-terminal Kinase (JNK), and the inhibitor of nuclear factor-kB kinase (Ikkβ). The activation of pro-inflammatory cytokines and NF-κB signalling pathway induce insulin and leptin resistance in the central nervous system (Akira et al., 2006). However, n-3 PUFA and their derivatives have well-known anti-inflammatory effects by reducing the intracellular inflammatory mediators (Cintra et al., 2012). For instance, HFD feeding enriched with fish oil (n-3 PUFA) for 2 months was found to reduce the hypothalamic levels of TNF-α, IL-6, and TRAF6, and increase the level of the anti-inflammatory cytokine IL-10 in male Wistar rats (Pimentel et al., 2013). A newly synthesized DHA derivative (CRBM-0244) was reported to exert anti-inflammatory properties by decreasing the activation of NF-κB

and related gene expression of pro-inflammatory mediators in lung tissue (Morin et al., 2011).

DHA, obtained directly from maternal milk or fish oil, is an omega-3 fatty acid that is a primary structural component of the human brain. It exerts beneficial effects in preventing the dysregulation of leptin signalling and the development of obesity (Vasickova et al., 2011, Pimentel et al., 2013). DHA derivative appears to exhibit therapeutic utility for obesity and associated metabolic disorders as naturally occurring n-3 PUFA by preventing weight gain, glucose intolerance, and decreasing lipids accumulation and adipose tissue inflammation with higher efficacy (Rossmeisl et al., 2009). In this study, we propose to examine the effect of central DHA and DHA derivative (α-ethyl DHA ethyl ester) on hypothalamic leptin sensitivity in short-term HFD mice. Energy intake, body weight gain, hypothalamic inflammation, and hypothalamic leptin JAK2-STAT3 and PI3K-Akt signalling in response to the administration of DHA and DHA derivative were investigated.

Materials and Methods:

Animals

80 Male C57BL/6J mice (10 weeks old, body weight: 25.35 ± 1.83 g) were obtained from the Animal Resource Centre (Perth, WA, Australia) and housed in environmentally controlled conditions (temperature 22 °C, 12 hour light/dark cycle). All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Experiment Protocols

After 1 week of acclimatization, mice were randomized into one of the eight groups: Lab chow +saline (LS), lab chow + leptin (LL), high fat + saline (HS), high fat + leptin (HL), high fat +DHA +saline (HDS), high fat + DHA +leptin (HDL), high fat +DHA derivative + saline (HDdS), high fat + DHA derivative +leptin (HDdL) (n=10/group). All mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An intracerebroventricular (icv) cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) as described in our previous study (Wu et al., 2014). The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track in the brain sections of each animal (Supplementary Fig. 2).

Energy homeostasis test

Seven days after the cannula implantation, The mice in LS and LL group were fed lab chow (5%, 3.21 kcal/g) (LC, Vella Stock feeds, Doonside, NSW, Australia), and the other five groups were fed high fat diets (60%, 4.58 kcal/g). The mice in the HFD groups were treated with icv injection of DHA, DHA derivative, or saline (3nmol/day/mouse) at the same time for two days (Abizaid and Horvath, 2008). Energy intake and body weight were measured after the HFD feeding for 48 hours. At the end of day 2, the mice receive either an icv injection of leptin (0.5 μg in 2 μl saline) or vehicle (2 μl saline) according the group design. As described previously (Ross et al., 2010), DHA (D2534, Sigma-Aldrich) and DHA derivative (α-ethyl DHA ethyl ester) were dissolved in 96% ethanol, dried using nitrogen gas, dissolved in 40% hydroxypropyl-b-cyclodextrin (HPB) (H107, Sigma-Aldrich), and stored at -20 °C. The

working solution contained 3 nmol DHA or DHA derivative for every injection (Yu et al., 2013).

Tissue collection

1 hour after the leptin and saline central injection, the mice were sacrificed by CO_2 asphyxiation. The brains were immediately collected, snaps frozen in liquid nitrogen, and stored at -80°C for further processing and analysis. At a temperature of -18°C, 500 µm frozen brain sections were cut using a cryostat from Bregma -0.58 mm to -2.72 mm according to a standard mouse brain atlas (Paxinos and Franklin, 2002). The mediobasal (MBH) and the paraventricular nucleus (PVN) of the hypothalamus were dissected from frozen coronal sections using a Stoelting Brain Punch (#57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) based on previously described coordinates (Yu et al., 2013).

Western blot analysis

Western blotting was performed on protein extracts from frozen tissue as described in our previous study (Wu et al., 2014). The expression of specific proteins was determined using the following antibodies: pJAK2 (sc-21870) (Santa Cruz Biotechnology, California), pSTAT3 (Tyr705) (#9145), SOCS3 (#2932), pAkt (#9271), phosphor-forkhead box protein O1 (pFOXO1) (#9461), TLR4 (#2219S), and pJNK (#9251) (Cell Signalling Technology Beverly, MA, USA). Bands corresponding to the proteins of interest were scanned and band density was analyzed using the Quantity One automatic imaging analysis system (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were normalized to β-actin, based on our previous studies (du Bois et al., 2012). Due to the small amount of tissue in the MBH and PVN of the hypothalamus, we used a previously-described modified multi-strip western blot, which

allows the detection of multiple proteins with a smaller sample size than the standard western blot (Yu et al., 2013).

RNA isolation and RT-PCR

Total RNA from the hypothalamus was extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA, USA) and reverse-transcribed to first-strand complementary DNA using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed in a 20 μl final reaction volume using a SYBR green I master on a Lightcycler 480 Real-time PCR System (F. Hoffmann-La Roche Ltd, Switzerland). Amplification was carried out at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. This was repeated for a total of 45 cycles. The mRNA expression levels of inflammatory molecules were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as the internal control. Expression levels for each gene were calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct$ sample - ΔCt reference) as described previously (Livak and Schmittgen, 2001).

Statistics

Data were analyzed using the statistical package SPSS 19.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and the post hoc Tukey–Kramer honestly significant difference (HSD) test were used to analyze central leptin sensitivity, hypothalamic leptin signalling molecules, and the mRNA expression of inflammatory cytokines. $p<0.05$ was regarded as statistically significant. Values are expressed as mean ± SEM.

Results:

1. icv DHA and DHA derivative reduce energy intake and body weight gain in HFD mice

Initially, male C57BL/6J mice were fed either a lab chow (5%, 3.21 kcal/g) or a HFD (60%, 4.58 kcal/g) for two days. The HFD significantly increased energy intake (+44.85%, $p<0.001$, Fig. 1A) and body weight gain (+149.90%, $p<0.001$, Fig. 1B) in 48 hours in comparison to the lab chow group. To examine the direct effect of central administration of n-3 PUFA and n-3 PUFA derivatives on the regulation of energy intake and body weight gain, all of the mice in the HFD groups were treated with icv injection of DHA or DHA derivative (α-ethyl DHA ethyl ester) (3 nmol/day/mice) for two days. After 2 days icv injection, DHA decreased energy intake (-15.22%, $p<0.01$, Fig. 1A) and body weight gain (-51.85%, $p<0.05$, Fig. 1B) compared to the HF group. DHA derivative decreased energy intake (-11.84, $p<0.05$, Fig. 1A) compared to the HF group. These results indicate that the central administration of DHA and DHA derivative alone exert an anorexigenic effect.

2. icv DHA and DHA derivative reduce hypothalamic inflammation

Cumulative evidence has shown the effect of high-fat diets on the induction of hypothalamic inflammation (De Souza et al., 2005). n-3 PUFA have well-known anti-inflammatory effects and inflammation-resolving actions (Cintra et al., 2012). To determine the direct impact of central treatment of DHA and DHA derivative on hypothalamic inflammation, the expression of pro-inflammatory markers and cytokines in the MBH and PVN of the hypothalamus was measured. Consistent with previous researches, HFD increased the hypothalamic activation of the inflammatory cytokines in

both the MBH and the PVN, including TNF-α (MBH: $p<0.05$, Fig. 2A; PVN: $p<0.05$, Fig. 3A), IL-1β (MBH: $p<0.01$, Fig. 2A; PVN: $p<0.05$, Fig. 3A), and IL-6 (MBH: $p<0.05$, Fig. 2A; PVN: $p<0.05$, Fig. 3A). This was accompanied by the up-regulation of some proteins involved in inflammatory signal transduction, such as TLR4 (both $p<0.05$, Fig. 2B&3B) and pJNK (both $p<0.05$, Fig. 2C&3C) in MBH and PVN. Administration of DHA centrally inhibited the mRNA expression of TNF-α (PVN: $p<0.05$, Fig. 3A), IL-1β (MBH: $p<0.05$, Fig 2A; PVN: $p=0.091$ Fig. 3A), IL-6 (both $p<0.05$, Fig. 2A&3A), and protein level of pJNK (MBH: $p<0.05$, Fig.2C) in the hypothalamus of HFD groups. Similarly, DHA derivative decreased the mRNA expression of TNF-α (both $p<0.05$, Fig. 2A&3A), IL-1β (both $p<0.05$, Fig. 2A&3A), IL-6 (both $p<0.05$, Fig. 2A&3A), and protein level of pJNK (MBH: $p<0.05$, Fig. 2C) in MBH and PVN nucleus. These data demonstrate that the central administration of DHA and DHA derivative exerts a potent anti-inflammatory response in the hypothalamus of HFD mice. SOCS3 is a member of the family of SOCS proteins that bind with their central SH2 domains to phosphor-tyrosine residues in the cytokine receptors (Howard and Flier, 2006). In the current study, the level of SOCS3 was significantly elevated in the MBH and PVN of the HFD mice (both $p<0.05$, Fig 2D&3D). This effect was inhibited by treatment with central DHA (MBH: $p<0.05$, Fig. 2D) and DHA derivative (MBH: $p<0.05$, Fig. 2D; PVN: $p<0.05$, Fig. 3D).

3. icv DHA and DHA derivative improve leptin JAK2-STAT3 and PI3K-Akt signalling in the hypothalamus

Leptin plays a key role in energy homeostasis through the JAK2-STAT3 and PI3K-Akt signalling in the hypothalamus (Plum et al., 2006, Myers, 2004). The effect of central administration of DHA and DHA derivative on these two signalling pathways in MBH

and PVN of hypothalamus was examined. In the lab chow feed groups, the level of pJAK2, pSTAT3, pAkt, and pFOXO1 was increased significantly by the leptin injection (supplementary Fig. 1). In the HF groups, the leptin-induced activation changes on above parameters were partly attenuated, except for pSTAT3 (in MBH) and pFOXO1 (in PVN) where increase were evidenced (both $p<0.05$, Fig. 4B&5D). The ratios of pSTAT3/STAT and pJAK2/JAK2 also demonstrate central leptin resistance significance in HFD mice. This suggests, to some trend, there is a central leptin resistance in HFD mice. However, the central administration of DHA significantly improved leptin signal transduction through the up-regulation of pJAK2 (MBH: $p<0.05$, Fig 4A; PVN: $p<0.05$, Fig. 4C), pSTAT3 (MBH: $p<0.05$, Fig 4B; PVN: $p<0.05$, Fig. 4D), and pAkt (MBH: $p<0.05$, Fig 5A; PVN: $p<0.05$, Fig. 5C) in the HFD group compared with vehicle injection. The central administration of DHA derivative also significantly improved pJAK2 ($p<0.05$, Fig. 4C), pSTAT3 ($p<0.05$, Fig.4D), and pAkt ($p<0.01$, Fig. 5C) in response to central leptin in the PVN but not in the MBH nucleolus. These data suggest that the central administration of DHA improves leptin signalling through the JAK2-STAT3 and PI3K-Akt pathways in both the MBH and the PVN, while DHA derivative only exerts this effect in the PVN. There is no alteration for pFOXO1 between DHA and DHA derivative treatment groups (Fig. 5).

Discussion:

Supplementation with n-3 PUFA has anti-inflammatory properties, providing a protective effect against the development of obesity (Schmitz and Ecker, 2008, Cintra et al., 2012). The present study determine to ascertain the acute effect of central administration of n-3 PUFA and n-3 PUFA derivatives on inflammatory response and

leptin signalling in the MBH and the PVN of hypothalamus. We provide the first evidence that DHA and DHA derivative improve hypothalamic leptin sensitivity and leptin signalling in HFD-fed mice. DHA and DHA derivative (α-ethyl DHA ethyl ester) are thus positioned to play an important role in the prevention of obesity.

It has been well established that n-3 PUFA are associated with the prevention of HFD-induced obesity in vivo and in vitro studies (Storlien et al., 1987, Reseland et al., 2001, Ukropec et al., 2003, Mori et al., 2004). However, the potential effect of central n-3 PUFA, especially n-3 PUFA derivatives on energy homeostasis is largely undiscussed. In the present study, HFD feeding induced a significant increase of energy intake and body weight gain than that in lab chow-fed mice. Importantly, the icv administration of DHA and DHA derivative decreased energy intake and body weight gain in HFD mice. This result is in agreement with previous study by Schwinkendorf 's group have shown that the central administration of DHA reduced food intake and body mass in male Sprague-Dawley rats (Schwinkendorf et al., 2011). Few reports have covered DHA derivative, and only one study has demonstrated that dietary DHA derivative (α-ethyl DHA ethyl ester) feeding for 4 months reduced the food intake and body weight gain in HFD mice, which is consistent with our finding (Rossmeisl et al., 2009). The present study demonstrates for the first time that central administration of DHA derivative exerts anorexigenic effect.

In addition to pharmacological and genetic approaches, dietary fatty acids could be attractive candidates for controlling the hypothalamic inflammation in the installation and progression of diet-induced obesity. It has been determined that both HFD

consumption and the central administration of SFA can induce an inflammatory response in the hypothalamus (Kleinridders et al., 2009, Milanski et al., 2009). However, whether the direct central action of n-3 PUFA and n-3 PUFA derivatives have a beneficial effect on hypothalamic inflammation is still unclear. In the current study, we initially administered the HFD (60%, 4.58 kcal/g) to mice. The result indicated that HFD for 2 days induced an acute pro-inflammatory response in the hypothalamus (as demonstrated by the increased mRNA expression of TNF-α, IL-1β, and IL-6), which is consistent with a recent study made by Thaler et al. (Thaler et al., 2012). In addition, the increased activation of the hypothalamic inflammatory molecules, TLR4, pJNK, and SOCS3 in the HFD is in agreement with the previous report that demonstrated HFD induced hypothalamic inflammation through TLR4-NF-κB signalling. On the other hand, the present study showed that the central administration of DHA and DHA derivative reduced the intracellular inflammatory cytokines and their mediators, including TNF-α, IL-1β, IL-6, and pJNK in hypothalamus. The result is in line with previous dietary n-3 PUFA reports (Pimentel et al., 2013, Milanski et al., 2009), and suggests that the exposure of central DHA and DHA derivative suppress the hypothalamic inflammatory profile. Combined with the inhibitive effect of the DHA derivative on TLR4 and following downstream parameter pJNK, our results predict that the central administration of DHA derivative might exert an anti-inflammatory function through TLR4/NF-κB pathway (Oh et al., 2010).

Defective JAK2-STAT3 signalling in the hypothalamus contributes to the development of HFD-induced central leptin resistance and obesity (Ghilardi et al., 1996, Munzberg et al., 2004, Bjornholm et al., 2007). Our results demonstrate that the central

administration of DHA and DHA derivative up-regulate pJAK2 and pSTAT3 expression at specific nuclei in the hypothalamus, which may represent a potential route to improve leptin signalling. Our finding is consistent with previous studies showing that the icv administration of n-3 PUFA (linolenic acid) improves hypothalamic leptin signal transduction via the JAK2-STAT3 pathway (Cintra et al., 2012). The mechanism for this amelioration effect has been further explored in the present study via SOCS3, a potent inhibitor of leptin signalling. A previous study has shown that SOCS3 inhibits JAK2, a tyrosine kinase believed to be required for all known leptin-dependent signalling pathways (Bjorbaek et al., 1999, Bjorbaek and Kahn, 2004a). In addition, it has also been proven that heterozygous SOCS3-deficient mice exhibit an enhanced activation of leptin-induced hypothalamic STAT3 phosphorylation (Howard et al., 2004). These SOCS3-deficient mice have an increased sensitivity to the weight-reducing effects of leptin, and they are resistant to the development of DIO. These findings suggest a critical role of SOCS3 in mediating hypothalamic leptin resistance via the JAK2-STAT3 pathway. The present study demonstrated that the level of SOCS3 was specifically elevated in the hypothalamus in HFD animals, and also that DHA and DHA derivative reduced hypothalamic SOCS3 levels. Therefore, both previous reports and the present results indicate that DHA and DHA derivative may exert its beneficial effect via inhibiting the level of SOCS3 through the JAK2-STAT3 signalling.

Another potential mechanism for the development of obesity is the impaired regulation of the PI3K-Akt pathway (Warne et al., 2011, Metlakunta et al., 2008). Both dietary and central administration of n-3 PUFA have been shown to improve the PI3K signalling transaction significantly by enhancing the protein level of pAkt and pFOXO1 (Pimentel

et al., 2012, Cintra et al., 2012). In line with previous studies, we demonstrated that the central administration of DHA and DHA derivative promoted leptin PI3K-Akt signalling (via pAkt and pFOXO1), suggesting a potential preventive effect on leptin resistance and obesity. In particular, this is the first evidence to show that the central administration of DHA derivative exerts a facilitative effect on leptin signalling in the hypothalamus. The K_{ATP} channel activation induced by fatty acids may be a possible mechanism for the effect of fatty acids on hypothalamic leptin signalling. It has been reported that n-3 PUFA are able to activate K(+)(ATP) channels in the neuron membrane (Hirafuji et al., 2003). And also K_{ATP} channel activation and leptin mediated phosphorylation of cellular signalling intermediates are PI3K dependent in ARC neurones (Mirshamsi et al., 2004). Taken together, our findings and previous reports may help to explain the potential mechanism of n-3 PUFA on leptin PI3K signalling. In our current study, compare with the effect of DHA on leptin signalling, the promoting effect of DHA derivative on leptin JAK2-pSTAT3 and PI3K-Akt signalling specifically exists in PVN but not in the ARC, indicating that the DHA derivative may be more sensitive to hypothalamic leptin in the PVN than in other nucleus.

Recent findings imply that hypothalamic inflammation is the possible mechanism underlying diet induced obesity and central insulin and leptin resistance (Cintra et al., 2012). Constitutive activation of hypothalamic Ikkβ has been reported to induce central leptin resistance and impaired leptin signalling by reducing STAT3 phosphorylation (Zhang et al., 2008b). In contrast, a pharmacological blockade of inflammatory signalling in the hypothalamus improved leptin sensitivity and pSTAT3 activation (Milanski et al., 2012). In this study, the central administration of DHA and DHA

derivative inhibited the hypothalamic inflammation by decreasing the genetic expression of the pro-inflammatory cytokines and inflammatory signalling molecules. This may contribute to the improved leptin sensitivity and restored hypothalamic leptin signalling through pSTAT3. Therefore, the current study indicates that the protective effect of n-3 PUFA and their derivatives on reducing inflammation is beneficial to the prevention of obesity (Cintra et al., 2012, Pimentel et al., 2013). Another potential beneficial mechanism of n-3 PUFA on obesity is the binding with GPR120, an important mediator of the anti-inflammatory and leptin-sensitizing effects of unsaturated fatty acids in the hypothalamus (Cintra et al., 2012). It has been proved that, in the hypothalamus of obese rats, n-3 PUFA is able to bind with GPR120, and GPR120 activates signal transduction through β-arrestin 2/TAB1, which switches-off the TLR4 and TNF-α inflammatory pathways (Cintra et al., 2012). However, a different viewpoint from another report suggests that n-3 FA derivatives were unable to stimulate GPR120 activation in cell assay (Oh et al., 2010). Therefore, further studies are warranted to confirm the detailed role and mechanism of DHA derivative in this pathway and DIO-induced central leptin resistance.

In summary, the present study demonstrates that DHA and DHA derivative exhibit beneficial effects on obesity and associated metabolic disorders. Exposure of central DHA and DHA derivative (α-ethyl DHA ethyl ester) can lead to significant anti-inflammatory response and clearly improve leptin JAK2-STAT3 and PI3K-Akt signalling pathways in the hypothalamus. Therefore, these compounds could be an attractive therapeutic target for the prevention and treatment of obesity, dyslipidemia,

and diabetes. Moving forward, greater consideration should be given to designing nutritional interventions that target multiple leptin signalling pathways.

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Figures:

Fig. 1

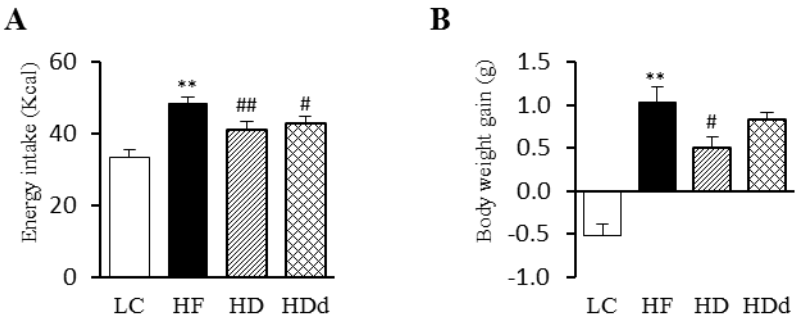
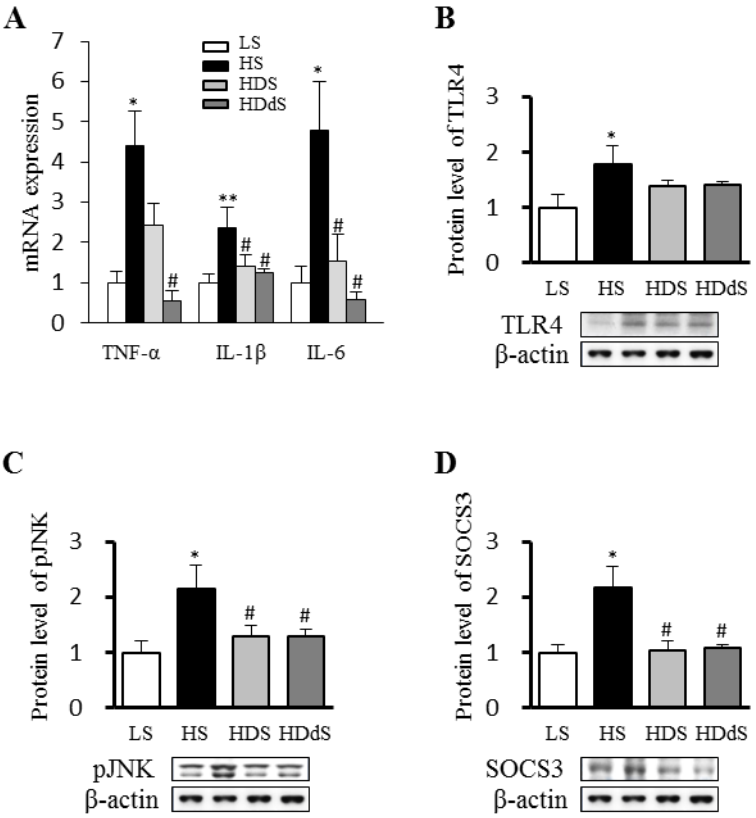


Fig. 2



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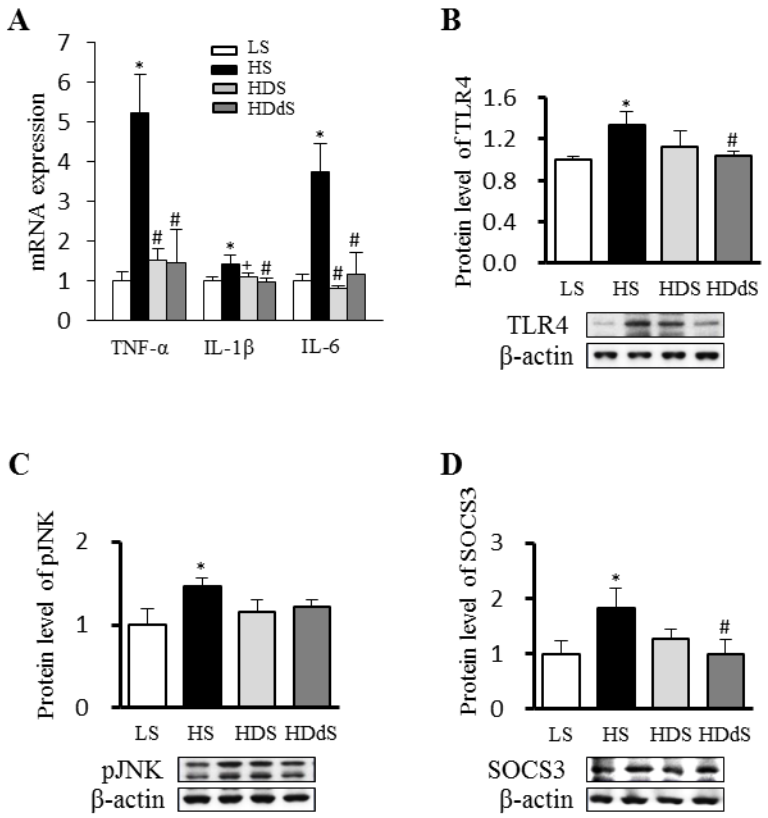
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Fig. 4

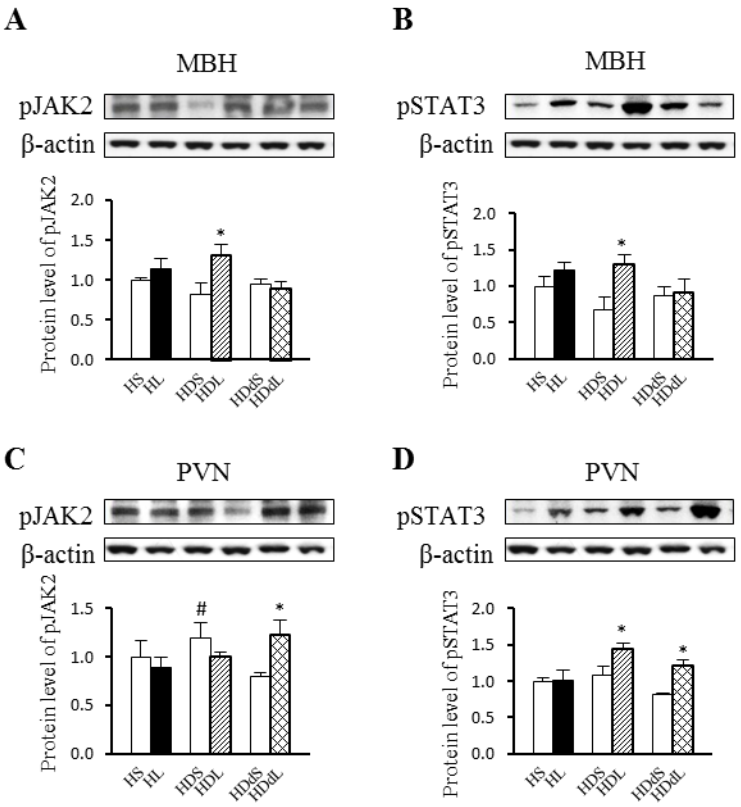
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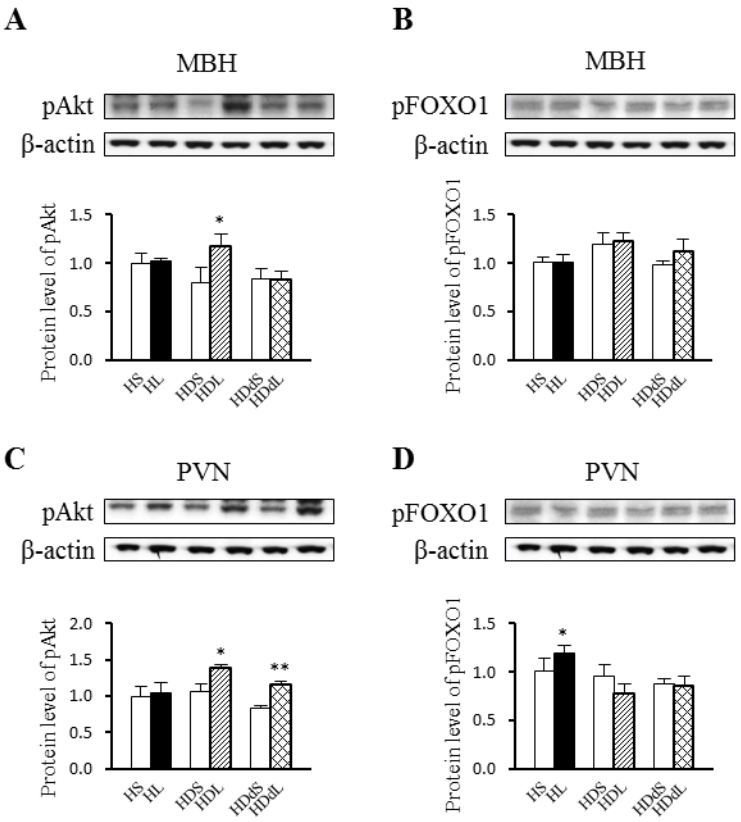
Fig. 5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Supplementary data:

Fig. 1

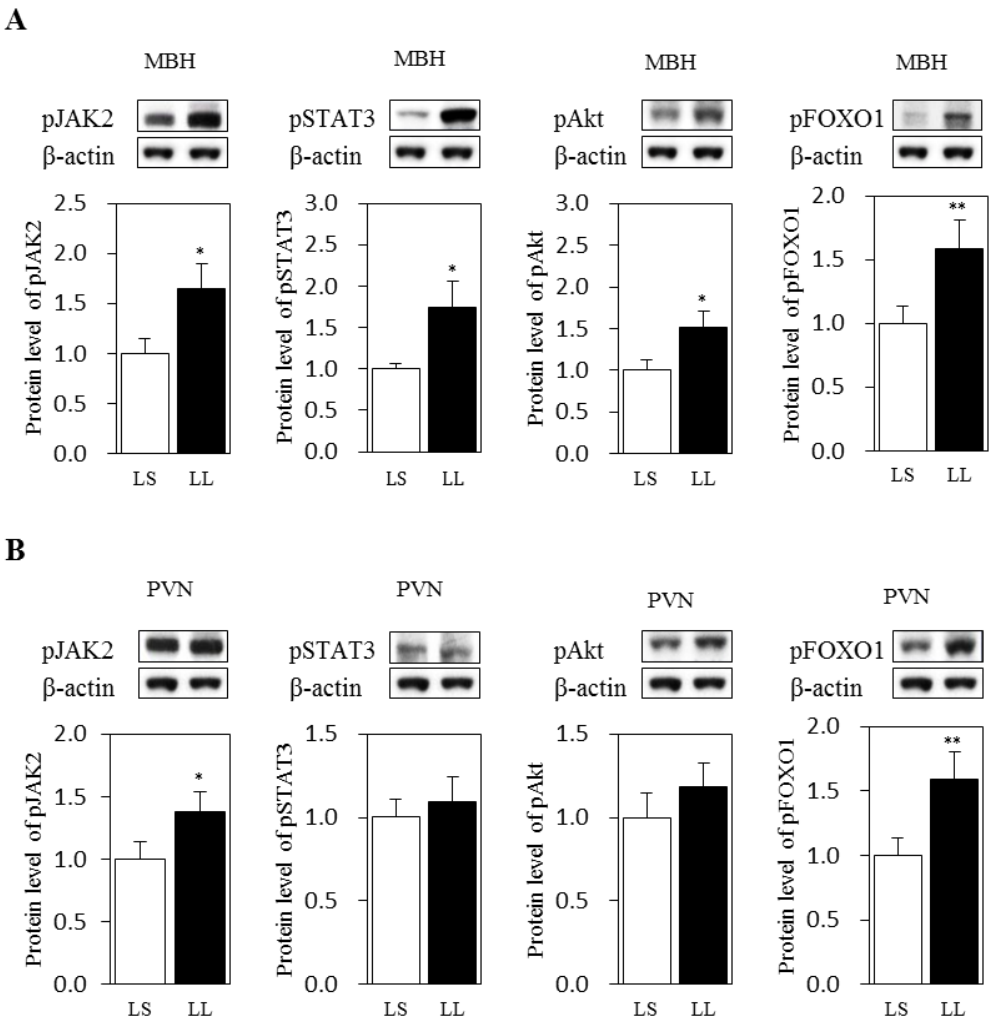

Fig. 1 Icv leptin action on JAK2-STAT3 and PI3K signaling in the hypothalamus. pJAK2, pSTAT3, pJAK2, pFOXO1 in the MBH (A) and PVN (B) of the hypothalamus were detected by western blot after the icv injection of leptin/saline in lab chow diet mice. *p<0.05, * * p<0.01 vs LS.

Fig. 2 The location confirmation of cannula implantation (same as Figure 4. in thesis)

Figure legends:

Fig. 1 The effect of icv DHA and DHA derivative on energy intake and body weight gain.

Energy intake (A) and body weight gain (B) for 48 hours were significantly increased in the HF-diet group, and inhibitory effects were induced by the icv injection of DHA and DHA derivative. $^{**}p < 0.01$ *vs* LC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* HF.

Fig. 2 The effect of icv DHA and DHA derivative on the inflammatory response in the MBH.

The mRNA expression of TNF-α, IL-1β, and IL-6 in the MBH was detected by RT-PCR. The protein level of TLR4 (B), pJNK (C), and SOCS3 (D) in the MBH was detected by western blot after the icv injection of DHA, DHA derivative, and vehicle in the HFD mice. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* LS; $^{\#}p < 0.05$ *vs* HS.

Fig. 3 The effect of icv DHA and DHA derivative on the inflammatory response in the PVN.

The mRNA expression of TNF-α, IL-1β, and IL-6 in the PVN was detected by RT-PCR. The protein level of TLR4 (B), pJNK (C), and SOCS3 (D) in the PVN was detected by western blot after icv injection of DHA, DHA derivative, and vehicle in the HFD mice. $^{*}p < 0.05$ *vs* LS; $^{\#}p < 0.05$ *vs* HS; $+ 0.05 < p < 0.1$ vs HS.

Fig. 4 icv DHA and DHA derivative improve leptin JAK2-STAT3 signaling in the hypothalamus.

Phospho-JAK2 (pJAK2) (A/C) and phospho-STAT3 (pSTAT3) (B/D) in the MBH and PVN were detected by western blot after an icv injection of leptin or saline followed by the icv injection of DHA, DHA derivative, and vehicle in the HFD mice. *p<0.05 *vs* saline injection, $^{\#}p$<0.05 *vs* HS.

Fig. 5 icv DHA and DHA derivative improve leptin PI3K-Akt signaling in the hypothalamus.

Phospho-Akt (pAkt) (A/C) and phospho-FOXO1 (pFOXO1) (B/D) in the MBH and PVN were detected by western blot after an icv injection of leptin or saline followed by the icv injection of DHA, DHA derivative, and vehicle in the HFD mice. *p<0.05, **p<0.01 *vs* saline injection.

Chapter Five

DHA and α-ethyl DHA ethyl ester improve hepatic glucose and lipid metabolism via central leptin regulation in HFD male mice (Study 4)

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DHA and α-ethyl DHA ethyl ester improve hepatic glucose and lipid metabolism via central leptin regulation in HFD male mice

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Abbreviated Title: DHA and DHA derivative improve hepatic energy metabolism

Key words: n-3 PUFA, docosahexaenoic acid, α-ethyl DHA ethyl ester, leptin resistance, hepatic glucose and lipid metabolism

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Abstract:

N-3 polyunsaturated fatty acids and their derivatives exert anti-obesity effects by improving leptin sensitivity and metabolic action in peripheral tissues. However, the precise role of these fatty acids on energy homeostasis and hepatic metabolism in the central nervous system is still unclear. This study sought to determine the influence of central docosahexaenoic acid (DHA) and α-ethyl DHA ethyl ester on energy intake and body weight gain, hepatic glucose and lipid metabolism in high-fat diet (HFD) mice. C57BL/6 mice were treated with intracerebroventricular (icv) injection of DHA, DHA derivative, or vehicle (3nmol/day), together with HFD feeding for 2 days. Acute central injection of DHA and DHA derivative exhibits an anorexigenic effect in HFD mice. Icv injection of DHA and DHA derivative improves the effect of central leptin in regulating hepatic glucose metabolism by down-regulating glucose transportation (GLUT2) and glycolysis (GK). DHA and DHA derivative also ameliorate hepatic lipid metabolism, which is mediated by the decreased activity of lipogenesis (FAS, SCD1, and SREBP-1c) and cholesterol synthesis (HMG-CoA reductase), and the increased activity of β-oxidation (PPARα, ACAT1, and ACOX). Icv injection of DHA enhanced the activation of TH in mediobasal hypothalamus, connecting the action of central leptin to hepatic metabolism. Central administration of DHA and DHA derivative reversed HFD-induced adiposity and improved leptin's regulation on hepatic glucose and lipid metabolism by increasing central leptin sensitivity. Thus, the administration of DHA and DHA derivative may provide realistic and alternative therapeutic strategies for the treatment of obesity and associated metabolic disturbances.

Introduction:

There is growing evidence that dietary lipid components are implicated in altering central leptin sensitivity, *leptin signal transduction* pathways, and the central regulation of hepatic glucose and lipid homeostasis (Obici et al., 2002, Warne et al., 2011, Morgan et al., 2004). Diets rich in saturated fatty acids are associated with decreased leptin sensitivity, obesity-induced defective leptin signaling in the hypothalamus, and dysregulation of peripheral metabolism, including hepatic steatosis, hyperglycemia, lipidemia etc. (Warne et al., 2011, Milanski et al., 2009). In contrast, n-3 polyunsaturated fatty acids (n-3 PUFA) and their derivatives exert a range of protective effects against high-fat diet (HFD)-induced leptin resistance, glucose intolerance, and hepatic metabolic abnormalities (Cintra et al., 2012, Clarke, 2001, Rossmeisl et al., 2009). Intracerebroventricular (icv) injection of an n-3 PUFA α-linolenic acid (ALA, C18:3, n-3) has been reported to reduce food intake and body adiposity and increase hypothalamic leptin sensitivity and leptin signaling in obese rodents (Cintra et al., 2012). The DHA derivative (α-ethyl DHA ethyl ester) has also been demonstrated to reduce plasma leptin level, glucose intolerance, and adiposity (Rossmeisl et al., 2009).

Docosahexaenoic acid (DHA, C22:6, n-3), a major fatty acid in n-3 PUFA, is found in maternal milk and marine fish oil. DHA is the predominant fatty acid of membrane phospholipids in the retina and brain matter of mammals (Guesnet and Alessandri, 2011). Dietary DHA exhibits therapeutic utility for obesity and metabolic syndrome, and restores hyperglycemia and hyperlipidemia in obese rodents (Bays, 2007, Rustan et al., 1992, Vasickova et al., 2011, Pimentel et al., 2013). Similarly, the DHA derivative α-ethyl DHA ethyl ester has shown promise for the treatment of HFD-induced obesity,

dyslipidemia and insulin resistance in rodents (Rossmeisl et al., 2009). We have recently shown that administration of DHA and α-ethyl DHA ethyl ester (DHA derivative) improves leptin signaling in the hypothalamus in HFD-fed mice (data not shown). However, whether DHA and DHA derivative are able to exert beneficial effects on the regulation of hepatic glucose and lipid metabolism via the central leptin signaling pathway is still unclear.

Leptin, an adipocyte-derived hormone, initiates negative feedback within the hypothalamus, including the restraint of food intake and stimulation of energy expenditure (Friedman, 2002). In times of excess energy storage, impaired responses or 'resistance' to the afferent input of leptin to the hypothalamus favour weight gain, fat accumulation, and peripheral metabolic dysregulation. This may in turn contribute to the development of obesity and type 2 diabetes. Previous studies indicate that defective signal transducers and activators of transcription 3 (STAT3) signaling (El-Haschimi et al., 2000, Munzberg et al., 2004) and phosphatidylinositol 3-kinase (PI3K) signaling (Metlakunta et al., 2008) play a critical role in the hypothalamic regulation of energy homeostasis and peripheral glucose and lipid metabolism during diet-induced obesity (DIO). Previous studies have shown that hypothalamic leptin mediates peripheral glucose and lipid metabolism by regulating the gene transcription of key enzymes involved in its metabolism (Pocai et al., 2005, Hidaka et al., 2002, Toyoshima et al., 2005, Shimomura et al., 1999, Prieur et al., 2008). For instance, icv leptin has been shown to inhibit glucose output, hepatic gluconeogenesis (e.g. glucose 6-phosphatase, G6Pase, and phosphoenolpyruvate carboxykinase, PEPCK), glucose transportation (e.g. glucose transporter 2, GLUT2), and glycolysis (e.g. glucokinase, GK) in HFD rodents

(Pocai et al., 2005, Hidaka et al., 2002, Toyoshima et al., 2005, Shimomura et al., 1999). In addition, other reports have demonstrated that leptin administration can inhibit hepatic lipogenesis, while promoting fatty acid oxidation and cholesterol transportation (Minokoshi et al., 2002, Rossetti et al., 1997). In particular, Prieur and colleagues have demonstrated that icv administration of leptin not only suppresses lipogenesis by decreasing acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-coenzyme A desaturase 1 (SCD1) mRNA expression, but also activates β-oxidation by increasing acyl-CoA oxidase (ACOX) and carnitine palmitoyl transferase 1 (CPT1) mRNA expression in the liver of *ob/ob* mice (Prieur et al., 2008). Finally, it has been suggested that leptin regulates peripheral energy metabolism through its action on autonomic nerves, particularly via the sympathetic nerve system, which transmits leptin signals to the liver and other tissues (Shi et al., 2013).

Taken together, this may support the hypothesis that n-3 PUFA and derivatives regulate hepatic glucose and lipid metabolism by regulating central leptin sensitivity and action. In the present study, we examined the effect of icv administration of DHA and DHA derivative on energy homeostasis, central leptin-regulated hepatic glucose and lipid metabolism, and hypothalamic sympathetic nervous system activity.

Materials and Methods:

Animals

80 Male C57BL/6J mice (10 weeks old, body weight: 25.35 ± 1.83 g) were obtained from the Animal Resource Centre (Perth, WA, Australia) and housed in environmentally controlled conditions (temperature 22 °C, 12 hour light/dark cycle). All

experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

Experiment Protocols

After 1 week of acclimatization, mice were randomized into one of the eight groups: Lab chow +saline (LS), lab chow + leptin (LL), high fat diet + saline (HS), high fat diet + leptin (HL), high fat diet +DHA +saline (HDS), high fat diet + DHA +leptin (HDL), high fat diet +DHA derivative + saline (HDdS), high fat diet + DHA derivative +leptin (HDdL) (n=10/group). All mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An icv cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) as described in our previous study (Wu et al., 2014). The accuracy of cannula implantation into the lateral ventricle was confirmed by examining the needle track in the brain sections of each animal (Supplementary Fig. 1).

Energy intake and body weight gain test

Seven days after the cannula implantation, the mice in the LS and LL group were fed lab chow (5%, 3.21 kcal/g) (LC, Vella Stock feeds, Doonside, NSW, Australia), and the other mice were fed HFD (60%, 4.58 kcal/g). The mice in the HFD groups were treated with icv injection of DHA, DHA derivative, or saline (1.5nmol/time/mouse, twice/day/mouse) at the same time for two days (Abizaid and Horvath, 2008, Yu et al., 2013). Energy intake and body weight were measured 48 hours after the beginning of HFD feeding. As described previously (Ross et al., 2010), DHA (D2534, Sigma-Aldrich) and DHA derivative (α-ethyl DHA ethyl ester) were dissolved in 96% ethanol,

dried using nitrogen gas, dissolved in 40% hydroxypropyl-b-cyclodextrin (HPB, H107, Sigma-Aldrich), and stored at -20 °C.

Tissue collection

After 2 days treatment, the mice were fasted overnight. On the third day, the mice were conducted with an icv injection of leptin/saline (0.5 μg in 2 μl saline). The blood glucose level was examined 1hour after injection using a glucometer (Alameda, CA). After the blood glucose level test, the mice were sacrificed by CO_2 asphyxiation. The brains and livers were immediately collected, snaps frozen in liquid nitrogen, and stored at -80°C for further processing and analysis. At a temperature of -18°C, 500 μm frozen brain sections were cut using a cryostat from Bregma-0.58 mm to -2.72 mm according to a standard mouse brain atlas (Paxinos and Franklin, 2002). The mediobasal (MBH) and the paraventricular nucleus (PVN) of the hypothalamus were dissected from frozen coronal sections using a Stoelting Brain Punch (#57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) based on previously described coordinates (Yu et al., 2013).

Western Blot analysis

Western Blot was performed on protein extracts from frozen tissue as described in our previous study (Wu et al., 2014). The expression of hypothalamic t yrosine hydroxylase (TH) proteins was determined using the following antibodies: Anti-TH (AB9983) (EMD Millipore, USA). Bands corresponding to the proteins of interest were scanned and band density was analyzed using the Quantity One automatic imaging analysis system (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were normalized to β-actin, based on our previous studies (du Bois et al., 2012). Due to the small amount of tissue in the MBH and PVN of the hypothalamus, we used a

previously-described modified multi-strip Western Blot, which allows the detection of multiple proteins with a smaller sample size than the standard Western Blot (Yu et al., 2013).

RNA isolation and RT-PCR

Total RNA from the hypothalamus was extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA, USA) and reverse-transcribed to first-strand complementary DNA using the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed in a 20 μl final reaction volume using a SYBR green I master on a Lightcycler 480 RT-PCR System (F. Hoffmann-La Roche Ltd, Switzerland). Amplification was carried out at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. This was repeated for a total of 45 cycles. The mRNA expression levels of inflammatory molecules were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as the internal control. Expression levels for each gene were calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct$ sample - ΔCt reference) as described previously (Livak and Schmittgen, 2001).

Statistics

Data were analyzed using the statistical package SPSS 19.0 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and the post hoc Tukey–Kramer honestly significant difference (HSD) test were used to analyze energy intake, body weight gain, blood glucose levels, and the mRNA expression of key enzymes involved in hepatic glucose and lipid metabolism. $p<0.05$ was regarded as statistically significant. Values are expressed as mean ± S.

Results:

Icv DHA and DHA derivative show anorexigenic function

Male C57BL/6J mice were fed lab chow (5%, 3.21 kcal/g) or high fat (60%, 4.58 kcal/g) diets for two days. The HFD led to a significant increase in energy intake (+44.85%, $p<0.001$, Fig. 1A) and body weight gain (+149.90%, $p<0.001$, Fig. 1B) in 48 hours in comparison to the lab chow group. To examine the direct effect of the central administration of n-3 PUFA and n-3 PUFA derivatives on energy regulation via the central nervous system, all mice in the HFD groups were treated with icv injection of DHA, DHA derivative (α-ethyl DHA ethyl ester), or vehicle (3nmol/day/mouse) for two days. Icv DHA reduced energy intake (15.22%, $p<0.01$, Fig. 1A) and body weight gain (51.85%, $p<0.05$, Fig. 1B). Similarly, icv injection of DHA derivative significantly reduced energy intake (-11.84%, $p<0.05$, Fig. 1A) but not body weight gain compared to the HF group. These results indicate that the central administration of DHA and DHA derivative have an anorexigenic effect in HFD mice.

Icv DHA and DHA derivative do not affect blood glucose levels

To determine the effect of central infusion of n-3 PUFA and n-3 PUFA derivatives on blood glucose level, a glucose level test was performed after the central administration of fatty acids. Compared with the lab chow diet mice, the blood glucose levels in all HFD feeding (for 2 days) groups were increased significantly ($p<0.01$, Fig. 2). However, there were no significant changes in blood glucose levels for the HFD groups that received icv administration of DHA or DHA derivative compared with vehicle injection. These results indicate that the blood glucose level was unaltered by central

DHA or DHA derivative administration, although it was enhanced by 2-days HFD feeding.

Icv DHA and DHA derivative ameliorate the central leptin-regulated hepatic glucose metabolism

To address whether the exposure of DHA and DHA derivative in central nervous system can regulate gene expression of enzymes involved in hepatic glucose metabolism, qRT-PCR assessment was conducted. Specifically, the genetic expression of the key enzymes of gluconeogenesis, glucose transportation, and glycolysis in the liver were examined. G6Pase and PEPCK are gluconeogenic enzymes. PEPCK is the rate-limiting enzyme that phosphorylates oxaloacetate to form phosphoenolpyruvate, G6Pase promotes the dephosphorylation of glucose-6-phosphate, and allows the release of newly synthesized glucose into the bloodstream. GLUT2 transports glucose from the liver to the bloodstream (Gould and Holman, 1993). GK is an enzyme that facilitates the phosphorylation of glucose to glucose-6-phosphate. In the present study, leptin unable to change the mRNA expression of G6Pase, PEPCK, GLUT2, and GK compared with the vehicle injection in the HFD groups (Fig. 3). After the fatty acids treatment, both DHA and DHA derivative increased the G6Pase mRNA expression (both $p<0.05$, Fig. 3A), which is inconsistent with previous studies. In addition, DHA also decreased the GLUT2 ($0.05<p<0.1$, Fig. 3C) and GK ($p<0.05$, Fig. 3D) mRNA expression in response to leptin compared with the vehicle injection. At the same time, DHA derivative decreased the GLUT2 mRNA expression in response to leptin compared with the vehicle injection ($p<0.05$, Fig. 3C). The results indicate that the icv administration of

DHA and DHA derivative improves some aspects of hepatic glucose metabolism in the presence of central leptin.

Icv DHA and DHA derivative improve the central leptin-regulated hepatic lipid metabolism

To determine the action of icv DHA and DHA derivative on the regulation of hepatic lipid metabolism in response to central leptin, we used qRT-PCR to examine the mRNA expression of enzymes involved in hepatic lipogenesis, lipid β-oxidation, and cholesterol metabolism. ACCα and FAS are critical enzymes for lipogenesis. SCD1 is a lipogenic enzyme responsible for the formation of monounsaturated fatty acids, the main precursors of triglycerides (Horton et al., 1998). Sterol and regulatory element binding protein-1c (SREBP-1c) is a transcription factor that promotes the expression of a number of lipogenic genes (Horton, 2002). In the HF group, leptin administration did not change the gene expression of FAS, SCD1, ACCα, and SREBP-1c compared with the vehicle injection (Fig. 4). This suggests that the HFD feeding may resist the action of leptin on lipogenesis (Fig. 4). Interestingly, FAS, SCD1, and SREBP-1c mRNA expression were decreased by DHA central administration in response to the leptin injection (all $p<0.05$, Fig. 4A&B&D). SREBP-1c mRNA expression was significantly reduced by the DHA derivative ($p<0.05$, Fig. 4D). DHA and DHA derivative alone increased the SCD1 mRNA expression significantly in the liver (both $0.05<p<0.1$, Fig. 4B). These results suggest that the central administration of DHA and DHA derivative improves the central leptin effect on lipogenesis.

ACOX and acetyl-CoA acetyltransferase 1 (ACAT1) are central enzymes involved in mitochondrial β-oxidation (Fournier et al., 1994). Peroxisome proliferator-activated

receptor alpha (PPARα) is a key regulator in the regulation of fatty acid β-oxidation and lipogenesis (Dreyer et al., 1993). In the HFD groups, the central leptin injection significantly increased the CPT1α mRNA expression (p<0.05, Fig. 5A). After the DHA pre-treatment, the mRNA expression of PPARα and ACAT1 was profoundly increased by leptin compared with the vehicle treatment (both p<0.05, Fig. 5C&5D). Similarly, DHA derivative increased the mRNA expression of ACOX (p<0.05, Fig. 5B) and ACAT1 (p<0.05, Fig. 5D) in response to the leptin injection in the HFD group. These results suggest that both DHA and DHA derivative ameliorate the central leptin modulation of β-oxidation in the liver.

In addition, the gene expression of key enzymes in cholesterol metabolism was examined. Apo lipoprotein A1 (ApoA1) is the main component of *high-density lipoprotein* and transports cholesterol and phospholipids from the body's tissues to the liver (Breslow et al., 1982). 3-hydroxy-3-methylglutaryl-coenzyme reductase (HMG-CoA reductase) is the key enzyme involved in the de novo synthesis of cholesterol. In the HFD group, the expression of HMG-CoA reductase was down-regulated by the central injection of DHA and DHA derivative compared with the vehicle injection in response to leptin (DHA: p<0.01, Fig. 5F; DHA derivative: p<0.05, Fig. 5F). There was no significant difference in the mRNA expression of ApoA1 between the groups (Fig. 5E).

Icv DHA and DHA derivative enhance hypothalamic TH expression

Hypothalamic TH has been suggested to connect central leptin action on sympathetic activity to peripheral energy homeostasis (Shi et al., 2013). To determine whether DHA and DHA derivative act on the hypothalamus and regulate leptin-mediated hepatic

metabolism through the sympathetic nervous system, the protein level of hypothalamic TH was examined. Central leptin significantly stimulated the TH activation compare with the vehicle group in the PVN of the hypothalamus ($p<0.01$, Fig. 6B). In the HFD group, DHA ($p<0.05$, Fig. 6A) increased the TH protein expression in MBH of the hypothalamus significantly while the DHA derivative unable to increase TH in the hypothalamus.

Discussion:

It is well-known that defective leptin signaling and dysfunction in the regulation of hepatic energy homeostasis induced by HFD contribute to the development of obesity, which appears to largely originate from the hypothalamic sensitization of leptin (Ghilardi et al., 1996, Warne et al., 2011). Dietary n-3 PUFA and n-3 PUFA derivatives exert beneficial effects in preventing central leptin resistance, obesity, and peripheral metabolic disturbances (Cintra et al., 2012, Rossmeisl et al., 2009). Our previous study has demonstrated that DHA and DHA derivative can prevent central leptin resistance and improve hypothalamic leptin signaling in HFD mice (data not shown). In the present study, we further explored the role and mechanism of DHA and DHA derivative on hypothalamic leptin's action in regulating hepatic glucose and lipid metabolism in HFD mice. The role of the hypothalamic sympathetic nervous system activity in the regulation of hepatic energy metabolism by DHA and DHA derivative was also investigated.

Several recent animal studies have suggested that n-3 PUFA have neuroprotective effects against HFD-induced insulin and leptin resistance and obesity (Flachs et al.,

2006, Cintra et al., 2012). Most of these studies focus on the dietary n-3 PUFA effects in preventing obesity. Only a few studies examine neuronal control of energy homeostasis and peripheral metabolism after acute injection of n-3 PUFA in central nervous system. The present study demonstrates that the central administration of DHA and DHA derivative exhibits anorexigenic function, and prevents the liposity induced by short-term exposure to HFD by reducing energy intake and body weight gain. Our results are consistent with previous findings showing that the central administration of DHA and ALA significantly enhanced the anorexigenic effect of leptin in both normal and obese rats (Cintra et al., 2012, Schwinkendorf et al., 2011). DHA derivative (α-ethyl DHA ethyl ester) has also been shown to exhibit a similar beneficial effect on obesity and associated metabolic traits as naturally occurring n-3 PUFA in C57BL/6 mice in reducing leptin secretion (Rossmeisl et al., 2009). In addition, the effects of n-3 PUFA on energy homeostasis through its regulation of hypothalamic neuropeptide expression may further support the findings from our study. Dietary n-3 PUFA implementation and substitution increase anorexigenic pro-opiomelanocortin (POMC) expression and decrease neuropeptide Y expression in the arcuate nucleus of the hypothalamus compared to a high-SFA diet in rodents (Dziedzic et al., 2007, Huang et al., 2004). Specifically, the central administration of DHA has been reported to have an anorexigenic effect by up-regulating the hypothalamic POMC gene expression in male Sprague-Dawley rats (Schwinkendorf et al., 2011). However, the role and mechanism of DHA and DHA derivative on anorexigenic effect via the regulation of hypothalamic neuropeptides requires further study.

In addition to the well characterized anorexigenic properties, hypothalamic leptin also plays an important role in the regulation of hepatic glucose metabolism by altering its key metabolic enzymes (Sivitz et al., 1997, Morton and Schwartz, 2011). Leptin has recently been reported to have inhibitory effects on hepatic gluconeogenesis (via G6Pase and PEPCK) (German et al., 2011), glycolysis (via GK) (Tang and Chen, 2010), and glucose transportation (via GLUT2) (Toyoshima et al., 2005). In the present study, the normal preventive effect of leptin on this glucose metabolism was impaired in HFD mice, which may attribute to attenuated hypothalamic leptin signaling in DIO (El-Haschimi et al., 2000, Metlakunta et al., 2008). Importantly, we found for the first time that icv injection of DHA decreased GLUT2 and GK mRNA expression in response to central leptin in HFD mice, suggesting that central DHA administration improves central leptin's action on hepatic glucose transportation and glycolysis. Thus DHA derivative shows a preventive effect on hepatic transportation in the present study. Our findings suggest that DHA and DHA derivative improve hepatic glucose metabolism and have anorexigenic effects via central leptin regulation way. Consistent with our finding, a previous study has shown that the central administration of n-9 PUFA oleic acid inhibited hepatic glucose metabolism partly via a neuronal control mechanism (Obici et al., 2002). However, another possible mechanism suggests that dietary lipids may act directly on central nervous system to regulate hepatic glucose metabolism via a nutrient metabolic mechanism that is independent of central leptin (Levin et al., 1999). Further studies are warranted to elucidate whether n-3 PUFA and n-3 PUFA derivatives modulate hepatic glucose and lipid metabolism via a direct nutrient metabolic mechanism or a neuroendocrine mechanism.

Central administration of leptin has been demonstrated to improve hepatic lipid metabolism through up-regulating fatty acid oxidation and down-regulating lipogenesis (Gallardo et al., 2007, Toyoshima et al., 2005). The effects of n-3 PUFA and n-3 PUFA derivatives on improving hepatic fatty acid β-oxidation have also been reported (Rossmeisl et al., 2009, Dobrzyn and Dobrzyn, 2006, Neschen et al., 2007). However, the effects of n-3 PUFA and n-3 UPFA derivatives on hepatic lipid metabolism in regulating central leptin's action are still unclear. In the present study, we showed that the gene expression of FAS, SCD1, and SREBP-1c were down-regulated by DHA, and SREBP-1c was down-regulated by DHA derivative respectively at the presence of leptin. This suggests that DHA and DHA derivative improve the effect of leptin on hepatic lipogenesis. In addition, the effect of leptin on fatty acid β-oxidation was also improved by icv injection of DHA and DHA derivative, as evidenced by the up-regulation of PPARα and ACAT1 mRNA expression by DHA as well as the increase of ACOX and ACAT1 gene expression by DHA derivative in the liver. Thus, our results showed for the first time that DHA and DHA derivative play a significant role in regulating leptin's action on hepatic lipid metabolism, particularly lipogenesis and fatty acid β-oxidation. The suppressive effect of leptin on cholesterol synthesis via HMG-CoA reductase in obesity has already been evidenced (Prieur et al., 2008). In the present study, the central administration of DHA and DHA derivative down-regulated the mRNA expression of HMG-CoA reductase in the liver, suggesting the central administration of DHA and DHA derivative improves central leptin's regulation on hepatic cholesterol synthesis. Taken together, our findings suggest that DHA and DHA derivative inhibit hepatic lipogenesis and cholesterol synthesis and improve hepatic

lipid oxidation. This may reduce lipid deposition in the liver, thereby alleviating the lipotoxicity and obesity induced by HFD.

Hypothalamic leptin Janus kinase 2 (JAK2)-STAT3 and PI3K-Akt signaling pathways play an important role in energy homeostasis (Plum et al., 2006, Myers, 2004). It has been suggested that hypothalamic JAK2-STAT3 signaling contributes to the dysregulation of energy intake and energy expenditure (El-Haschimi et al., 2000, Munzberg et al., 2004), while the PI3K-Akt signaling pathway significantly regulates the hepatic glucose and lipid metabolism in DIO (Metlakunta et al., 2008). It remains unknown whether n-3 PUFA modulate hepatic glucose and lipid metabolism via the hypothalamic leptin signaling pathway. Our recent study has demonstrated that the central administration of DHA and DHA derivative improved both JAK2-STAT3 and PI3K-Akt signaling in the hypothalamus. Given the effects of DHA and DHA derivative in improving leptin's action on hepatic glucose and lipid metabolism as shown in the present study, we speculate that DHA and DHA derivative exert beneficial effects on regulating hepatic glucose and lipid metabolism by improving leptin STAT3 and PI3K signaling in the hypothalamus.

The mechanism by which leptin signal from the hypothalamus modulates peripheral glucose and lipid metabolism is an area of current research (Shi et al., 2013). It has been suggested that the sympathetic nervous system plays a key role in connecting central leptin signals from the hypothalamus to the liver and other peripheral tissues (Morton, 2007). For instance, it has been reported that leptin acts in the hypothalamus to suppress lipogenesis via activating sympathetic nervous system in both liver and white adipose

tissues (Buettner et al., 2008, Warne et al., 2011). The expression of TH (a rate-limiting enzyme for the synthesis of catecholamines) in the hypothalamus has been identified as a major candidate and indicator for the regulation of sympathetic outflow in normal or obese rodents (Li et al., 2009, Shi et al., 2013). In the present study, we showed for the first time that DHA increased leptin-induced activation of TH in MBH of the hypothalamus, suggesting that DHA improves the function of leptin-activated sympathetic outflow. This finding may connect the effects of n-3 PUFA in regulating hepatic glucose and lipid metabolism with hypothalamic sympathetic nervous system. However, further valid evidence is needed to prove this mechanism.

In conclusion, the present study demonstrates that central administration of DHA and DHA derivative reduces energy intake and body weight gain and improves central leptin's action in regulating hepatic glucose and lipid metabolism. Our findings raise the possibility that fatty acids-induced regulation of hepatic energy homeostasis attribute to the leptin sensitivity alteration within the hypothalamus. It also provides a valid animal model for the effect of central administration of fatty acids on peripheral glucose and lipid metabolism. The fact that the central administration of DHA and DHA derivative improves central leptin's effect on energy homeostasis and hepatic glucose and lipid metabolism sheds a light on the possibility of nutritional intervention with DHA and DHA derivative as novel therapeutic targets for the prevention and treatment of obesity and associated metabolic disturbances.

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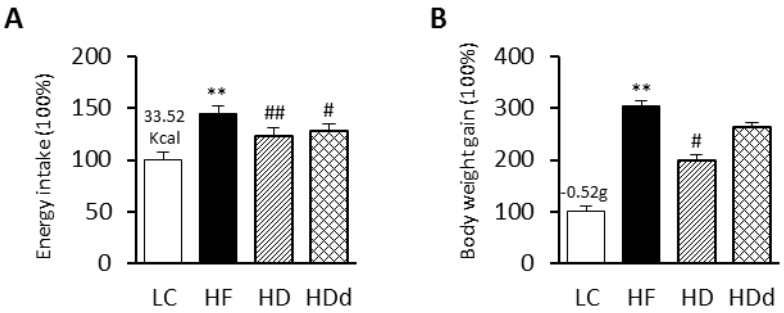
Figures:

Fig. 1



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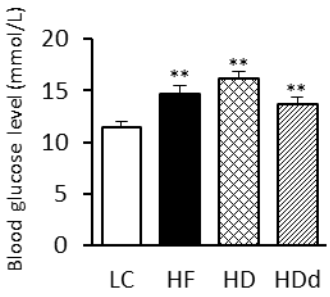

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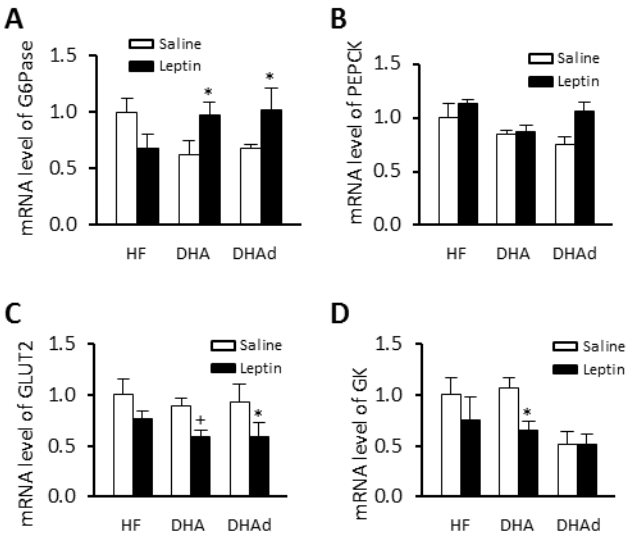

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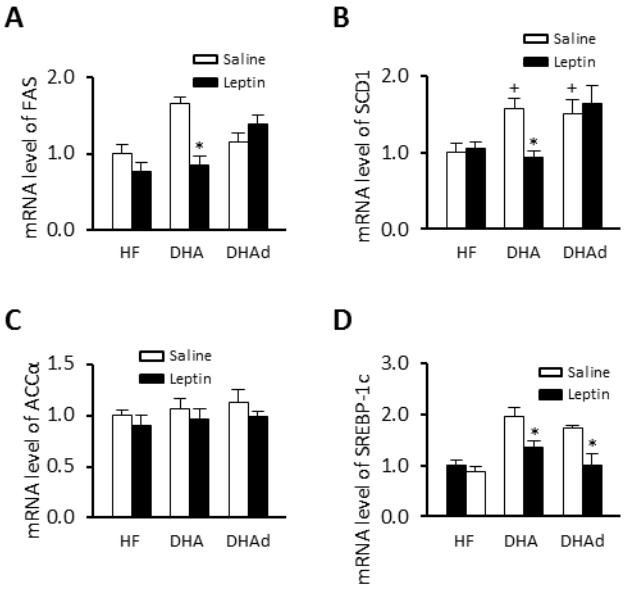
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Fig. 5

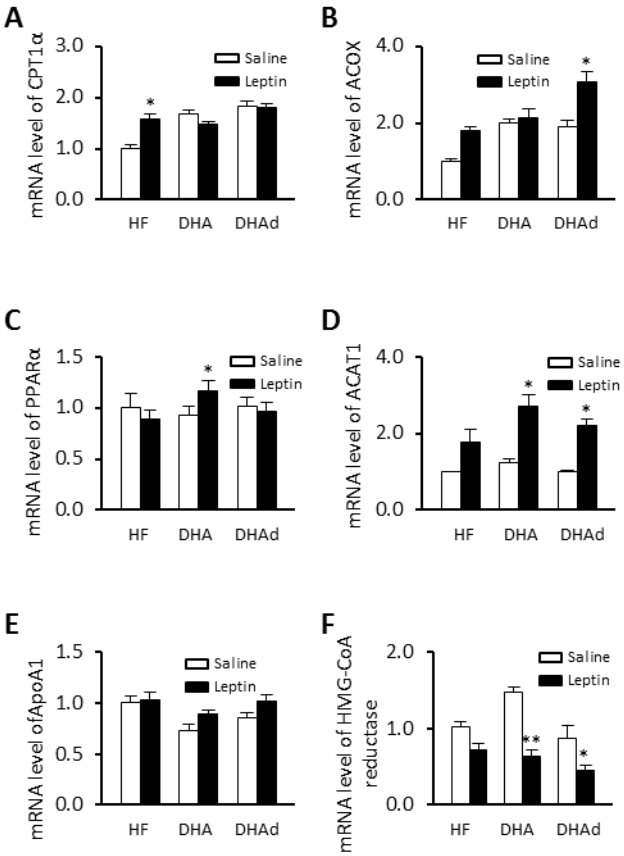
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Fig. 6

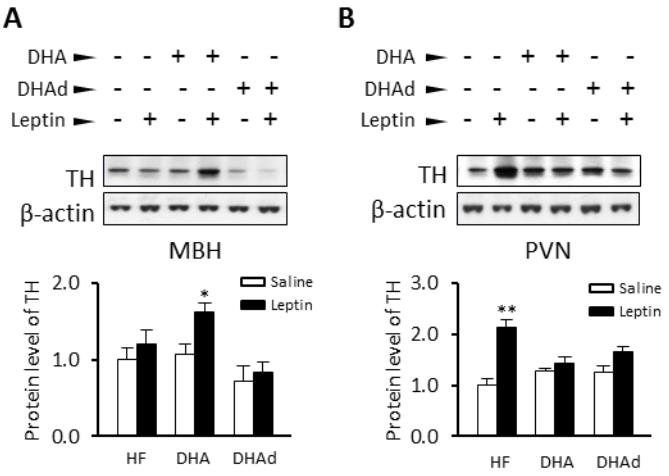
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Figure legends:

Fig. 1 Effects of icv DHA and DHA derivative on energy intake and body weight gain

Energy intake (A) and body weight gain (B) for 48 hours were examined in mice after treated with the icv injection of DHA, DHA derivative, or vehicle, together with lab chow or high fat diet feeding for two days. $^{**}p<0.01$ *vs* LC, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* HF; LC: lab chow feeding; HF: high fat diet feeding; HD: high fat diet feeding + DHA; HDd: high fat diet feeding + DHA derivative.

Fig. 2 Effects of icv DHA and DHA derivative on blood glucose level

After overnight fasting, the mice were conducted with icv injection of leptin or saline. The blood glucose level was examined 1 hour after leptin/saline injection in mice treated with an icv injection of DHA, DHA derivative, or vehicle for two days. $^{**}p<0.01$ *vs* LC. LC: lab chow feeding; HF: high fat diet feeding; HD: high fat diet feeding + DHA; HDd: high fat diet feeding + DHA derivative.

Fig. 3 Effects of icv DHA and DHA derivative on mRNA expression of genes involved in hepatic glucose metabolism

The mRNA levels of G6Pase (A), PEPCK (B), GLUT2(C), and GK (D) in the liver were measured by quantitative real-time PCR in HFD-fed mice treated with an icv injection of leptin or saline after the icv injection of DHA, DHA derivative, or vehicle for 2 days. $^{*}p<0.05$ *vs* saline injection, $+0.05<p<0.1$ *vs* saline injection.

Fig. 4 Effects of icv DHA and DHA derivative on mRNA expression of genes involved in hepatic lipogenesis

The mRNA levels of FAS (A), SCD1(B), ACCα (C), SREBP-1c(D) in the liver were measured by quantitative real-time PCR in HFD-fed mice treated with an icv injection of leptin or saline after the icv injection of DHA, DHA derivative, or vehicle for 2 days. $^{*}p<0.05$ *vs* saline injection, $+0.05<p<0.1$ *vs* HS.

Fig. 5 Effects of icv DHA and DHA derivative on mRNA expression of genes involved in hepatic fatty acid β-oxidation and cholesterol metabolism

The mRNA levels of CPT1α (A), ACOX (B), PPARα (C), ACAT1 (D), ApoA1 (E), and HMG-CoA reductase (F) in the liver were measured by quantitative real-time PCR in HFD-fed mice treated with an icv injection of leptin or saline after the icv injection of DHA, DHA derivative, or vehicle for 2 days. $*p<0.05$, $**p<0.01$ *vs* saline injection.

Fig. 6 Effects of icv DHA and DHA derivative on TH protein level in the hypothalamus

The level of TH protein expression in the MBH (A) and PVN (B) of the hypothalamus was detected by Western Blot in HFD-fed mice treated with an icv injection of leptin or saline after the icv injection of DHA, DHA derivative, or vehicle for 2 days. $*p<0.05$, $**p<0.01$ *vs* saline injection. MBH: mediobasal hypothalamus, PVN: paraventricular nucleus.

Chapter Six

6.1 Overall Discussion

The present series of studies demonstrate that exposure of distinct fatty acids in the central nervous system leads to different effects on central leptin sensitivity, hypothalamic leptin signalling pathway, and central leptin regulated hepatic metabolism. Moreover, these studies indicate that the influences of distinct fatty acids on central leptin sensitivity and action are associated with hypothalamic inflammation. The knowledge obtained from these studies may lead to practical dietary interventions with the proper use of leptin sensitising or insensitising fatty acids to control type II diabetes and obesity. This chapter will provide a general discussion of the key findings of the present PhD project, and the potential mechanism of central leptin sensitivity alteration induced by fatty acids. A detailed discussion of each study has been included in the end of chapters 2 - 5. The main findings of this thesis are summarised in Table. 2.

Table 2. Summary of the main findings of chapter 2-5

	Function	Parameter	PA	ARA	DHA	DHA de
Central leptin sensitivity	Energy homeostasis	FI or EI	– 1,4,16h	–4,16,24h	↓	↓
		BWG	~	~	~	↓
	MBH	pJAK2	–	–	†	~
		pSTAT3	–	–	†	~
		SOCS3	~	–	NA	NA
		pAkt	–	–	†	~
		pFOXO1	–	–	~	~
	PVN	pJAK2	–	–	*†	†
		pSTAT3	–	–	†	†
		SOCS3	–	–	NA	NA
		pAkt	–	–	†	†
		pFOXO1	–	–	†	~
Hepatic glucose metabolism	Gluconeogenesis	G6Pase	–	–	–	–
		PEPCK	–	–	~	~
	Glucose transportion	GLUT2	–	–	*†	†
	Glycolysis	GK	~	–	†	~
Hepatic lipid metabolism	Lipogenesis	FAS	–	–	†	~
		SCD1	–	–	†	~
		ACCa	–	~	~	~
		SREBP-1c	NA	NA	†	†
	Fatty acid-oxidation	CPT1α	NA	NA	~	~
		PPARα	NA	NA	†	~
		ACOX	–	~	~	†
		ACAT1	–	~	†	†
	Cholesterol metab	ApoA1	~	~	~	~
		HMG-CoA reductase	–	–	†	†
Hypothalamic infalmmation	MBH	TNF-α	↑	↑	~	↓
		IL-1β	~	~	↓	↓
		IL-6	~	~	↓	↓
		pIkBα	↑	~	NA	NA
		pJNK	NA	NA	↓	↓
		TLR4	NA	NA	~	~
		SOCS3	~	~	↓	↓
	PVN	TNF-α	↑	↑	↓	↓
		IL-1β	↑	↑	*↓	↓
		IL-6	~	↑	↓	↓
		pIkBα	~	↑	NA	NA
		pJNK	NA	NA	~	~
		TLR4	NA	NA	~	↓
		SOCS3	↑	↑	~	↓
Sympathetic nervous system	MBH	TH	~	–	†	~
	PVN	TH	–	–	~	~

†, the function in response to leptin is improved; –, the function in response to leptin is inhibited; ~, no significant change; ↑, the level is increased; ↓, the level is decreased; *, the significance is $0.05<p<0.1$; NA: data not available.

- **The effects of distinct fatty acids on central leptin sensitivity**

In Study 1 and Study 2, it was reported that the central administration of PA and ARA decreased central leptin sensitivity by preventing central leptin action in decreasing food intake and body weight gain. In line with previous reports, the findings confirm that centrally administration of SFA, in particular PA, induce central leptin resistance in rodents (Lin et al., 2000, Benoit et al., 2009, Posey et al., 2009). The studies demonstrate for the first time that central administration of ARA is sufficient to trigger cellular responses and induce central leptin resistance. Additionally, in Study 3 and Study 4, we provide evidence to indicate that central administration of DHA and DHA derivative show anorexigenic effect by decreasing energy intake and body weight gain under short-term HFD feeding. Although the body weight changes in these series of studies are less significant than those of energy intake, they still show a trend of changes between the vehicle groups and the fatty acids treatment groups in relation to the percentage change. The findings suggest that diet-induced leptin sensitivity changes can interfere with the catabolic effects of leptin on energy intake and body weight gain. Although one previous peripheral study demonstrated that HFD of DHA derivative (α-ethyl DHA ethyl ester) exhibited a higher efficacy on obesity and associated metabolic traits as natural n-3 PUFA (Rossmeisl et al., 2009). Unexpectedly, compared to DHA, DHA derivative did not exert a greater effect on reducing energy intake and body weight in our study. Our results show the direct central effect of n-3 PUFA on energy intake and body weight gain in mice. Potential future studies will allow for further exploration of the precise mechanism of action for natural n-3 PUFA and n-3 PUFA derivative.

In addition to the effect on energy homeostasis, the central leptin sensitivity alteration induced by distinct fatty acids was further reinforced by the changes in the hypothalamic leptin signalling mediator molecules in the present thesis. Study 1 and Study 2 found that the icv PA and ARA led to impaired hypothalamic leptin STAT3 and PI3K signalling, evidenced by the down-regulation of the phosphorylation of JAK2, STAT3, Akt, and FOXO1, and the up-regulation of SOCS3. In contrast, in Study 3, the central administration of DHA and DHA derivative improved leptin JAK2 and PI3K signalling in the hypothalamus. This effect is accompanied by enhanced pJAK2, pSTAT3, and pAkt, and decreased SOCS3 in HFD mice. The findings are in accordance with previous studies, and confirm the inhibitory effects of SFA (Howard et al., 2004, Munzberg et al., 2004, Zhang et al., 2008b) and the improved effects of n-3 PUFA (Cintra et al., 2012) on these two leptin signalling pathways. The current findings also show novel evidence for the first time about the effects of n-6 PUFA and n-3 PUFA derivatives on leptin STAT3 and PI3K signalling pathways in the hypothalamus. These findings may provide new therapeutic candidates for the prevention of central leptin resistance and obesity.

Furthermore, as a negative regulator of leptin signalling, the role of SOCS3 in the hypothalamus in regulating central leptin sensitivity has been demonstrated in the present studies. Previous studies confirmed that neuronal deletion of SOCS3 improved leptin sensitivity and conferred resistance to DIO. Therefore, SOCS3 could be a promising target of leptin-sensitizing therapies. The role of SOCS3 in regulating hypothalamic leptin signalling in response to fatty acids is important to determine the mechanism of leptin resistance under HFD condition. It was found that the hypothalamic SOCS3 level was increased by icv injection of PA and ARA in normal

mice (in Study 1 and Study 2), while it was decreased by DHA and DHA derivative treatment in HFD mice (Study 3) in the absence of leptin. Previous studies have demonstrated that the over-expression of SOCS3 in the hypothalamus induced by a HFD contributes to central leptin resistance and obesity (Enriori et al., 2007, Munzberg et al., 2004). On the other hand, the inhibition of SOCS3 in neurons increases hypothalamic pSTAT3 and the anorexigenic effect of leptin, and protects from the development of DIO (Zhang et al., 2008a). Therefore, the combination of previous studies and my findings suggests that the increase of hypothalamic SOCS3 level induced by PA and ARA may contributes to the impaired leptin STAT3 signalling, and the decrease in the hypothalamic SOCS3 level induced by DHA and DHA derivative contributes to improved leptin STAT3 signalling in the hypothalamus. Taken together, it is speculated that PA and ARA decrease central leptin sensitivity, and DHA and DHA derivative increase central leptin sensitivity, in part via negative feedback from SOCS3 to the JAK2-STAT3 signalling mechanism in the hypothalamus. However, since SOCS3 is multi-functional, its level in the hypothalamus does not only reflect changes in leptin sensitivity, and the role of SOCS3 in the regulation of leptin sensitivity after DHA and DHA derivative treatment deserves further investigation.

In addition, my results suggest that central administration of fatty acids mediates central leptin sensitivity and action by regulating two leptin key signalling pathways. These findings reveal the important role of leptin STAT3 and PI3K signalling in regulating central leptin sensitivity in response to the injection of fatty acids. However, we cannot exclude the possibility that other leptin signalling pathways are also involved in this process. Previous studies have demonstrated that hypothalamic leptin AMP kinase signalling is impaired under HFD, which was associated with the development of DIO

(Martin et al., 2006). Therefore, more studies are warranted to explore the role of other leptin signalling pathways in the hypothalamus (such as AMPK signalling) in regulating central leptin sensitivity and action in response to the distinct fatty acids.

One study in humans showed that daily ingestion of fish oil (DHA) over a 12-week period reduced energy intake but not body weight in overweight and obese women, compared with supplementation with oleic acid (OA) rich oil (Harden et al., 2014). Despite their importance, long-term studies of such effects are relatively scarce. It would therefore be of benefit to further examine the long-term effects of n-3 PUFA on obesity related disorders in animals and humans.

Importantly, we provide clear evidence to show the effects of distinct fatty acids on leptin signalling pathways in specific MBH and PVN regions of the hypothalamus. According to the changes of leptin signalling parameters in the MBH and PVN, it was found that both MBH and PVN nucleus are responsive to central PA, ARA, and DHA administration, and the PVN but not the MBH is responsive to central DHA derivative administration. This finding suggests that distinct biological actions of leptin in response to distinct fatty acids are mediated by different brain nuclei.

- **The effects of distinct fatty acids on central leptin in the regulation of hepatic glucose and lipid metabolism**

Since the central leptin sensitivity alteration induced by different fatty acids has already been shown, we further demonstrated whether the deficient or improved leptin sensitivity will cause consequent suppressive or improved effect on hepatic energy metabolism. The results from Study 1 and Study 2 showed that central administration of PA and ARA may impair the regulation of hepatic glucose and lipid metabolism by

leptin. Further, in Study 4, we demonstrated that DHA and DHA derivative may improve central leptin's action in mediating hepatic glucose and lipid metabolism. Previous study has provided well-established evidence showing that leptin plays an important role in hepatic glucose and lipid metabolism (Hidaka et al., 2002). My findings suggest that attenuation of hepatic glucose and lipid metabolism induced by PA/ARA or improvement by DHA/DHA derivative may be partly attributed to the deficient or improved central leptin sensitivity. Although further evidence is still needed, my findings provide novel evidence towards the critical role of distinct fatty acids in regulating hepatic metabolism via central leptin regulation.

To determine how fatty acids regulate energy metabolism from central nervous system to the liver, the expression of TH (as the sympathetic nerve system activity indicator) in the hypothalamus was investigated. The results showed that the leptin-regulated activation of TH in the hypothalamus was decreased by PA and ARA, while that was increased by DHA. These findings indicate that PA and ARA have an inhibitory effect, while DHA show an improved effect on central leptin action in regulating hypothalamic sympathetic activity. Combine with the inhibitory effect of central leptin on hepatic glucose and lipid metabolism by PA and ARA, or improved effect from DHA and DHA derivative in our findings, it suggests that fatty acids differentially regulate hepatic energy metabolism via sympathetic nervous system tone. The findings are in accordance with a previous study which showed that the sympathetic activity was reduced by HFD, which was attributed to the attenuated hypothalamic leptin PI3K signalling, and induced hepatic metabolic dysfunction in the obese mice (Warne et al., 2011).

Blood lipid levels (eg. cholesterol, triglycerides, and free fatty acids) are important constituents of the lipid fraction of the human body, and reflect the functional changes

of hepatic lipid metabolism. A number of studies have reported the blood lipid metabolism in obese rodents is markedly impaired, and that leptin treatment can improve the dyslipidemic profile and affect hepatic lipid metabolism (Prieur et al., 2008). The effect of central administration of fatty acids on lipid profile in blood should be examined in further studies to confirm functional improvement.

- **The effects of distinct fatty acids on hypothalamic inflammation**

It has been suggested that hypothalamic inflammation is the predominant potential mechanism of central leptin resistance and obesity underlying DIO (De Souza et al., 2005, Milanski et al., 2009, Zhang et al., 2008b). More specifically, SFA and n-6 PUFA show potent pro-inflammatory properties, while n-3 PUFA and their derivatives have well-known anti-inflammatory effects (Cintra et al., 2012, Pimentel et al., 2013, Morin et al., 2011). To determine the mechanism of central leptin sensitivity and hepatic metabolism changes, the hypothalamic inflammation in response to different fatty acids was investigated. Our results (Study 1 and Study 2) show that central PA and ARA administration exhibit potent pro-inflammatory effects in the hypothalamus. DHA and DHA derivative were found to inhibit HFD-induced hypothalamic inflammation by decreasing the genetic expression of the pro-inflammatory cytokines and inflammatory signalling molecules (study 3). Consistent with previous studies, our findings confirm the effects of SFA and n-3 PUFA on hypothalamic inflammation (Milanski et al., 2009, Cintra et al., 2012). The findings also provide new evidence for the pro-inflammatory effects of n-6 PUFA and the anti-inflammatory effects of n-3 PUFA derivatives in the hypothalamus. Previous studies have demonstrated that the activation of the inflammatory IKK-β/NF-κB pathway and increase of hypothalamic cytokines can affect central leptin sensitivity and signalling via mediating the leptin signalling mediators (e.g.

pJAK2, pSTAT3, IR etc.) (Zhang et al., 2008b). Together with the changes of hepatic metabolism induced by fatty acids via regulating leptin sensitivity, both previous research and our present findings suggest that the activation and attenuation of central inflammation via IKK-β/NF-κB signalling contribute to impaired or improved leptin sensitivity and the corresponding alteration of peripheral metabolism.

In addition, the hypothalamic inflammatory signalling pathways, including IKK-β/NF-κB, TLR4, and JNK signalling in response to distinct fatty acids contribute novel data towards the understanding of central leptin sensitivity and action meditation. I demonstrated for the first time that icv injection of DHA derivative down-regulated the expression of TLR4, accompanied by reduced levels of cytokines (e.g. TNF-α, IL-1β, and IL-6) in the hypothalamus. This finding suggests that the role of DHA derivative in regulating central inflammation is associated with hypothalamic TLR4/NF-κB signalling. This finding is consistent with previous evidence that has demonstrated the anti-inflammatory effect of DHA derivative (Morin et al., 2011). However, the specific mechanism of DHA derivative on central inflammation, especially via the TLR4/NF-κB signalling pathway, needs to be further explored in the future.

More intriguingly, this thesis demonstrated for the first time that both DHA and DHA derivative decreased the activation of pJNK in the hypothalamus (Study 3), which suggests that the JNK signalling pathway may be involved in the hypothalamic inflammation induced by DHA and DHA derivative. This may provide a new potential mechanism for central n-3 PUFA and n-3 PUFA derivatives to influence hypothalamic inflammation. Since previous studies have shown that the JNK inflammatory pathway is implicated in the activation of NF-κB signalling, TLR4 activation, and SOCS3 gene expression (De Souza et al., 2005, Milanski et al., 2009). Further studies are warranted

to explore the role and mechanism of JNK signalling in the development of central inflammation, central leptin resistance, and obesity induced by DHA and DHA derivative as well as other types of fatty acids.

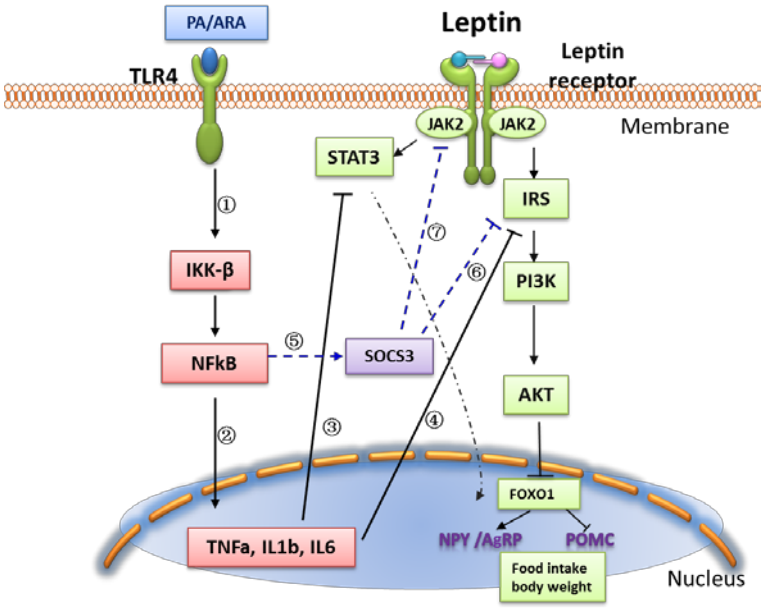

Figure 7. Effects of PA and ARA on central leptin resistance

The proposed mechanisms of central leptin resistance induced by PA/ARA: ① Increased levels of PA/ARA induce intracellular signalling cascades, in part via TLR4, which activates TLR4 signalling. This stimulates IKK-β/NF-κB inflammatory signalling, and ② induce a series of inflammatory responses, including an increase of pro-inflammatory cytokines. ③ The activation of IKK-β/NF-κB and increased levels of cytokines in the hypothalamus may inhibit the activation of STAT3 ④ and phosphorylation of IRS, and induce central leptin resistance. Alternatively, PA/ARA activates the transcription factor NF-κB, ⑤ which induces the over-expression of the leptin signalling negative regulator SOCS3. SOCS3 prevents leptin signalling via ⑥ interferes with phosphorylation of the IR ⑦ and activation of pJAk2.

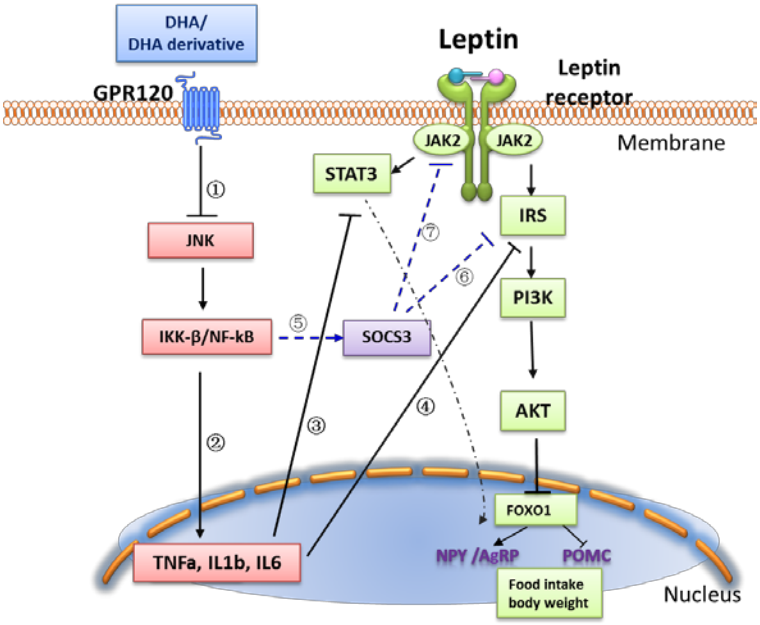

Figure 8. Effects of DHA and DHA derivative on central leptin resistance

The proposed mechanisms of increased central leptin sensitivity induced by DHA/DHA derivative: ①DHA/DHA derivative may bind to their receptors in the membrane, and block the phosphorylation and activation of JNK, and ② further inhibit the activation of IKK-β/NF-κB signalling and the increase of inflammatory cytokines. DHA/DHA derivative may improve central leptin sensitivity and action by ③ increasing activation of STAT3 ④ and the phosphorylation of IRS. Alternatively, DHA/ DHA derivative may improve the central leptin sensitivity and action by ⑤ preventing the expression of SOCS3, and subsequently inhibiting the suppressive effect of SOCS3 on ⑥ phosphorylation of IR ⑦ and activation of pJAk2.

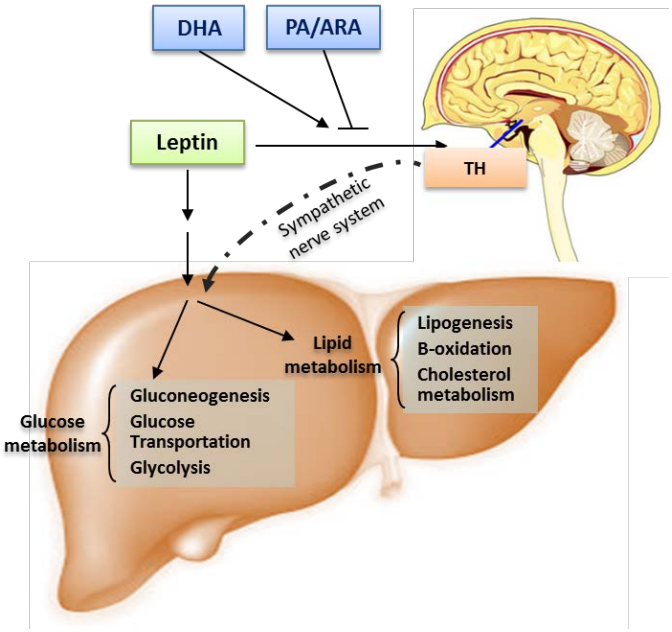

Figure 9. Effects of fatty acids on central leptin-regulated hepatic glucose and lipid metabolism

Exposure of fatty acids in the central nervous system may increase or decrease central leptin sensitivity, which changes the leptin regulation of hypothalamic sympathetic activity by regulating the activity of TH. The signals from TH-positive neurons directly project to the brainstem autonomic regions, and send efferent signals to the spinal cord and exert autonomic control in the liver to regulate hepatic glucose and lipid metabolism.

6.2 Recommendations for Future Research

Based on the findings in the present thesis, recommendations for future research are listed below.

- **Hypothalamic leptin signalling - Target**

The present study unveils the critical role of leptin-induced STAT3 signalling and PI3K signalling in the hypothalamus in regulating energy homeostasis and hepatic

metabolism. The deficient and resistant leptin signalling in response to distinct fatty acids has been proven to be an important contributor to obesity, diabetes, and associated metabolic disturbance. Our findings suggest that the restoration or improvement of hypothalamic leptin STAT3 and PI3K signalling are important objectives of the therapy for these conditions.

In addition, the present study has proven the negative regulatory effect of SOCS3 in hypothalamus. However, the specific role and mechanism of SOCS3 in regulating leptin STAT3 signalling and action in response to distinct fatty acids exposure in the central nervous system has still not been thoroughly elucidated. Further studies are warranted to investigate the direct effect of fatty acids on the JAK2-STAT3-SOCS3 signalling pathway by using pharmacological inhibition or the genetic ablation of SOCS3, as well as other possible targets of upstream of the JAK2-STAT3 signalling pathway.

- **Hypothalamic inflammation signalling pathway-Target**

Our findings suggest that dietary fatty acid-induced inflammatory response in the hypothalamus plays a critical role in mediating central leptin resistance and obesity. Previous evidence has also indicated that the pharmacological inhibition of TLR4 could reduce hypothalamic inflammation and result in prevention on food intake and improvement in central leptin sensitivity and associated peripheral metabolism in DIO rats. Based upon current understandings, several inflammatory mediators, especially TLR4, pro-inflammatory cytokines, and IKK-β/NF-κB signalling, are proposed to be promising therapeutic targets for the treatment of DIO. Our results also provide potential therapeutic methods to use nutrients as a treatment to prevent central inflammation in DIO.

In addition, although the activation of hypothalamic inflammation by distinct fatty acids has been proved in vitro, solid evidence for the effects of fatty acids in activating neuronal inflammation in vivo is scarce. Therefore, in vivo studies are needed to detect whether microglia are indeed the primary responders to dietary fatty acids, and determine if they can be triggered as an inflammatory response upon HFD feeding. The roles of TLR4-dependent intracellular signalling mechanisms in regulating the inflammation of microglia and astrocytes in response to distinct fatty acids also remain unknown.

- **Fatty acid sensing in more brain regions**

It was found in this present thesis that leptin signalling in both the MBH and PVN of the hypothalamus have been impaired by central injection of PA and ARA, and been improved by DHA. DHA derivative shows similar beneficial effects as DHA, but they only exist in PVN region. This suggests that different brain regions show different response to dietary fats, and exhibit different functional effects in regulating central leptin action. In further studies, some unsolved questions, such as the effects of dietary fatty acids on the leptin signalling pathway in other brain regions (e.g. hippocampus, brain stem), need further exploration.

In addition, to better understanding the mechanism of hypothalamic response and action to different fatty acids, more experiments of long-term fatty acids treatment as well as established obesity after long-term HFD consumption in animals should be built into further studies.

- **Sympathetic nervous system**

The findings in the present study indicate that central PA and ARA suppress leptin-mediated hypothalamic TH activation, while DHA is able to improve leptin-regulated TH activation. This may connect the regulations of different fatty acids hepatic glucose and lipid metabolism with hypothalamic sympathetic nervous system. However, further valid evidence is needed to prove this mechanism. Therefore, the role and mechanism of the hypothalamus autonomic way, especially the sympathetic nervous system, in regulating peripheral metabolism underlying distinct fatty acids warrants further exploration.

6.3 Significance

I demonstrate that the exposure of fatty acids in the central nervous system induces central leptin sensitivity changes that diminish or improve the ability of leptin to negatively influence food intake, body weight gain, and hepatic energy metabolism. The findings raise the possibility that fatty acid-induced changes of central leptin sensitivity contribute to body energy homeostasis and hepatic glucose and lipid metabolic regulation. With the high worldwide prevalence of obesity, identification of the molecular basis for these effects will provide new insight into the mechanisms of central leptin resistance, obesity and associated metabolic disturbances.

Typical Western diets are currently characterized by high levels of SFA and n-6 PUFA, and low levels of n-3 PUFA. In particular, n-6 PUFA are present at high levels in many common cooking oils and processed foods. Nutrient treatment is a new intervention used to regulate body energy homeostasis and prevent obesity. Identification of the influence of dietary fat on central leptin sensitivity and hepatic metabolism may yield

novel approaches to the improvement of the pharmacological and dietary management (e.g. regulate the n-3 PUFA to n-6 PUFA ratio) for preventing obesity and associated metabolic disturbances.

Moreover, in the present thesis, I demonstrate that distinct fatty acids modulate central leptin sensitivity by impairing or improving specific STAT3 and PI3K signalling pathways in the hypothalamus. This effect is accompanied by transcription changes of key molecular mediators in the hypothalamus, such as pJAK2, pSTAT3, SOCS3, pAkt, and pFOXO1. A better understanding of the mechanisms by which distinct fatty acids influence specific leptin signalling in the hypothalamus might lead to the identification of novel therapeutic targets for the prevention and treatment of obesity.

My findings revealed that the central administration of SFA and n-6 PUFA stimulates pro-inflammatory effects in normal mice, while n-3 PUFA and their derivatives exert anti-inflammatory properties in the hypothalamus under HFD feeding. The findings support a valid animal model in which cellular exposure to excess nutrients, particularly dietary fatty acids, trigger cellular inflammation and leptin resistance in the central nervous system. Furthermore, the findings suggest that, in addition to pharmacological and genetic approaches, nutrients can be attractive candidates for controlling hypothalamic inflammation, which in turn contributes to the prevention and treatment of obesity.

6.4 Conclusions

In conclusion, the present study demonstrates that the acute central administration of PA and ARA plays a causal role in central leptin resistance, and prevents the anorexigenic

effect of leptin by decreasing central leptin sensitivity. This decrease of central leptin sensitivity is concomitant with impaired hypothalamic leptin JAK2-STAT3 and PI3K-Akt signalling, and the pronounced attenuation of leptin-regulated hepatic glucose and lipid metabolism. The potent pro-inflammatory effects in the hypothalamus activated by PA and ARA may contribute to central leptin resistance, adiposity, and hepatic metabolic disturbances. In contrast, DHA and DHA derivative exhibit beneficial effects on energy homeostasis and associated hepatic metabolic disorders by increasing central leptin sensitivity in HFD mice. Central exposure of DHA and DHA derivative show an anorexigenic effect, accompanied by ameliorated leptin JAK2-STAT3 and PI3K-Akt signalling pathways in the hypothalamus in HFD mice. The anti-inflammatory effects in the hypothalamus are induced by DHA and DHA derivative, which may be one of the mechanisms to account for the ameliorated central leptin sensitivity, leptin signalling, and hepatic energy metabolism. Therefore, PA, ARA, DHA and DHA derivative all play a significant role in the regulation of central leptin on energy homeostasis and peripheral energy metabolism.

The reports showed above indicate that the specific type of dietary fat is capable of inducing or preventing central leptin resistance by affecting central leptin sensitivity at both food intake and hypothalamic leptin signalling molecule level. The different inflammatory signalling pathways, including IKK-β/NK-κB, TLR4/NK-κB, and JNK pathway, in response to central dietary fatty acids are involved in the development of leptin resistance. This helps to identify the potential biological mechanism of central leptin resistance, obesity, and associated metabolic disturbances. Therefore, these compounds could be attractive therapeutic targets for the prevention and treatment of obesity, diabetes, and dyslipidaemia. Moving forward, greater consideration should be

given to designing nutritional interventions that target multiple leptin signalling and inflammation signalling pathways.

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APPENDIX

Appendix A-Chapter 2 Supplementary

Supplement 1 Statement from co-authors

This is to attest that the PhD candidate, Licai Cheng, contributed significantly to the investigation (Cheng, L., Yu, Y., Szabo A., Wu Y., Wang H., Camer D., Huang, X.-F. (2015). Palmitic acid induces central leptin resistance and impairs hepatic glucose and lipid metabolism in male mice. *Journal of nutritional biochemistry*, 2015 May 26 (5): 541-548.): designed and performed the experiment work, analysed the data, interpreted results, and wrote the manuscript. Yinghua Yu contributed to discussions, and reviewed and edited the manuscript. Yizhen Wu and Hongqin Wang contributed to data analysis. Ms. Danielle Camer for her editorial reading of the manuscript. Alexander Szabo and Xu-Feng Huang contributed to the experimental design and reviewed and edited the manuscript.

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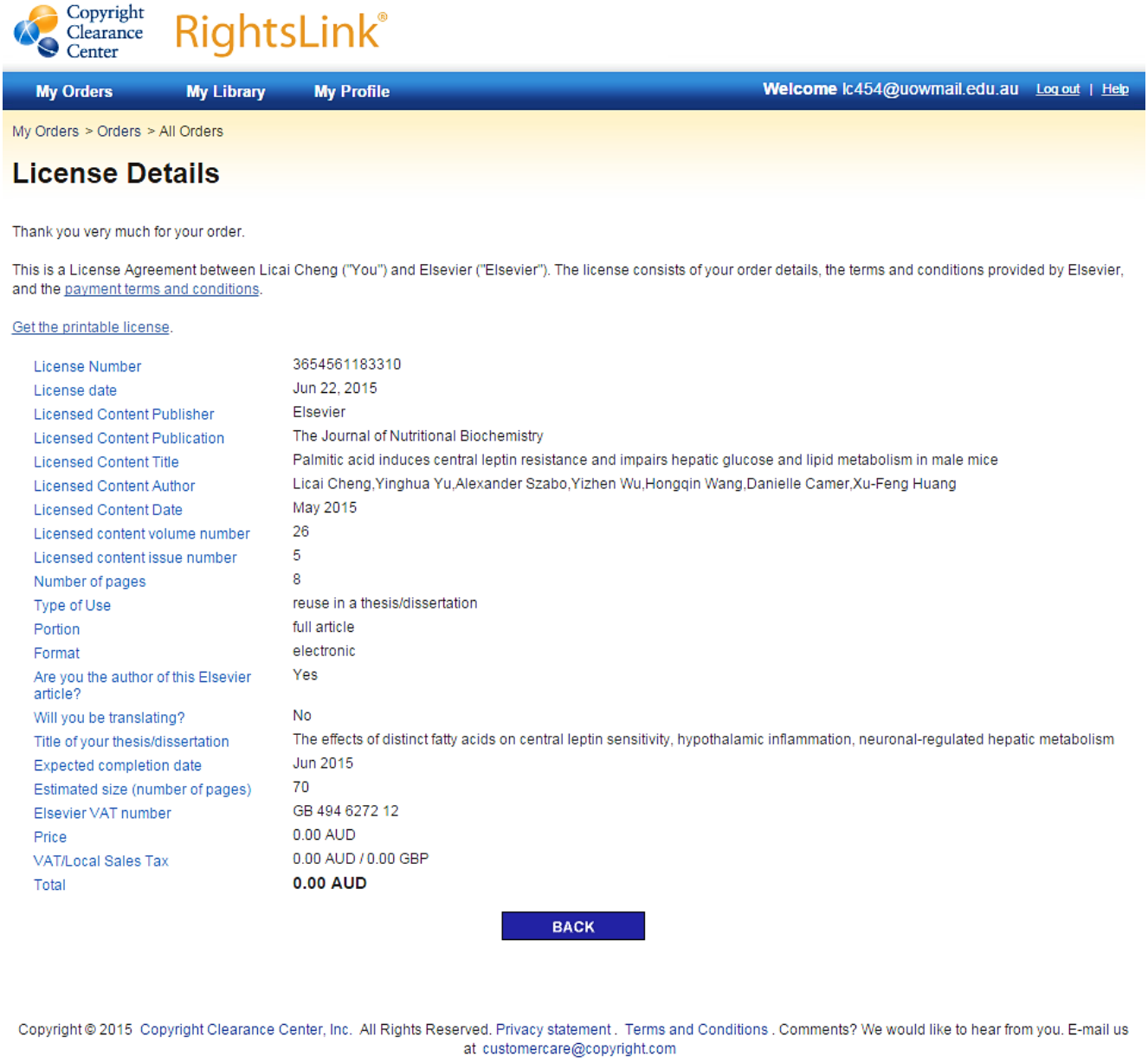

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Supplement 1 Statement from co-authors

This is to attest that the PhD candidate, Licai Cheng, contributed significantly to the investigation (Cheng, L., Yu, Y., Zhang Q. Szabo A. Wang H., Huang, X.-F. (2015). Arachidonic acid impairs hypothalamic leptin signalling and hepatic energy homeostasis in mice. *Molecular and cellular endocrinology*, 2015 April. 25(411): c12-18.): designed and performed the experiment work, analysed the data, interpreted results, and wrote the manuscript. Yinghua Yu contributed to discussions, and reviewed and edited the manuscript. Qingsheng Zhang contributed to critical comment and editorial revision of the manuscript. Hongqin Wang contributed to data analysis. Alexander Szabo and Xu-Feng Huang contributed to the experimental design and reviewed and edited the manuscript.

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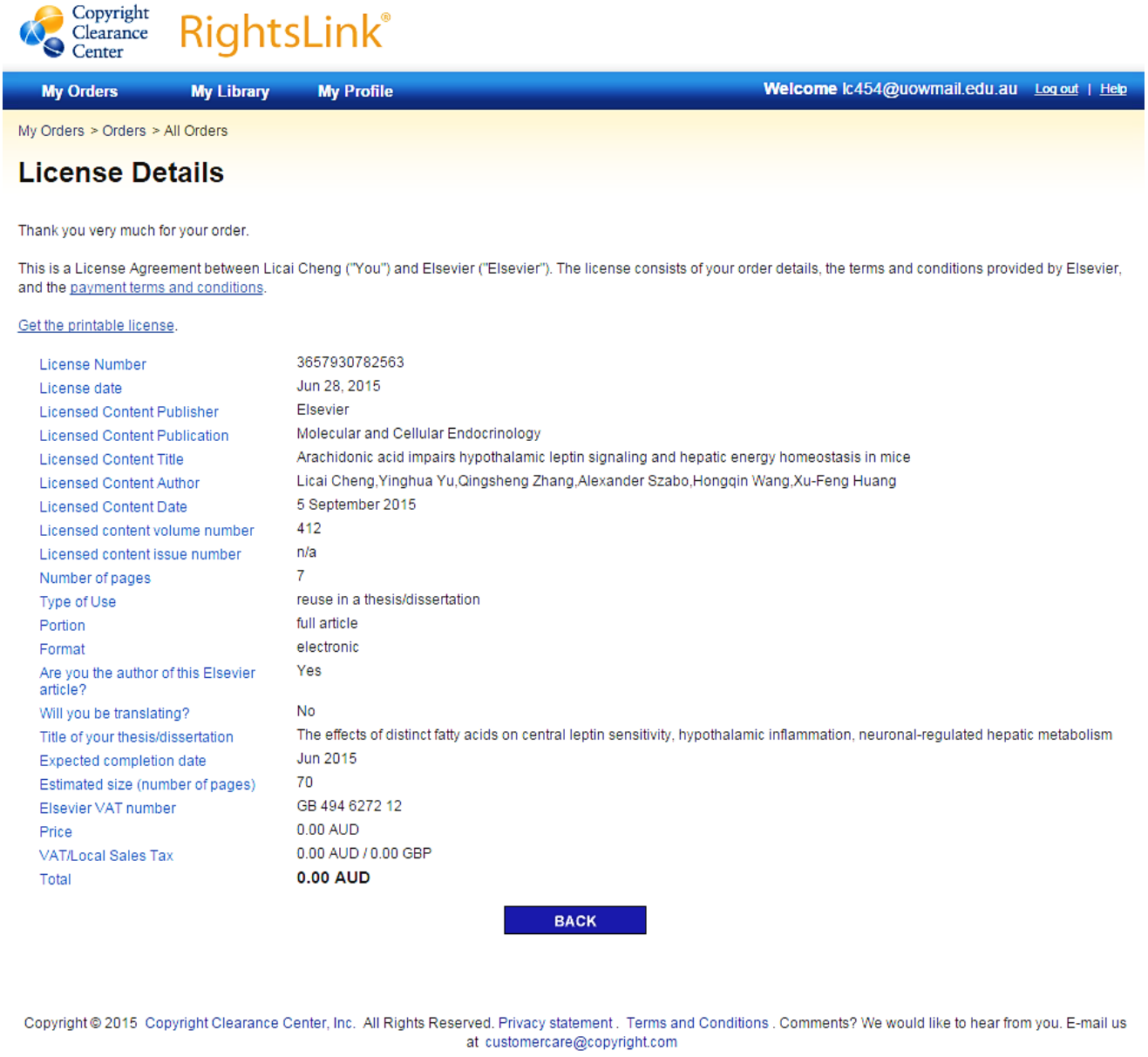

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