Identification of central mechanisms underlying statin-induced changes in consummatory behaviour in rats

A thesis

submitted in fulfilment

of the requirements for the degree

of

Master of Science (Research)

at

The University of Waikato

by

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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2017

1 Abstract

Simvastatin is a cholesterol lowering statin whose adverse effects include an increase in appetite and, consequently, obesity. This is counterproductive to the otherwise beneficial outcomes of simvastatin on metabolic and cardiovascular health. The mechanisms underlying simvastatin-induced hyperphagia are unknown. This thesis investigated whether central mechanisms contribute to simvastatin-induced increase in appetite by using a laboratory rat model. First, the effect of intracerebroventricularly (ICV) administered simvastatin on the energy-driven intake of 'bland' chow, and on reward-motivated consumption of palatable solutions was determined. The data indicated that ICV simvastatin moderately increases ingestion of energy-dense chow, but it does not affect consumption of calorie-dilute and non-caloric palatable sucrose or saccharin solutions. It suggests that simvastatin acting directly at the brain level elevates intake of energy, while being ineffective in stimulating eating for reward. Surprisingly, rats injected ICV with simvastatin also consume significantly more water after water deprivation, which points to a relationship between centrally acting simvastatin and thirst-related processing. In the second part of this project, the effect of an orexigenic dose of ICV simvastatin on neuronal activation in consumption-related hypothalamic sites was investigated. Simvastatin elevated c-Fos immunoreactivity, which serves as a marker of neuronal activation, in the arcuate and paraventricular nuclei. These two sites have a profound influence on the regulation of energy intake and energy balance, as well as an effect on water balance. It can be concluded that simvastatin increases intake of energy and of water, and that it likely exerts its action through the hypothalamic paraventricular and arcuate nuclei.

"*A nana is warm hugs and sweet memories. She remembers all of your accomplishments and forgets all of your mistakes*." – Barbara Cage

This thesis is dedicated to my nana, Winifred Isgrove, for her friendship, her support and her unwavering encouragement for me to chase my dreams. I am one step closer to those now nana, so thank you for believing in me. Nga mihi arohanui.

2 Acknowledgments

Throughout my journey of graduate study these last two years, I would not have been able to achieve as such as I have without the support of some key people. My research has very much been a group effort, and I am extremely thankful to those who have helped me along the way.

To my supervisor, Pawel Olszewski and to Anica Klockars I owe my deepest appreciation. Pawel and Anica have given me direction, support and encouragement during my master's research. They have provided me with opportunities outside my research to expand my expertise, and I will always be grateful for the time they have dedicated to helping and teaching me.

I would like to thank the PhD students and technician in my lab, Sarah Gartner, Oscar Klockars and Kerry Allen. Sarah and Ocsar taught me many of the lab protocols and techniques I used throughout my research, as well as allowing me to gain insight on their own research projects. Kerry was always willing to help in any way she could and was a great help during my research.

To the other master's students in my lab, Kathryn Laloli, Laura Mccoll and Crystal Gerring, thank you for the welcomed distractions in the office. We have come a long way from first year, but five years down the track we have finally made it.

Last but not least, to my friends, family and my fantastic fiancé Jack Keeys, thank you for your unwavering support, reassurance and patience. I don't know how I would have got through this year without you all.

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5 List of Abbreviations

LDL-C: low density lipoprotein cholesterol

CVD: cardiovascular disease

BW: body weight

IL-1: interleukin 1

MCP-1: monocyte chemoattractant protein 1

SMC: smooth muscle cell

HDL-C: high density lipoprotein cholesterol

HMGR: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase

SREBP: sterol regulatory element-binding proteins

ACAT: acyl coenzyme A cholesterol acyltransferase

ER: endoplasmic reticulum

SCAP: SREBP cleavage-activating protein

S1P: site 1 protease

S2P: site 2 protease

bHLH: NH2-terminal bHLH-Zip domain

SRE: sterol response element

INSIG-1: insulin-induced gene 1 protein

INSIG-2: insulin-induced gene 2 protein

CETP: cholesterol ester transfer protein

VLDL: very low density lipoprotein

HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A

NAD: nicotinamide adenine dinucleotide

BMI: body mass index

SNP: single nucleotide polymorphism

BBB: blood brain barrier

CNS: central nervous system

FI: food intake

EE: energy expenditure

CCK: cholecystokini

GLP-1: glucagon-like peptide-1

PYY: peptide YY

HPA: hypothalamic-pituitary-adrenal axis

NAc: nucleus accumbens

VP: ventral pallidum

VTA: ventral tegmental area

NTS: nucleus of solitary tract

AP: area postrema

DMNV: dorsal motor nucleus of the vagus nerve

LHA: lateral hypothalamic area

α-MSH: α-melanocyte-stimulating hormone

MC4-R: melanocortin 4 receptor

SOP: supraoptic nuclei

DMH: dorsomedial hypothalamic nucleus

VMH: ventromedial nucleus of hypothalamus

ARC: arcuate nuclei

PVN: paraventricular nuclei

MCH: melanin concentrating hormone

NPY: neuropeptide Y

AgRP: agouti-related protein

POMC: pro-opiomelanocortin

CART: cocaine- and amphetamine-regulated transcript

CSF: cerebral spinal fluid

ICV: intracerebral ventricle

GABA: y-aminobutyric acid

Y1: to neuropeptide Y receptors type 1

Y5: to neuropeptide Y receptors type 5

CRH: corticotropin-releasing hormone

TRH: thyrotropin-releasing hormone

ACTH: adrenocorticotropic hormone

T3: triiodothyronine

T4: thyroxine

MCH-1: melanin concentrating hormone 1 receptor

MCH-2: melanin concentrating hormone 2 receptor

mg/mL: milligram per millilitre

DMSO: dimethyl sulfoxide

nmol: nanomole

µL: microlitre

TBS: tris-buffered saline

H_2O_2: hydrogen peroxide

BAT: brown adipose tissue

6 Identification of central mechanisms underlying statin-induced changes in consummatory behaviour in rats

6.1 Introduction

Statins are a common class of drug prescribed to maintain proper cholesterol levels due to their efficiency at reducing low density lipoprotein cholesterol (LDL-C) (Weng, Yang, Lin, & Tai, 2010). They have been shown to decrease LDL-C levels by between 40 to 60%, making them a great tool to fight cardiovascular disease (CVD) and related adverse effects (Law, Wald, & Rudnicka, 2003)

Statins are used by a significant proportion of the population, with 20% of American adults being prescribed statins at some point in their lives due to high cholesterol levels, and a further 200 million people using them worldwide for the same reasons (Blaha & Martin, 2013; Mercado et al., 2015).

These drugs are prescribed for two reasons: genetic predisposition to CVD, and leading a lifestyle that is conducive of CVD. The genetic analyses and characterisation of CVD risk alleles is becoming more comprehensive with the use of genetic markers. Genetic markers include previously discovered gene groups such as many of the apolipoprotein (apolipoprotein A1, A4, B, C3 and E) and interleukin genes (interleukin 4 and 6) (Reis et al., 1997; Stavljenic-Rukavina, 2002). More recent discoveries of genetic markers for CVD include endocan, urate transporter GLUT9, osteoprotegerin and LDL receptor gene (Balta et al., 2015; Goldstein & Brown, 2009; Mallamaci et al., 2015; Singh, Kumar, Tewari,

& Agarwal, 2017). The use of genetic markers has made it easier to identify individuals susceptible to CVD, and has led to the identification of polymorphisms in genes which contribute to CVD. Due to the advancement of genetic marker use, over 1100 mutations in the LDL receptor gene have been found which contribute to the dysfunction of the LDL receptor (Goldstein & Brown, 2009). The mutations within the LDL receptor gene are associated with the most common CVD promoting disease, familial hypercholesterolemia (Austin, Hutter, Zimmern, & Humphries, 2004). Familial hypercholesterolemia is a hereditary condition where the ability to metabolise LDL-C is impaired (Goldstein & Brown, 2009). In addition, genetic markers have also identified a range of polymorphisms which are associated with other diseases that contribute to CVD such as coronary heart disease and cerebrovascular disease (Bhatnagar, Wickramasinghe, Williams, Rayner, & Townsend, 2015).

Statins are required for people whose impaired physiology are unable to properly metabolise LDL-C, and lifestyle choices can exacerbate the outcome from genetic predisposition to CVD. Dietary choices such as high sodium consumption, high processed meat consumption and low calcium in the diet may intensify genetic predisposition to CVD (Graudal, Hubeck-Graudal, & Jürgens, 2012; Micha, Wallace, & Mozaffarian, 2010; Slinin, Foley, & Collins, 2005).

Although these drugs are vital for people who fall into this category, they have also been associated with adverse off-target effects. These effects described by patients range from having a mild impact on health and wellbeing, such as nausea and fatigue, to effects considered critical for positive health outcomes such muscle atrophy and increased obesity risk (Redberg, 2014; Tomlinson & Mangione, 2005).

Increased risk to develop obesity has gained a great deal of attention. While the risk of developing CVD due to genetic components and lifestyle choice is decreased by statin use, energy dysregulation causing weight gain is also observed and may not only counteract this beneficial outcome, but exacerbate the effects of CVD. The increase in body weight (BW) may reflect an influence simvastatin, a type of statin, has on appetite which needs to be further investigated.

Therefore, it is imperative to understand how these drugs work and the mechanisms behind their off-target effects. Consequently, we can then gain knowledge of how to mitigate an increase in BW caused by simvastatin, and instead promote its intended use.

To uncover the mechanisms behind simvastatin induced weight gain, this thesis explores the underlying molecular pathways behind CVD development and how statins fit into this system. Both the peripheral and central mechanisms behind food intake will be discussed, as well as the neural pathways behind eating for energy and eating for pleasure. Ultimately, this thesis attempts to explain how simvastatin is influencing food intake with possible suggestions around reversing or reducing the increase in food intake, and therefore weight gain.

6.2 Atherosclerosis, cholesterol regulation and statin function

6.2.1 The progression of atherosclerosis

CVD is becoming increasingly common; thus, it is crucial that statins are utilised in order to reduce complications that arise from atherosclerosis (Ministry of Health, 2016). Atherosclerosis is a gradual process where plaques are formed beneath the endothelium of arteries (P. Libby, Ridker, & Hansson, 2011). These plaques narrow the arteries, putting stress on the cardiovascular system and increasing the risk of a major coronary event or stroke (Jurkovitz, Abramson, Vaccarino, Weintraub, & McClellan, 2003). The most common hypothesis for the progression of atherosclerosis is the oxidative modification hypothesis. This was first proposed by Michael S Brown and Goldstein (1983) who showed chemical modifications to LDL could increase their affinity for macrophage uptake. Research that lead on from this initial finding suggests oxidation of LDL-C has a role in the initiation of atherosclerosis and is partially responsible for the formation of fatty plaques (Steinberg, 2009).

The mechanisms behind the progression of atherosclerosis is continuously being updated with new research. However, the current explanation states that the progression of atherosclerosis begins with LDL-C passing through the endothelium and accumulating in the tunica intima via vesicles, leaky junctions or both (Figure 1) (Dabagh, Jalali, & Tarbell, 2009). Vesicular transportation of LDL-C is hypothesised to occur via endocytosis, fluid endocytosis (non-receptor dependent) and transcytosis by endothelial cells (Snelting-Havinga et al., 1989; Vasile, Simionescu, & Simionescu, 1983). In addition to this, the cell turnover – leaky junction hypothesis suggests that during mitosis and apoptosis of endothelial cells, LDL-C can pass through the weakened endothelium due to its

increased permeability (Lin, Jan, & Chien, 1990; Weinbaum, Tzeghai, Ganatos, Pfeffer, & Chien, 1985). The LDL-C then becomes oxidised through one of several different methods such as reacting to lipoxygenase, copper and ceruloplasmin, iron or peroxynitrite (Parthasarathy, Raghavamenon, Garelnabi, & Santanam, 2010). Oxidised LDL-C (OxLDL-C) can then initiate the release of cytokines such as interleukin-1 (IL-1) (Bonow, Mann, Zipes, & Libby, 2011). IL-1 increases expression of chemoattractants such as monocyte chemoattractant protein 1 (MCP-1) which attracts monocytes, and adhesion molecules which capture monocytes on endothelial cell membranes (Blankenberg, Barbaux, & Tiret, 2003; Bonow et al., 2011; Tedgui & Mallat, 1999). This assists the crossing of monocytes into the tunica intima where they are exposed to stimuli which initiate their differentiation into macrophages (Moore & Freeman, 2006). These macrophages are then stimulated to express scavenger receptors which bind to OxLDL-C and engulf them (Moore & Freeman, 2006). However, the macrophages that have engulfed OxLDL-C become 'overwhelmed' and instead of breaking down the lipids, they turn into foam cells which accumulate within the tunica intima, contributing to the development of atherosclerosis (Suzuki, Kurihara, Takeya, & Kamada, 1997). Macrophages within the tunica intima that express scavenger receptors also display antigens from the OxLDL-C which attracts T cells (Hansson, 2001). In the centre of the mass these foam cells can undergo apoptosis where they deposit more LDL-C, this is termed the necrotic core (Ramji & Davies, 2015). At this stage, the fatty streak is formed, which is the first visible sign of atherosclerosis and health issues can begin to arise (Steinberg, 2009). After this, smooth muscle cells (SMC) from the tunica media then divide into new SMCs which migrate into the tunica intima (Blankenberg et al., 2003;

Tedgui & Mallat, 1999). The SMCs continue dividing in the tunica intima, forming a fibrous cap with collagen, which allows the plaque to become stabilised, progressing from a fatty streak to a fibrofatty lesion (Blankenberg et al., 2003). The plaque contains a matrix made from SMCs which traps and accumulates foam cells and LDL-C. The matrix is then encased by a cap of SMCs and collagen. Once this is established, SMCs can undergo apoptosis which enables more LDL-C through the endothelium into the tunica intima (Blankenberg et al., 2003; Tedgui & Mallat, 1999). The established plaque narrows blood flow, and if the fibrous cap becomes damaged, thrombosis is likely to occur as the contents of the plaque spill out into the blood stream (Bentzon, Otsuka, Virmani, & Falk, 2014). Additional cholesterol from dietary choices can also increase the amount of circulating cholesterol (Graudal et al., 2012; Micha et al., 2010; Slinin et al., 2005). However, this alone is not likely to initiate atherosclerosis but can worsen pre-existing underlying metabolic conditions such as familial hypercholesterolemia.

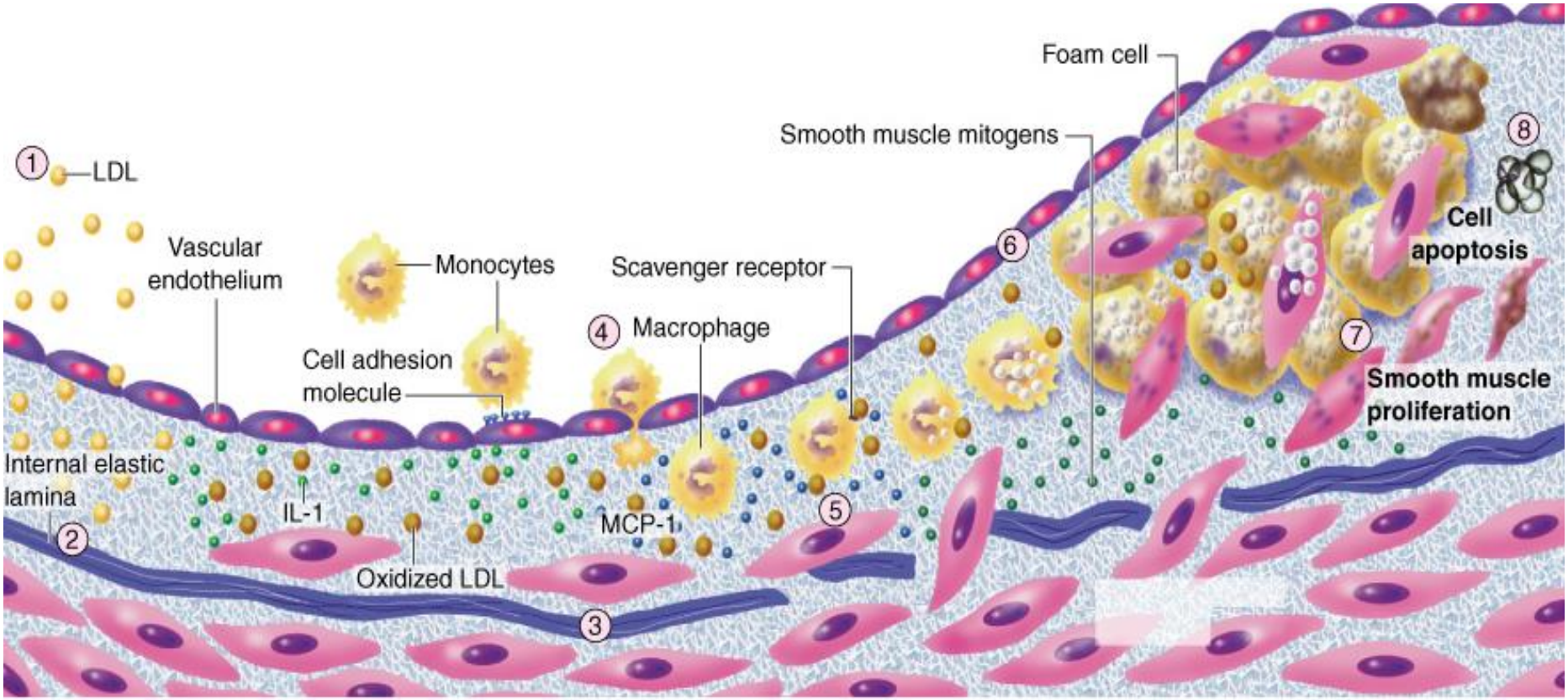

Figure 1. Progression of atherosclerosis and the different components that form a plaque. 1: LDL-C passes through the endothelium. 2: LDL-C in the tunica intima become oxidised. 3: OxLDL-C trigger the expression of cytokines and cell adhesion molecules which attract monocytes. 4: Monocytes enter the tunica intima. 5: Monocytes differentiate into macrophages which express scavenger receptors to engulf LDL-C. 6: 'Overwhelmed' macrophages become foam cells. 7: SMCs migrate from the tunica media into the tunica intima. 8: An established plaque can undergo apoptosis of SMCs. Reprinted from "Adhesion Molecules and Atherosclerosis" by S. Blankenberg, S. Barbaux, L. Tiret, 2003, *Atheroscleorisis, 170(2),* p. 194. Copyright 2003 by Elsevier Ireland Ltd.

In addition to LDL-C concentration, factors such as inflammation and immune response may also contribute to atherosclerosis.

More recently, atherosclerosis is being viewed as a chronic inflammatory disorder (Hotamisligil, 2006). Cytokines released by macrophages and T cells have several modulatory effects on surrounding cells, and in atherosclerotic environments can alter endothelial cell function, SMC proliferation and thrombosis (Liao & Laufs, 2005). Furthermore, inflammation has been suggested to have a role in the initiation and progression of atherosclerosis (Peter Libby, Ridker, & Maseri, 2002). During initiation, inflammatory signals from selectins are involved in increasing the number of adhesion molecules on the endothelial cells, attracting more monocytes (Peter Libby et al., 2002). Throughout the subsequent progression of atherosclerosis, immune cells and surrounding cells such as SMCs within the forming plaque produce a range of chemokines, interleukins and cytokines (Peter Libby et al., 2002). These produce a pro-inflammatory environment and help uphold progression of the plaque (Ramji & Davies, 2015). There is evidence to suggest inflammation has a major role in the development of atherosclerosis, although its role in LDL-C oxidisation is still unknown (Peter Libby et al., 2002; Ramji & Davies, 2015).

The role of immune cells in atherosclerosis is complimentary to inflammation. In the past, immune cells have been described only as a part of the plaque mass (Hansson & Libby, 2006). But more recently, immune cells, particularly macrophages, are seen as a main contributor to atherosclerosis which may have some role in initiation (Ramji & Davies, 2015). In the past, macrophages were thought to be the only immune cells involved in atherosclerosis. But it has now been discovered that macrophages attract T cells which also contribute to

atherosclerosis by promoting inflammation (Hansson, 2001; Hansson & Libby, 2006). Macrophages attract T cells by presenting foreign antigens, which in this case is the negatively charged macromolecules on OxLDL-C which macrophages use scavenger receptors to identify (Hansson, 2001). This illustrates that both the innate and adaptive immunity have a role in atherosclerosis and more research needs to be done to uncover the extent of and reason for their contribution (Hansson & Libby, 2006; Peter Libby et al., 2002).

Although the progression of atherosclerosis has been split into three hypotheses (oxidation of LDL-C, inflammation and immune response), the progression of atherosclerosis is likely to be a combination of all these. All three explanations are shown to play some role in atherosclerosis and have significant of overlap. This suggests the three hypotheses and their interactions with one another are likely the main drivers of atherosclerotic development (Galkina & Ley, 2009).

While cholesterol may seem detrimental to health, it is an essential lipid which is crucial for the normal operation of cells (Goldstein & Brown, 2009). With proper function of cholesterol regulation in healthy individuals, atherosclerosis is not likely to occur.

6.2.2 Cholesterol production and regulation

Cholesterol is a vital lipid for several reasons. It is a main component of cell membranes which gives them their fluidity, is the precursor for many steroid hormones and has a significant role in the development of myelin sheaths around axons (Goldstein & Brown, 2009). Cholesterol needs to be transported in lipoproteins within the blood stream because it is hydrophobic. The purpose of LDL-C is to transport cholesterol from the liver where plenty is produced, to cells

within the body which require it. LDL-C is only one of the fractions of lipoproteins, with five others that are used to carry various lipids (Goldstein & Brown, 2009). High density lipoprotein cholesterol (HDL-C) is another key fraction which delays the progression of atherosclerosis as it collects cholesterol from the vascular system to be transported to the liver, converted to bile acids and excreted (Gordon et al., 1989; Sanossian, Saver, Navab, & Ovbiagele, 2007). The cholesterol is esterified during uptake by a LDL and once it reaches a cell which requires more cholesterol than it can produce, it is uptaken through receptor mediated endocytosis (Figure 2) (Maxfield & Wüstner, 2002) . However, the molecular structure of LDL-C makes it more susceptible to oxidation (Goldstein & Brown, 2009). Once the LDL-C is brought into the cell, lysozymes break down the lipoprotein, releasing the cholesterol. Cholesterol then has several regulatory effects within the cell, including reducing the amount of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR) – a key enzyme for cholesterol production – and decreasing the number of LDL receptors which is controlled by sterol regulatory element-binding proteins (SREBPs). Cholesterol also causes an increase in acyl coenzyme A cholesterol acyltransferase (ACAT). These three regulatory steps ensure a negative feedback loop and maintain lipid homeostasis within cells (Goldstein & Brown, 2009).

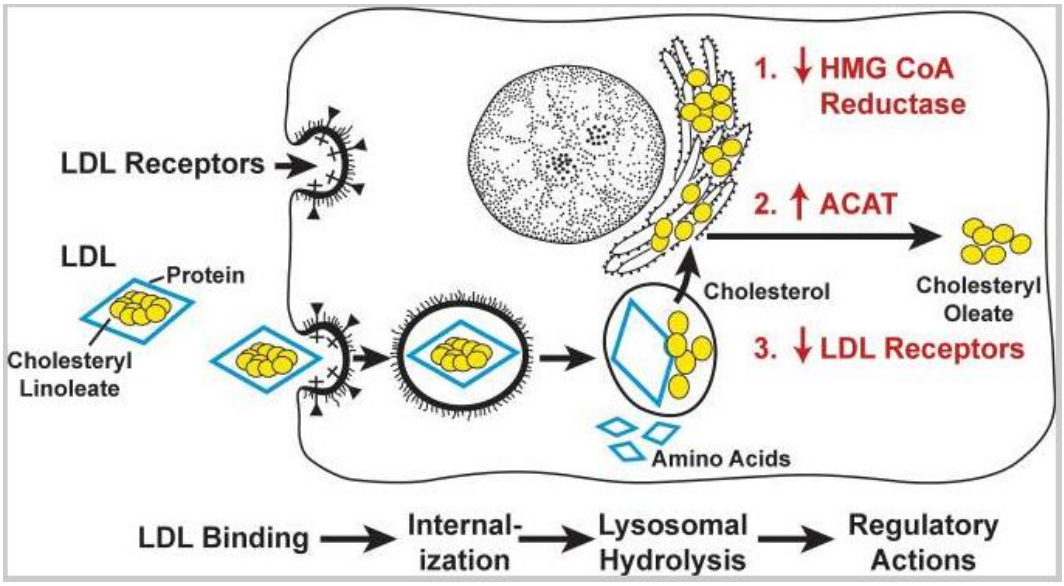

Figure 2. LDL-C is brought into the cell via receptor mediated endocytosis. Once the LDL-C has been internalised, lysozymes are hypothesised to break down the amino acids releasing the free cholesterol. The release of cholesterol has several downstream effects on lipid homeostasis. Reprinted from "The LDL Receptor" by J. L. Goldstein and M. S. Brown, 2009, *Atheroclesrosis, Thrombosis and Vascular Biology, 29(4),* p. 431 - 438. Copyright 2009 by American Heart Association, Inc.

SREBPs ensure the maintenance of lipid homeostasis within cells by increasing transcription of cholesterol producing enzymes during low cholesterol levels in cells (Michael S. Brown & Goldstein, 1999; DeBose-Boyd, 2008). SREBPs are located in the membrane of the endoplasmic reticulum (ER) and when released via two separate cleavages, have downstream effects which increase cholesterol production (Figure 3) (DeBose-Boyd, 2008). SREBP cleavage-activating protein (SCAP) is the cholesterol detecting protein located in the ER which binds to SREBPs in response to low cholesterol levels. The SCAP/SREBP complex migrates to the golgi body for proteolytic processing, where the first cleavage of SREBP by Site-1 protease (S1P) cuts the SREBP into two halves, both of which remain attached to the golgi body (DeBose-Boyd, 2008). Site-2 protease (S2P) then cuts one of the halves, the NH2-terminal bHLH-Zip domain (bHLH), from the golgi body membrane so it can bind to a sterol response element (SRE) in the

nucleus. In the nucleus, bHLH influences the transcription of cholesterol production target genes such as the HGMR and LDL-receptor gene (Shimano, 2001). When there is a high concentration of cholesterol present, the binding of SCAP to another ER protein called insulin-induced gene 1 protein (INSIG-1) and insulin-induced gene 2 protein (INSIG-2) inhibits its binding to SREBP, therefore inhibiting the production of cholesterol (Michael S. Brown & Goldstein, 1999; Yabe, Brown, & Goldstein, 2002; Yang et al., 2002). HMGR, ACAT and LDL receptor are three key components of cholesterol regulation (DeBose-Boyd, 2008).

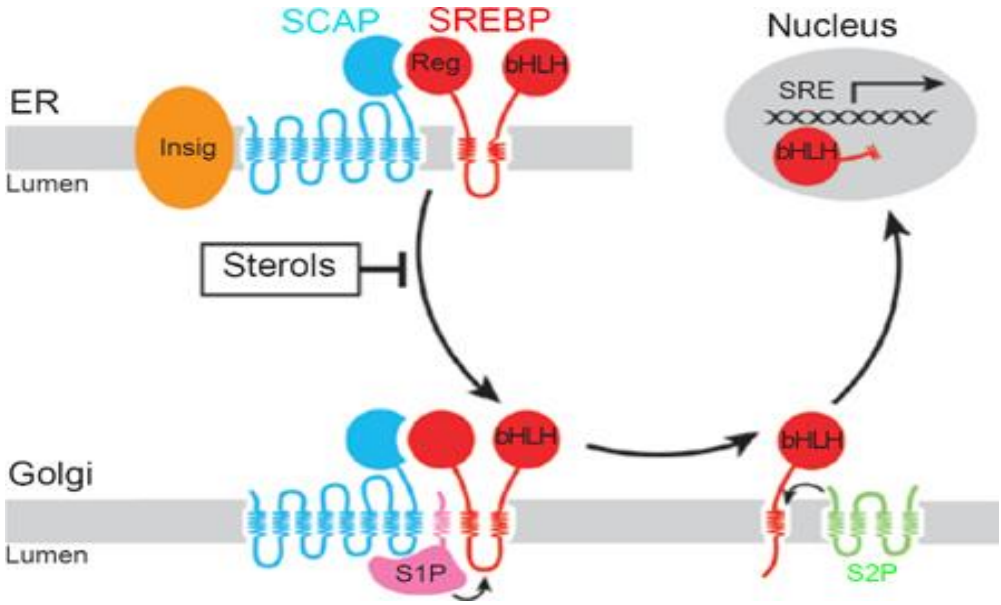

Figure 3. The regulation of cholesterol production through the SREBP pathway. In the presence of cholesterol, SCAP binds to SREBP in the ER membrane. SCAP/SREBP then migrates to the golgi body where two cleavages are made. bHLH then binds to SRE in the nucleus., which influences the transcription of cholesterol related genes. Reprinted from "Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase" by R. A. DeBose-Boyd, 2008, *Cell Research, 18,* p. 609-621. Copyright 2008 by Nature Publishing Group.

HMGR is an enzyme which catalyses the rate limiting step in the mevalonate pathway, the conversion of HMG CoA to mevalonate (Buhaescu & Izzedine, 2007). The mevalonate pathway is responsible for the production of cholesterol.

Through multiple steps, acetyl-CoA is used to create desmosterol and 7-dehydrocholestrol which together form cholesterol (Figure 4) (Buhaescu & Izzedine, 2007). When cholesterol concentrations are low in cells, SREBPs are activated which increases the transcription rate of HMGR (DeBose-Boyd, 2008). However, during high concentrations of cholesterol, SREBP activation is supressed as described above (DeBose-Boyd, 2008). With an abundance of cellular cholesterol, the degradation of HMGR is also increased up to three fold to further reduce cholesterol production (Gil, Faust, Chin, Goldstein, & Brown, 1985; Goldstein & Brown, 1984).

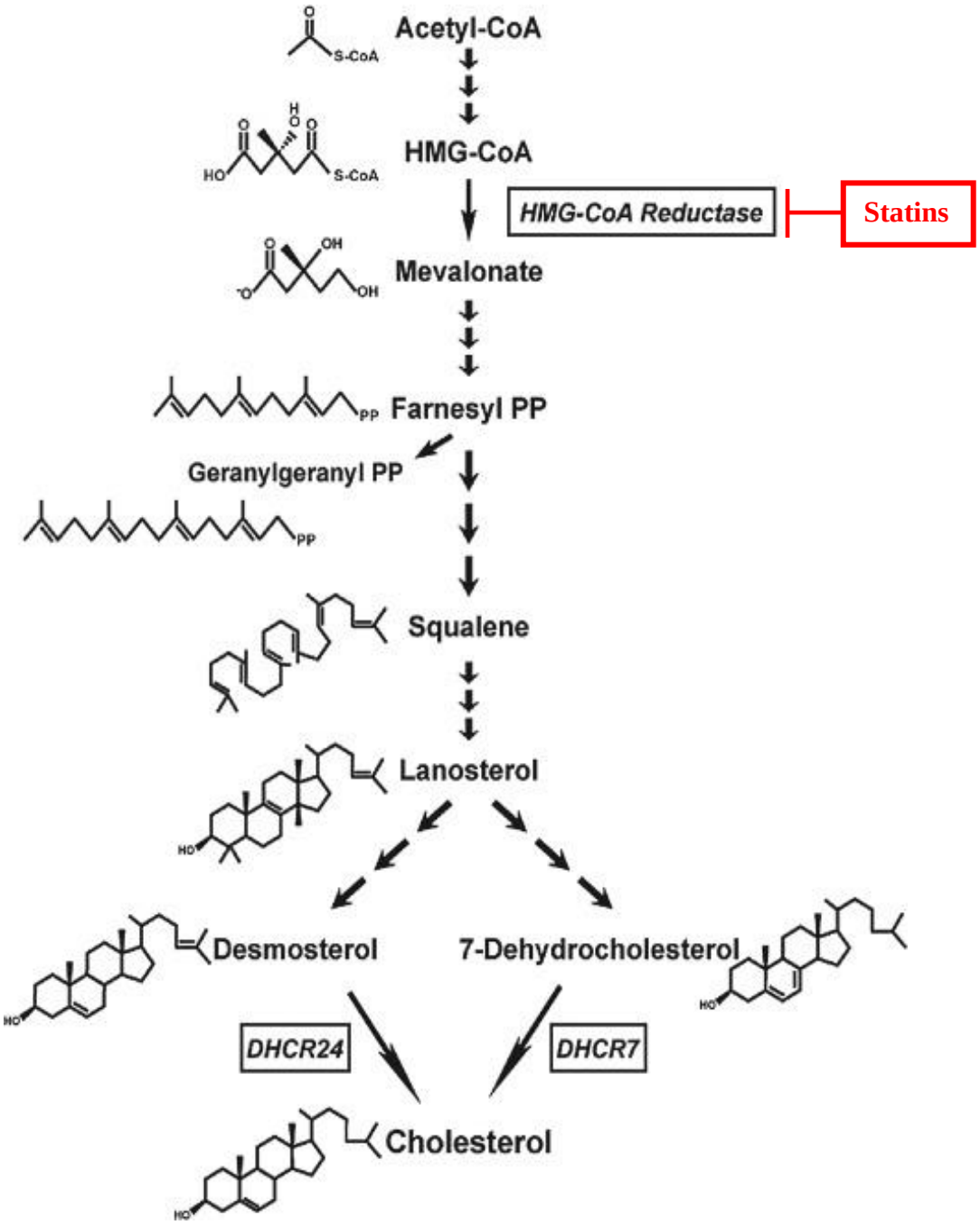

Figure 4. A simplified schematic of the mevalonate pathway; the pathway responsible for cholesterol synthesis. Modified from "Advances in the physiological and pathological implications of cholesterol: Cholesterol in health and disease" by V. A. Cortes, D. Busso, P. Mardones & A. Rigotti, 2013, *Biological Reviews, 88(4),* p. 825-843. Copyright 2013 by

ACAT is an enzyme which catalyses the reaction of cholesterol to cholesterol esters (Chang, Li, Chang, & Urano, 2009). When cholesterol is abundant in the cell, it directly activates ACAT to convert excess cholesterol into cholesterol ester

to be stored as a reservoir (Goldstein & Brown, 2009). High levels of cholesterol ester in the blood stream can aid atherosclerosis because cholesterol ester transfer protein (CETP) exchanges cholesterol ester from HDL, for triglycerides in very low density lipoproteins (VLDL) (Okamoto et al., 2000). This can increase the amount of VLDL and LDL in the blood stream which contribute to atherosclerosis, and decrease the amount of circulating high density lipoprotein (HDL), which is crucial for cholesterol excretion (Okamoto et al., 2000).

LDL receptors are produced in the rough ER and released into the cytoplasm where they undergo a conformational change before they can become active in the cell membrane (Goldstein & Brown, 1984). Like HMGR, when there are low concentrations of cholesterol in the cell, SREBPs can increase the transcription of LDL receptors (Goldstein & Brown, 2009). This controls the number of LDL receptors available to bring in exogenous cholesterol, preventing an over accumulation in the cell (Goldstein & Brown, 2009).

However, when dysfunction does occur in these regulation mechanisms, statins can be used to manage cholesterol production and prevent the onset of CVD related adverse effects (Blaha & Martin, 2013).

6.2.3 Molecular function of statins

The benefits of statin use to decrease LDL-C is well debated in the literature, with overwhelming evidence showing that the overall health benefits of stains outweigh their side effects (Blaha & Martin, 2013; Di Sciascio et al., 2009; Koh, Sakuma, & Quon, 2011; Mihaylova et al., 2012; Stroes, 2005; Weng et al., 2010).

Statins are able to lower LDL-C levels because they inhibit HMGR (Stroes, 2005). At the active site of HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A

(HMG-CoA) binds to the HMG-CoA-binding region, with nicotinamide adenine dinucleotide (NAD) also required to bind to the coenzyme-A-binding site for production of mevalonate (Friesen & Rodwell, 2004). Statins inhibit HMGR by binding to the HMG-CoA-binding region and blocking its access to HMG-CoA, due to their structural similarity (Figure 5) (Friesen & Rodwell, 2004; E. S. Istvan & J. Deisenhofer, 2001). Statins and HMG-CoA therefore compete against each other for access to the active site (E. S. Istvan & J. Deisenhofer, 2001). All statins inhibit HMGR by blocking its active site, however there are subtle differences in the way they bind due to their different molecular structures (Stroes, 2005). The side chains of some statins have also been shown to block a portion of the coenzyme-A-binding site, having a higher efficiency for lowering LDL-C due to partial inhibition of coenzyme binding as well as HMG-CoA binding (Figure 5) (Friesen & Rodwell, 2004). Because HMGR is the rate limiting enzyme in the mevalonate pathway, targeting HMGR is effective for impeding the production of cholesterol from the liver and decreasing the amount of circulating LDL-C. With lower levels of cholesterol, expression of LDL-receptors on cells increases which removes LDL-C from the bloodstream without giving it a chance to contribute to atherosclerosis (Figure 4) (Stroes, 2005).

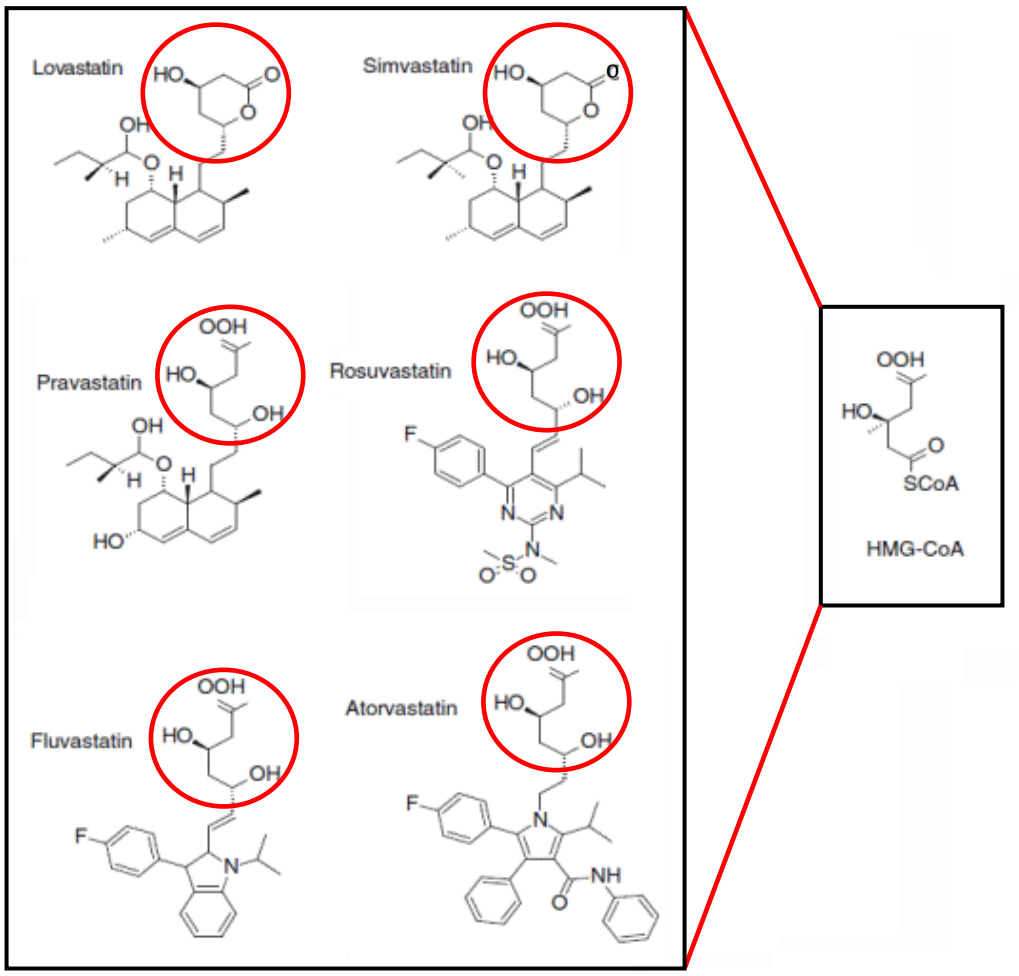

Figure 5. Molecular structure of six different statins depicting the hydroxyl group which binds to the HMG-CoA-binding region. The statins are able to compete with HMG-CoA for the HMG-CoA-binding region due to their structural similarity. Modified from "*Biotransformation of waste biomass into high value biochemicals*" by S. K. Brar, G. S. Dhillon & C. R. Soccol, 2014, New York, USA: Springer. Copyright 2014 by Springer Science and Business Media New York.

Statins have also been observed to have other LDL-C reducing effects in addition to HMGR inhibition, such as a reduction in CETP activity (McTaggart & Jones, 2008). Although statins are not thought to be direct inhibitors of CETP, some studies have suggested they may have some effect on CETP regulation (Guerin, Lassel, Le Goff, Farnier, & Chapman, 2000; McTaggart & Jones, 2008). CETP is a protein which exchanges cholesterol ester from HDL for triglycerides in VLDL as explained above. Guerin et al. (2000) suggest that atorvastatin, a type of statin, significantly decreases CETP plasma concentration, therefore decreasing the

amount of cholesterol ester that is exchanged between HDL and VLDL. This increases the amount of HDL and decreases the amounts of VLDL and LDL in circulation. However, more research is required in this area of statin function and only a small number of statins such as atorvastatin have been shown to have this effect (McTaggart & Jones, 2008).

In more recent research, statins have also been suggested to decrease the risk of CVD events independent of their influence on LDL-C. Studies have demonstrated a decrease in the risk of major cardiovascular events in people who have 'normal' LDL-C levels, as well as having an almost immediate effect on decreasing the incidence of heart attack after coronary revascularization surgery, irrespective of blood LDL-C concentration (Di Sciascio et al., 2009; Mihaylova et al., 2012).

There are three potential reasons why statins decrease CVD risk irrespective of LDL-C. Firstly, when circulating levels of LDL-C are low, atherosclerotic plaques can still be present. Statin therapy may be able to reduce the size and therefore the risks associated with plaques (Makris, Lavida, Nicolaides, & Geroulakos, 2010; Tawakol et al., 2013; Vale et al., 2014). Secondly, statins may also be able to increase the amount of circulating HDL, therefore excreting more cholesterol by influencing CETP activity. Lastly, statins are hypothesised to have a non-inflammatory influence which disrupts plaque formation, altering the 'pro-atherosclerotic' environment (Antonopoulos, Margaritis, Lee, Channon, & Antoniades, 2012; Subramanian et al., 2013). This was supported by an observed decrease in the number of inflammatory cells within a plaque after statin therapy, however the mechanisms behind this are not fully understood (Liao & Laufs, 2005).

Along with the health benefits statins come with, the use of some statins seem to propel behavioural changes which lead to increased BW, counterproductive for the treatment of underlying metabolic dysfunction statins (Aguirre et al., 2013; Athyros & Mikhailidis, 2014; Redberg, 2014; Sugiyama et al., 2014; Swerdlow et al, 2015). This counteracts the benefits statins have and can not only decrease the effectiveness of statins, but also worsen prognosis.

6.3 Increase of appetite and body weight by statin use

The use of statins has been shown to increase BW and adiposity in several studies (Aguirre et al., 2013; Athyros & Mikhailidis, 2014; Redberg, 2014; Sugiyama, Tsugawa, Tseng, Kobayashi, & Shapiro, 2014; Swerdlow et al.). Sugiyama et al. (2014) demonstrated an increase in calorie and fat intake in adult humans after statin use, and showed statin users have a faster increase of body mass index (BMI) than non-statin users. The increase in BW has been shown in several different types of statins (Aguirre et al., 2013; Athyros & Mikhailidis, 2014; Redberg, 2014; Sugiyama et al., 2014; Swerdlow et al, 2015).

Swerdlow et al. (2015) demonstrated that single nucleotide polymorphisms (SNPs) in human DNA which effect the function of HMGR showed similar effects in BW increase when statins are taken in humans. This suggests that statins causing disruption to HMGR may be one of the contributors to an increase in BW (Swerdlow et al, 2015).

Statins have also been shown to cause a difference in body fat accumulation, with Aguirre et al. (2013) suggesting subcutaneous adipose tissue increases a significant amount after only six weeks of statin use in rat models.

However, there are several different types of statins, and their differences can have alternative influences on physiology. Some physiological differences include variation in bioavailability, LDL-C and triglyceride lowering efficiency and their ability to increase HDL levels (Huffman, 2002; Schaefer et al., 2004). Some statins have also been suggested to cause changes in adipose development, with hydrophilic statins shown to be better at preventing lipid accumulation in particular areas of the body such as renal tubules when compared to lipophilic

statins (Gotoh et al., 2013). There are also differences in statin efficiency between different genders, ethnicities and age (Dean, 2010).

6.4 Simvastatin

There are several different types of statins with lovastatin, pravastatin, simvastatin, atorvastatin, pravastatin and fluvastatin being the main ones discussed in the literature (Jones, Kafonek, Laurora, & Hunninghake, 1998; Schaefer et al., 2004; Shepherd, Hunninghake, Barter, McKenney, & Hutchinson, 2003). They all have subtle differences; lovastatin is a 'natural' drug synthesised from fungi, and pravastatin and simvastatin are slight variations from lovastatin; while atorvastatin, fluvastatin and rosuvastatin are made synthetically (Chong, Seeger, & Franklin, 2001; Schachter, 2005). There are also differences in their molecular properties where atorvastatin, lovastatin and simvastatin are lipophilic and pravastatin, rosuvastatin and fluvastatin are more hydrophilic (Gotoh et al., 2013). Lipophilic statins are able to cross the blood brain barrier (BBB), which suggests they may potentially have an effect in the central nervous system (CNS). Simvastatin and lovastatin are also the only two statins which are not taken in their active state, making them (Istvan & Deisenhofer, 2001). They must first be hydrolysed when ingested to form the active hydroxyl acid which binds to the HMGR active site (Istvan & Deisenhofer, 2001).

Simvastatin has been classified as the second most effective statin at decreasing LDL-C after atorvastatin, however a larger number of mild adverse side effects have been identified by patients using atorvastatin such as nausea, myalgia and joint pain (Schaefer et al., 2004; Wierzbicki et al., 1999). Because atorvastatin is most effective it is the most commonly prescribed statin in New Zealand with 8.9% (284,262) of the population taking atorvastatin, and 7.1% (226,984) of the population taking simvastatin (Best Practice Advocate Centre New Zealand, 2014).

Although simvastatin has been found as a safe long term treatment for CVD, a few papers have shown that simvastatin increases food intake (FI) and BW (Carr, 2014; Palus et al., 2013). Majority of research papers show conflicting results, arguing that simvastatin use causes a decrease BW (Mansouri, Khodayar, Tabatabaee, Ghorbanzadeh, & Naghizadeh, 2015; Reinehr & Andler, 2004). However, it has been suggested that research showing a decrease in FI and BW induced by simvastatin is flawed because simvastatin possibly has an effect on energy expenditure (EE) (Cleanthous et al., 2011; Derosa, Mugellini, Ciccarelli, Rinaldi, & Fogari, 2002; Khan, Hamilton, Mundy, Chua, & Scherer, 2009; Kretzer, Maria, Guido, Contente, & Maranhão, 2016). An increased EE suggests that if control and treatment animals are being fed the same amount of food, treatment animals would lose BW due to an increased metabolism (Khan et al., 2009). Majority of research has also shown that simvastatin inhibits adipogenesis *in vitro,* however Khan et al. (2009) suggested that during *in vivo* studies, simvastatin may increase the differentiation of adipocytes (Mäuser, Perwitz, Meier, Fasshauer, & Klein, 2007; Nishio, Tomiyama, Nakata, & Watanabe, 1996; Song et al., 2003; Tomiyama, Nishio, & Watanabe, 2001). This demonstrates the difference between *in vivo* and *in vitro* research, where there may be pleiotropic effects *in vivo* that *in vitro* studies cannot detect. Although there is still debate in the literature, recent research is skewed towards simvastatin increasing BW and perhaps adiposity, and explanations for potentially incorrect opposing findings are being uncovered (Carr, 2014; Cleanthous et al., 2011; Khan et al., 2009; Kretzer et al., 2016; Palus et al., 2013). It is still unclear what is causing this change in FI and EE, and little research has been done on the mechanisms behind an observed increase in BW by simvastatin use. Because simvastatin is a lipophilic statin and

is able to cross the BBB, it could potentially have both a peripheral effect, which has been well studied, and a central effect which is less defined.

6.5 Peripheral vs central mechanisms effecting food intake

FI has both peripheral and central influences and is defined as the amount of food ingested within a given time period (Lenard & Berthoud, 2008). The regulation of FI along with EE are main components determining BW.

6.5.1 Peripheral regulation of food intake

The peripheral regulation of FI includes gastrointestinal information from mechanical and hormonal signalling. Gastrointestinal mechanoreceptors play a role in satiety and controls meal size and frequency (Cummings & Overduin, 2007; Houpt, 1982). During a meal, gastric distension triggers mechanoreceptors to send sensory information to the brain. These regions are then able to use neuronal pathways to secrete hormones causing a decrease in FI. The gut itself also has enteroendocrine cells which secrete gut peptides when food is ingested. This decreases appetite and increases satiety, therefore concluding meal time (Cummings & Overduin, 2007; Houpt, 1982). Such gut peptides include cholecystokinin (CCK), glucagon-like peptide-1(GLP-1) and peptide YY (PYY) (Cummings & Overduin, 2007; Houpt, 1982; Lenard & Berthoud, 2008). There are also hormones which control the increase of appetite when meal times are approaching, which is the role of a hormone called ghrelin (Klok, Jakobsdottir, & Drent, 2007). Ghrelin is secreted by the gastrointestinal system when the stomach is empty, eliciting hunger and promoting fat storage. Secretion is stopped once the stomach becomes stretched, which shows ghrelin plays a key role in meal initiation (Klok et al., 2007).

Leptin is another hormone involved in peripheral FI regulation and is secreted by adipose tissue. Leptin is able to cross the BBB and effect the hypothalamus, decreasing FI and increasing EE as fat reserves become higher (Klok et al., 2007).

Leptins role has been described as an intended pathway to prevent obesity, as more adipose tissue accumulates, more leptin is secreted to decrease FI. However, long term exposure to high leptin levels decreases its effectiveness due to decreased abundance in leptin receptors (Halaas et al., 1997). Leptin is also a key component in central regulation by binding to leptin receptors within the hypothalamus (Hayes et al., 2010).

The adrenal gland has also been shown to secrete hormones, such as cortisol, which effect FI. Cortisol is a steroid hormone which is most commonly associated with the stress response elicited by the hypothalamic-pituitary-adrenal (HPA) axis (George, Khan, Briggs, & Abelson, 2010). Cortisol has been suggested to increase FI during the short initial stages of the stress responses, with long term stress causing a decrease in appetite (Appelhans, Pagoto, Peters, & Spring, 2010; George et al., 2010).

Insulin is secreted by the pancreas in response to high blood glucose levels, and is able to reduce FI by crossing the BBB and binding to insulin receptors in the hypothalamus, similarly to leptin (Schwartz, Woods, Porte, Seeley, & Baskin, 2000). Insulin has also been shown to have an effect on EE by influencing fat deposition (Schwartz et al., 2000).

It is noteworthy to mention that although these mechanisms take place in the periphery, the periphery is not a closed circuit. The periphery sends sensory information to the CNS which propagates FI responses.

6.5.2 Central regulation of food intake

Central regulation is the overarching control mechanism of FI regulation, with three main areas of the brain responsible; the cortico-limbic, caudal brainstem and

hypothalamic systems (Figure 6) (Lenard & Berthoud, 2008). Neural pathways and molecules in these areas have roles in both eating for pleasure and energy, however, the cortico-limbic system is more associated with eating for pleasure, while neural pathways and molecules in the caudal brain stem and hypothalamus, in the context of this study, are more associated with eating for energy.

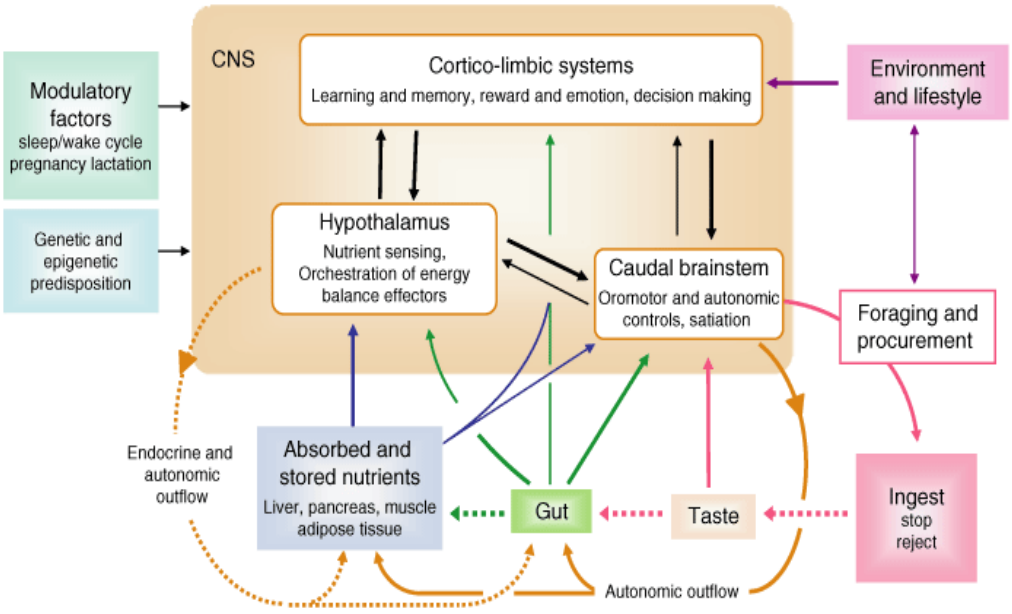

Figure 6. Simplified schematic of the three brain systems associated with the control of food intake and energy expenditure; cortico-limbic, caudal brainstem and hypothalamic regions. Reprinted from "Central and Peripheral Regulation of Food Intake and Physical Activity: Pathways and Genes" by N. R. Lenard & H. Bethoud, 2008, *Obesity, 16(S3),* p. S11-S22. Copyright 2008 by John Wiley and Sons.

6.5.2.1 Central regulation of food intake for pleasure

Cortico-limbic systems in this context are involved in reward circuits which encourage the intake of palatable food. Some of the areas involved in this response are the nucleus accumbens (NAc), ventral pallidum (VP) and ventral tegmental area (VTA). The main drivers of food hedonics are opioids, dopamine and serotonin (Lenard & Berthoud, 2008).

The ingestion of palatable foods regardless of hunger was an evolutionary benefit because foods high in sugars and fats are required for normal physiological

function (Mela, 2006). These foods were usually rare, so when they were found, consuming them in high amounts was beneficial for survival (Mela, 2006). However, in our current obesogenic environment, eating for pleasure is no longer important for survival and promotes weight gain (Lenard & Berthoud, 2008). There are three systems which drive hedonic eating; opioids, dopamine and serotonin systems.

Opioids are a family of peptides including endorphins, enkephalins and dynorphins (Kelley et al., 2002). Opioids are important in the neurophysiology of addiction, and it is a well-supported hypothesis that opioids increase hedonic FI, although the exact mechanisms remain undetermined (Berridge, 1996; Calcagnetti & Reid, 1983; Kelley et al., 2002; Saper, Chou, & Elmquist, 2002). Studies have demonstrated that opioid drugs such as morphine increase hedonic FI while naloxone, a drug used to block opioid receptors, reduces the preference for palatable foods (Frenk & Rogers, 1979). The NAc is thought to have a key role in this pathway because it has a high abundance of opioid receptors, and was therefore used to gain information on opioid receptor potency (Trezza, Damsteegt, Achterberg, & Vanderschuren, 2011). It was discovered that mu-opioid receptors, one of the three opioid receptor types, was most potent at inducing hedonic eating. Therefore opioids with high affinity for these receptors (enkephalins and beta-endorphin) have the most effect on hedonic FI (Trezza et al., 2011). However, the ventral striatum has been labelled as a key brain region for opioid control of hedonic eating, which has been shown to regulate desirability of palatable foods in order to increase FI (Kelley et al., 2002; Saper et al., 2002).

The dopamine system has been more difficult to relate to hedonic eating regulation. Dopamine neurons within the NAc are involved in addiction similarly

to opioid receptors, and therefore were hypothesised to be involved in hedonic eating also (Saper et al., 2002). However, early studies where the NAc was damaged but hedonic eating still occurred suggested dopamine did not have a key role in hedonic eating regulation (Balleine & Killcross, 1994). Furthermore, the inability to produce dopamine in mice caused them to die from starvation which could be reversed by administration of tyrosine hydroxylase, the rate limiting enzyme for biosynthesis of dopamine (Szczypka et al., 2001). It is now widely accepted that both dopamine and the NAc have some role in hedonic eating regulation, however the system is much more complex than being isolated to only a few molecules in these brain regions, making it difficult to study. It has been suggested that although dopamine is expressed during exposure to rewarding stimuli, dopamine release is highest after initial exposure to novel reward (Hajnal & Norgren, 2001). As a reward food item becomes more familiar, less dopamine is released having a smaller effect on hedonic FI (Hajnal & Norgren, 2001; Schultz, 1998). Perhaps this is why damage to the NAc was observed to have no effect on hedonic eating during studies because it is not essential for hedonic eating to occur, suggesting the relationship between dopamine and hedonic reward is more complicated than what was initially assumed (Saper et al., 2002).

Serotonin has also been shown to have an effect on both eating for energy and pleasure, however this system is much less studied than the opioid and dopamine systems (Saper et al., 2002). Serotonin has been shown to modulate FI regulation by increasing activation of POMC/CART neurons within the ARC, causing a decrease in FI for energy (Meguid et al., 2000; Saper et al., 2002). However, serotonin also stimulates POMC/CART neurons to produce beta-endorphin (Heisler et al., 2002; Kelley et al., 2002; Saper et al., 2002). Beta-endorphin binds

to mu-opioid receptors, the most potent receptor for inducing hedonic FI, modulating the initiation of the reward response (Heisler et al., 2002; Kelley et al., 2002; Saper et al., 2002). Serotonin has also been shown to decrease the preference for high fat and high sugar diets, even though their presence in the hypothalamus increases beta-endorphin levels (Blundell, Lawton, & Halford, 1995; Flaherty, Turovsky, & Krauss, 1994). Although current research points towards serotonin having an overall decrease in FI both for energy and pleasure, there are still conflicting results of what serotonins role is in hedonic eating and more research needs to be done to confirm this role (Heisler et al., 2002; Meguid et al., 2000; Saper et al., 2002).

However, eating for pleasure is only one of the forms of central regulation of FI. The CNS also has a crucial role in the regulation of eating for energy through neural pathways and molecules within brain regions such as the caudal brainstem and hypothalamus.

6.5.2.2 Central regulation of food intake for energy

Areas within the brainstem have a role in appetite regulation. This includes the nucleus of solitary tract (NTS), area postrema (AP) and dorsal motor nucleus of the vagus nerve (DMNV). NTS and AP both have leptin receptors, which when activated cause a decrease in FI (Hayes et al., 2010). The NTS is also integrated into the neural pathways of hypothalamic regions such as the lateral hypothalamic area (LHA), which has a key role in food intake for energy (Hayes et al., 2010). Because the AP is located outside the BBB, it has the unique ability it respond to peripheral hormones which are not able to cross the BBB such as CCK and GLP-1 (Saper et al., 2002). The DMNV receives information from the periphery via the vagus nerve to decrease FI, and has also been shown to decrease FI through the

binding of melanocortin's such as α-melanocyte-stimulating hormone (α-MSH) to melanocortin 4 receptor (MC4-R) (Williams, Kaplan, & Grill, 2000). This suggests the DMNV is able to effect FI without any input from the vagal nerve (Williams et al., 2000). Although the brainstem has a key role in appetite regulation and relaying information, the hypothalamus has been labelled as another brain region whose neural pathways and molecules are crucial for FI and EE regulation.

Neural pathways and molecules associated with the hypothalamus integrate information from the periphery and stimulate the pituitary gland to release hormones which impact FI. This includes the neural pathways and molecules associated with several brain regions including the supraoptic nuclei (SOP), dorsomedial hypothalamic nucleus (DMH), ventromedial nucleus of hypothalamus (VMH), arcuate nuclei (ARC), LHA and paraventricular nuclei (PVN).

The SOP has projections into the posterior pituitary gland which transports vasopressin and oxytocin for release into the bloodstream (Jezova, Michajlovskij, Kvetnansky, & Makara, 1993). Projections from the PVN are also able to do this (Jezova et al., 1993). Activation of DHN causes a decrease in FI, and BW as well as a decrease in thirst (Bellinger & Bernardis, 2002). VMH has a role in satiety, where electrical stimulation has been shown to decrease FI (Bellinger & Bernardis, 2002). LHA has been termed a key brain area for the regulation of hunger, as stimulation of the LHA increases FI as well as water consumption (Stuber & Wise, 2016). The LHA uses two hormones, orexin and melanin concentrating hormone (MCH) to create changes in FI regulation. It has also been suggested to have a key role in the reward system where orexin fibres are able to

innervate dopamine neurons within the VTA (Berthoud & Münzberg, 2011; Saper et al., 2002; Stuber & Wise, 2016). The ARC is a key brain region associated with the regulation of appetite, containing two populations of neurons which have been associated with either an increase or decrease in FI, depending on the presence of effector molecules such as leptin or insulin (Katherine A Simpson, Martin, & Bloom, 2008). In the context of eating for energy, a more thorough discussion of the ARC, PVN and LHA is warranted due to their crucial roles in central regulation on FI.

In the ARC, there are two key populations of neurons; neuropeptide Y/ agouti-related protein (NPY/AgRP) and pro-opiomelanocortin/ cocaine- and amphetamine-regulated transcript (POMC/CART) neurons (Cone, 1999). Both have receptors which bind leptin and insulin. With a high concentration of leptin/ insulin, NYP/AgRP inhibition and POMC/CART excitation have a anorexigenic effect (decreases FI). In contrast, low concentration of leptin/ insulin, NYP/AgRP excitation and POMC/CART inhibition has an orexigenic effect (increases FI) as well as an increase in fat deposition (Figure 7) (Cone, 1999). Leptin and insulin can cross the BBB and are found at the same concentration in blood plasma as they are in cerebral spinal fluid (CSF) (Cone, 2005; Oswal & Yeo, 2007). Leptin's effects in the ARC have also been shown to influence glucose metabolism and locomotor activity as well as FI (Coppari et al., 2005). POMC/CART neurons are able to effect FI because POMC is cleaved to produce melanocortin's such as α-MSH, which bind to MC4-Rs in the PVN, decreasing FI (Arora, 2006). The release of AgRP has been shown to block the binding of α-MSH to MC4-R in the PVN as a secondary regulation mechanism when NPY/ AgRP neurons are active (Schwartz et al., 2000). These MC4-R receptors have been found in more places

throughout the brain, which have an effect on eating control such as the NAc which then innervates to the LHA (Saper et al., 2002). CART is usually co-expressed with POMC and shows anorexigenic effects when administered directly into the hypothalamus (Lau & Herzog, 2014). However, CART administered ICV has been shown to have orexigenic effects demonstrating CART may have several different purposes within different areas of the brain (Lau & Herzog, 2014).

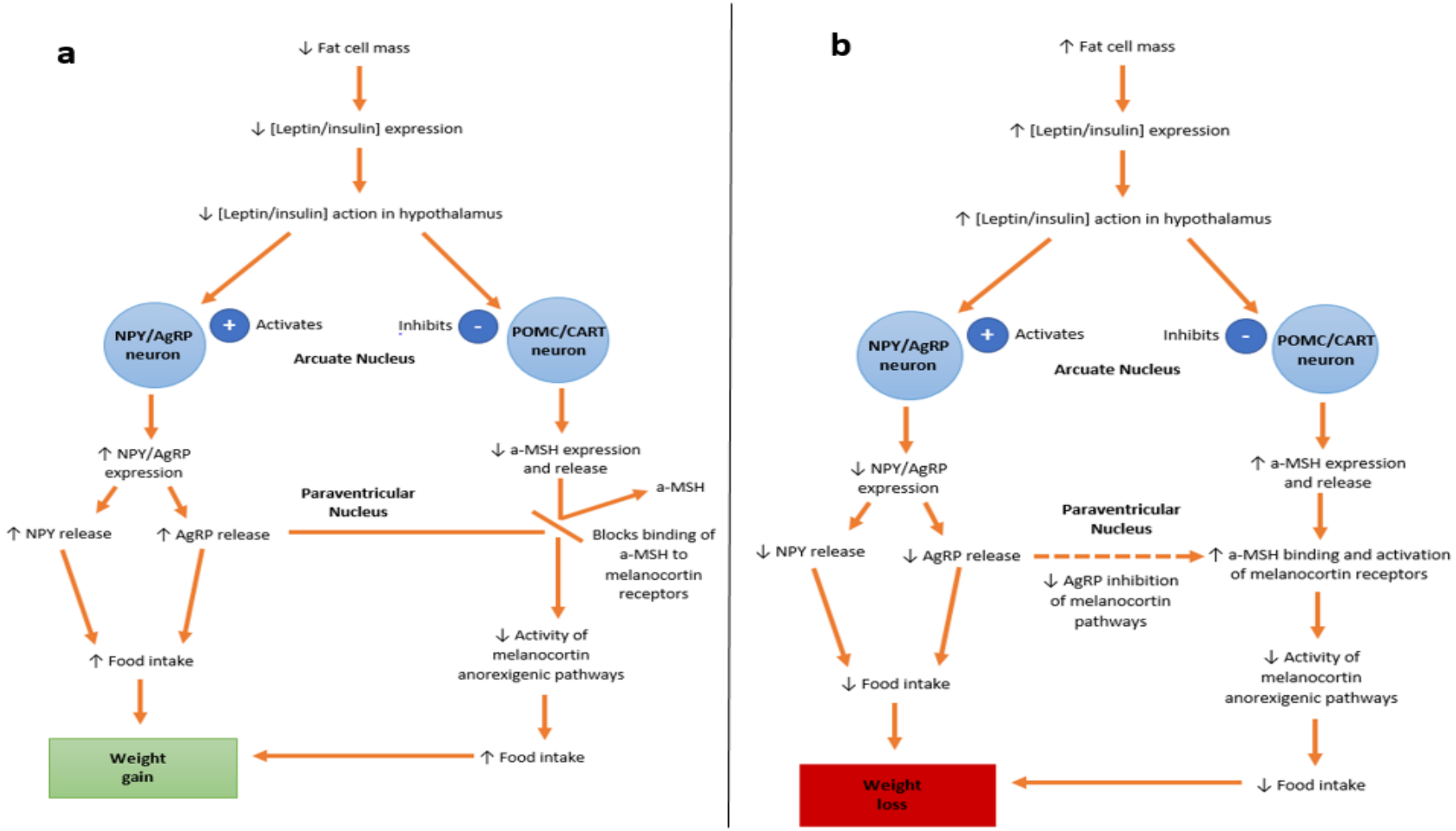

Figure 7. Food intake control in ARC and PVN with leptin and insulin expression. A. the molecular pathway utilized to increase food intake during low leptin levels. B. the molecular pathway utilized to decrease food intake during high leptin levels. Modified from "Central nervous system control of food intake" by M. W. Scwartz, S. C. Woods, D. Porte, R. J. Seeley & D. G. Baskin, 2000, *Nature, 404*, p. 661-671. Copyright 2017 by Macmillan Publishers Ltd, part of Springer Nature.

During a lack of leptin, when NYP/AgRP neurons are activated along with AgRP blocking the binding of α-MSH to MC4R, it also causes production of y-aminobutyric acid (GABA) which inhibits POMC neurons from decreasing FI (Lenard & Berthoud, 2008). NPY then binds to neuropeptide Y receptors type 1 (Y1) and neuropeptide Y receptors type 5 (Y5) in the PVN (these receptors are also found in the DMN and VMH), causing an increase in FI (Kalra, Dube, Sahu, Phelps, & Kalra, 1991; Nguyen et al., 2012; Schwartz et al., 2000).

The groups of neurons in the ARC can also be effected by ghrelin and PYY. Ghrelin has been shown to activate neurons in the ARC to stimulate appetite, however, ghrelin has not been shown to cross the BBB (Cowley et al., 2003). Despite this, small populations of specialised cells in the brain are able to secrete the ghrelin which effects the central regulation of FI. The effect and importance of ghrelin in the brain is much less studied than the effect of peripheral ghrelin (Cowley et al., 2003). PYY is secreted from L-cells in the intestine to decrease FI, however PYY can cross the BBB binding to Y2 receptors in the ARC, inhibiting NPY/AgRP neurons and can stimulate POMC/CART neurons (Karra, Chandarana, & Batterham, 2009; Le Roux & Bloom, 2005). NPY/AgRP and POMC/CART neurons in the ARC project to other brain regions such as PVN, LHA and DHN where they can elicit their responses (Katherine Anne Simpson, Martin, & R Bloom, 2009).

There are two main cell groups in the PVN; parvocellular and magnocellular sections (Hill, 2012) . Parvocellular neurons transport neuropeptides to the median eminence which initiate the production of hormones from the anterior pituitary. In the anterior pituitary, stimulatory hormones such as corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) cause the release of

effector hormones into the blood stream. These effector hormones include adrenocorticotropic hormone (ACTH) which controls the adrenal axis and thyrotropin-stimulating hormone (TSH) which controls the thyroid axis, as well as dopamine which control the reproduction axes and somatostatin which controls growth and development (Geerling, Shin, Chimenti, & Loewy, 2010; Hill, 2012). The whole pathway from innervation of ARC neurons by peripheral stimuli, to release of hormones from the pituitary gland is one of the ways FI and EE is regulated. Using the thyroid axis as an example of this pathway, when leptin levels are high and NPY/AgRP neurons are activated, this sends a signal to the PVN to release TRH (Figure 8) (Nillni, 2010). Parvocellular neurons transfer the signal elicited by TRH to the anterior pituitary where TSH is produced and released into the bloodstream. TSH will then stimulate the thyroid gland to produce hormones triiodothyronine (T3) and thyroxine (T4) which increase metabolism and EE (Nillni, 2010).

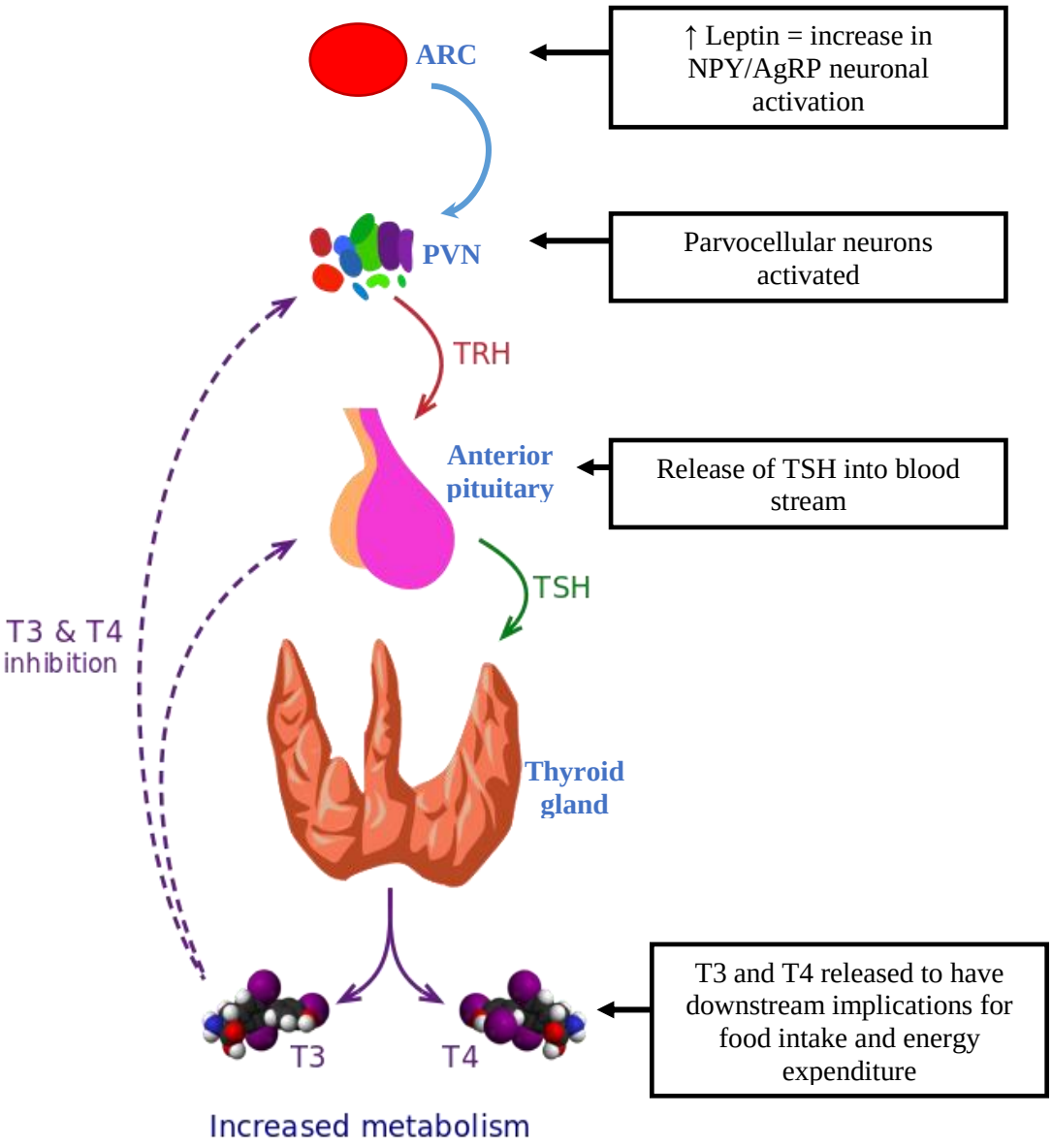

Figure 8. Hypothalamic-pituitary-thyroid axis showing the central pathway leading to increased metabolism and the negative feedback loop of T3 and T4 on TRhH release. Modified from "Thyroid" by I.A. Sherazi, 2014, *Zaki Centre*.

The adrenal axis has a similar process, where NPY/AgRP neurons release CRH from the PVN, which in turn stimulates ACTH from the anterior pituitary. This stimulates the adrenal gland to release cortisol into the blood stream initiating the stress response and overall decreasing FI (Kohno et al., 2008). All the separate axes contribute to an overall increase or decrease in FI depending on the signals given from the periphery and brain regions. These axes can also reiterate the complexity of the central regulation on FI. The hormones released into the blood

stream such as ACTH and TSH have more functions than only influencing FI, which makes it difficult to narrow their involvement down to only one aspect of the wide range of roles they are involved in. For example, although TSH elicits an increase in EE, it also has a key role in brain maturation and growth (Glass et al., 1987). The magnitude of connectivity makes dissecting out one function of a hormone or neuron problematic. This can have implications on the results of studies, because observations that are unable to be explained, or that are not consistent, may be due to pleiotropic effects these hormones and neurons have.

The magnocellular neurons act as a control centre for endocrine functions. These neurons along with neurons from the SON project to the posterior pituitary gland where they can either release vasopressin or oxytocin into the blood stream (Schwartz et al., 2000). Oxytocin has many roles once it is released related to lactation, parturition and bonding (Schwartz et al., 2000). However, its release from the pituitary has also shown downstream effects decreasing FI, and it has been suggested that AgRP is able to inhibit the release of oxytocin (Atasoy, Betley, Su, & Sternson, 2012). Vasopressin targets the kidneys for short or long term increases in water retention by the regulation of aquaporins, and increases thirst (Knepper, 1997). Meyer, Langhans, and Scharrer (1989) suggested vasopressin also decreases FI, however this has not been able to be replicated in following studies (Gulati & Sharma, 1993; Racotta, Soto-Mora, Palacios, & Quevedo, 1995).

The LHA facilitates a second common central pathway for energy regulation which is the orexin and MCH pathway. Orexins release causes an increase in FI (Tsujino & Sakurai, 2013; Yamanaka et al., 2000). Therefore, high concentrations of leptin or glucose hyperpolarise orexin neurons making it more difficult for

them to produce action potentials, while low levels of glucose and ghrelin depolarise orexin neurons making it easier to produce action potentials (Tsujino & Sakurai, 2013; Yamanaka et al., 2000). There are two types of orexin; orexin-A and orexin-B. There are also two orexin receptors; orexin-1 and orexin-2 receptors (Rodgers, Ishii, Halford, & Blundell, 2002). Orexin-A binds to both receptors, while orexin-B binds mostly to orexin-2 receptor (Sakurai et al., 1998). Orexin-A has the most influence on FI with much less evidence supporting any role of orexin-B in FI regulation (Sakurai et al., 1998). Orexin has been shown to project to NYP/AgRP neurons to cause an increase in FI through the melanocortin pathway (Rodgers et al., 2002). However, POMC/CART neurons from the ARC also have projections which connect to the orexin neurons in the LHA, showing the relationships between orexins and the melanocortin pathway is complicated including several different, parallel pathways (Saper et al., 2002; Volkoff & Peter, 2000). Orexins are also implicated in the reward system with projections to dopamine neurons within the VTA and serotonin neurons within the dorsal raphe nucleus (Rodgers et al., 2002). Orexin increases metabolism and EE which seems to be controlled by binding to orexin-2 receptor (Barson, Morganstern, & Leibowitz, 2013). Because orexin can affect the melanocortin pathway and is able to respond to peripheral signals such as leptin, glucose and ghrelin, it suggested orexins role is in food seeking and promoting FI, especially if it is palatable (Barson et al., 2013). Perhaps this suggests orexin has some role in the initiation of FI during meal times as well as more long term effects on energy metabolism and food preference (Barson et al., 2013). MCH is the other hormone within the LHA and also causes an increase in FI (Tritos et al., 1998). There are two MCH receptors; melanin-concentrating hormone 1 (MCH-1) and melanin-concentrating

hormone 2 (MCH-2) receptors. MCH-1 has been shown to play a key role in increasing FI which is separate from the melanocortin pathway (Shearman et al., 2003). There is substantial evidence to show that MCH increases FI, and also increases energy conservation suggesting MCH has a role in reinforcing food consumption and ongoing FI (Barson et al., 2013). However, much less is known about the biochemical interactions MCH has with other neuropeptides and how it causes these changes (Barson et al., 2013).

Both orexins and MCH increases FI with differing effects on EE. They have been observed to have an effect both on eating for pleasure and energy, although their degree of importance in these regulatory systems are unknown. However, LHA neurons are projected into several areas in the brain other than the hypothalamus and therefore the effect, purpose and function of orexin and MCH in the LHA are likely diverse. This may explain why their biochemical pathways are still largely unknown.

Though there are both peripheral and central mechanisms responsible for controlling FI regulation, neither systems would be able to function in isolation. Cross talk between the periphery and CNS is crucial for the proper regulation of homeostasis (Figure 9). However, the CNS is the overarching regulation mechanism which uses sensory inputs from the periphery to cause changes in homeostasis.

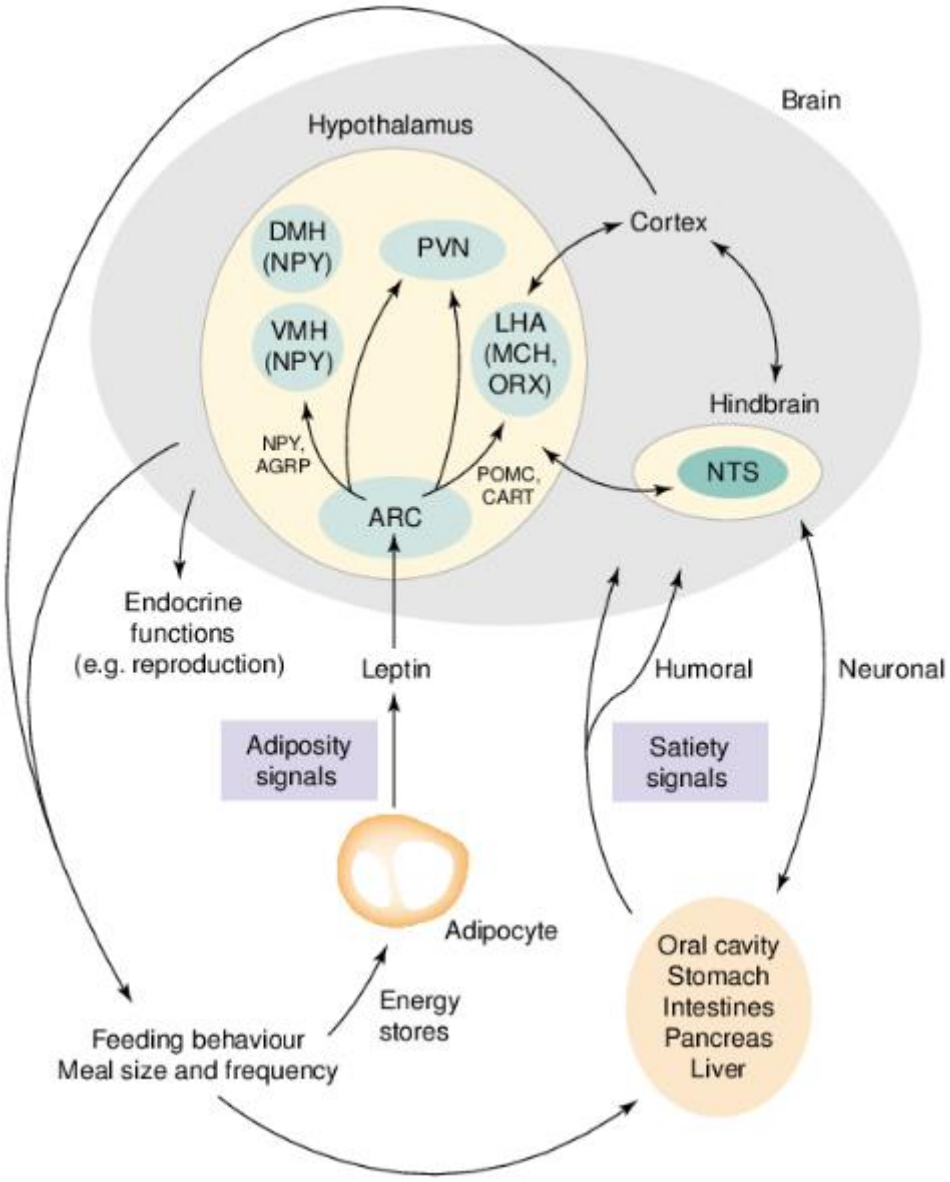

Figure 9. The regulation of food intake and energy expenditure is controlled by both peripheral and central mechanisms. There is a lot of complexity between these two systems which are often referred to as separate systems when cross talk between these systems ensures proper functioning of homeostasis. Reprinted from "Pharmacotherapy of obesity: targets and perspectives" by A. Chiesi, C. Huppertz & K. G. Hofbauer, 2001, *Cell press, 22(5),* p. 247-254. Copyright 2017 by Elsevier Science Ltd.

Eating for pleasure and for eating for energy are now thought to be two intertwined processes, where the same brain regions, neurons and molecules are able to influence both eating for pleasure and energy (Saper et al., 2002). There are many examples of this interlink, for example, leptin is one of the main

hormones used for regulating eating for energy. However, it has also been demonstrated to inhibit sweet taste cells on the tongue, influencing hedonic regulation at the most peripheral level (Saper et al., 2002). Another example is how POMC/CART neurons are crucial for the proper functioning of the melanocortin pathway, but also have the ability to produce beta-endorphin. Although they can be split apart and explained in isolation, it is significant to note that these systems function in conjunction with one another which adds more complexity to their effects on FI and EE.

6.6 Rationale and aims

There are many unknowns in the literature about central biochemical pathways which effect FI and EE, but the role of simvastatin on FI and EE has never before been explored. The goal of this research was to determine if simvastatin was causing an increase in FI in rat models, and to understand whether the mechanisms behind this increase was due to peripheral or central influences. This gains insight into the biochemical pathways involved. The three research aims which helped answer this goal were: 1. Determine the difference in energy driven FI between rats treated with simvastatin and controls (experiments one and two), 2. Determine the difference in pleasure driven FI between rats treated with simvastatin and controls (experiment three), and 3. Determine whether FI differences are driven by eating for energy or pleasure supported by both FI and c-Fos data (experiments one, two and five). These were achieved by doing a range of food intake studies as well as brain activation c-Fos immunoreactivity studies. FI for energy was investigated by looking at the differences in chow intake between deprived and non-deprived simvastatin and control groups. FI for pleasure was investigated by identifying diets preferred by rats administered with simvastatin, and by studying c-Fos activation in rat brains. During the study, water intake was measured. With the findings from this research, I hope to get a step closer to the development of statins which have less counterproductive side effects and have a more accurate target on the main problem, the reduction of cholesterol.

6.7 Materials and methods

6.7.1 Animals

The experiments were performed on 12-week-old male Sprague Dawley rats, single housed in standard plastic cages with wire lids, which were cleaned weekly. They were kept in a temperature- (22 ± 1 °C) and humidity-controlled room with a 12:12 light:dark cycle (lights on at 07:00). The animals had unrestricted access to food (Sharpes Feed) and tap water unless stated otherwise as part of experimental procedure. All experiments had approval from the University of Waikato Ethics Committee.

6.7.2 Cannulation

Animals were anaesthetised using ketamine (64.29 mg/mL) and xylazine (7.15mg/mL). A cannula was implanted stereotaxically in all rats for injection of simvastatin or DMSO into a lateral cerebral ventricle. With the rat's head in a stereotaxic frame, a hole was drilled into the skull, 1.6 mm lateral and 0.6 mm caudal to bregma, and a 22-gauge ICV guide cannula lowered with its tip 3.5 mm below the skull surface. The cannula was then fixed in place with dental acrylic and three small screws secured in the skull. Injections were given via a 28-gauge cannula, with its tip 1 mm below the guide cannula tip, connected via polythene tubing to a microsyringe. Animals were allowed one week to recover before being used in experiments.

6.8 Experiment design and analysis: Food intake

6.8.1 Experiment one: Effect of Simvastatin on deprivation induced chow intake.

20 male Sprague Dawley rats, of similar age and BW, were divided into four groups of five rats. The animals were deprived of food for 12 hours and then given ICV injections between 10:00 and 11:00. The control group was given a 3µl ICV infusion of 100% dimethyl sulfoxide (DMSO). The remaining three groups were given a 3µl ICV infusion of either 10, 30 or 100 nmol simvastatin, using DMSO as a vehicle. All animals had free access to food immediately after ICV infusion and food consumption was measured one, two and four hours after ICV infusion. All data was corrected for animal BW, and statistical analysis of the data comprised of a one-way ANOVA followed by the Fisher least significance test. *P* values <0.05 were considered significantly different. All statistical analysis was done using DeltaGraph software.

6.8.2 Experiment two: Effect of Simvastatin on overnight chow intake

20 male Sprague Dawley rats, of similar age and BW, were divided into two groups of ten rats. The control group was given a 3µl ICV infusion of DMSO, and the treatment group was given a 3µl ICV infusion of 30 nmol simvastatin (lowest effective dose) using DMSO as a vehicle, between 18:00 and 19:00. All animals had free access to food and food consumption was measured one, two, three, four, six and 12 hours after ICV infusion. All data was corrected for animal BW, and statistical analysis of the data included a student's t test. *P* values <0.05 were considered significantly different. All statistical analysis was done using DeltaGraph software.

6.8.3 Experiment three: Effect of Simvastatin on deprivation induced palatable food intake

20 male Sprague Dawley rats, of similar age and BW, were divided into two groups of ten rats. The control group was given a 3µl ICV infusion of DMSO, and the treatment group was given a 3µl ICV infusion of 30 nmol simvastatin using DMSO as a vehicle, between 10:00 and 11:00. All animals were then given access to either 20% sucrose solution, 0.2% saccharin solution or sweetened milk (26% milk powder (Dairy Goat Co-operative skim milk powder) and 10% sucrose) following infusion, in three separate experiments, and liquid consumption was recorded after two hours. All data was corrected for animal BW, and statistical analysis of the data included a student's t test. *P* values <0.05 were considered significantly different. All statistical analysis was done using DeltaGraph software.

6.8.4 Experiment four: Effect of Simvastatin on deprivation induced water consumption

20 male Sprague Dawley rats, of similar age and BW, were divided into two groups of ten rats. The animals were deprived of water for 12 hours and then given ICV injections between 10:00 and 11:00. The control group was given a 3µl ICV infusion of DMSO, and the treatment group was given a 3µl ICV infusion of 30 nmol simvastatin using DMSO as a vehicle. All animals then had free access to water and water consumption was measured two hours after ICV infusion. All data was corrected for animal BW, and statistical analysis of the data included a student's t test. *P* values <0.05 were considered significantly different. All statistical analysis was done using DeltaGraph software.

6.9 Experiment design and analysis: Brain activity

6.9.1 Experiment five: Effect of simvastatin on c-Fos immunoreactivity

20 male Sprague Dawley rats, of similar age and BW, were divided into two groups of ten rats. The control group was given a 3µl ICV infusion of DMSO, and the treatment group was given a 3µl ICV infusion of 30 nmol simvastatin using DMSO as a vehicle, between 10:00 and 11:00. Prior to the study, rats had free access to food and water.

6.9.2 Euthanasia and perfusion

Between 60 and 90 minutes after ICV infusion animals were deeply anaesthetised with an intraperitoneal injection of 3mL 35% urethane. They were then perfused with 100 ml of saline followed by 500 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were excised and postfixed overnight in PFA at 4 °C.

6.9.3 Immunohistochemistry

We studied the density of c-Fos immunoreactivity nuclei on nine brain sites involved in food intake: PVN, SOP, DMH, VMH, ARC, LHA, NTS, DMNV and AP.

Coronal sections of 60 µm were cut on the Vibratome (Leica). They were processed as free-floating sections for standard immunohistostaining against c-Fos. The tissue was treated for 10 min in 3% H_2O_2 in 10% methanol (in TBS, pH 7.4–7.6) and incubated overnight at 4 °C in the rabbit anti-c-Fos antibody (1:15000; Synaptic Systems). Subsequently, sections were incubated for one hour at room temperature in the goat-anti-rabbit antibody (1:400, Vector Laboratories), and then in the avidin-biotin complex (1:400; Vector Laboratories) for an hour. Peroxidase was visualised with a 15 minute incubation in 0.05%

diaminobenzidine, 0.01 H_2O_2, and 0.2% nickel sulfate. All incubations were done in a mixture of 0.25% gelatin (Sigma) and 0.5% Triton X-100 (Sigma) in TBS. Intermediate rinsing was done four times with TBS. Sections were mounted on gelatinised slides, dried, dehydrated in ascending concentrations of ethanol, soaked in xylene, and embedded in Entellan.

The number of Fos positive nuclear profiles in the regions of interest was counted on 4–5 sections per animal using Scion Image software. Densities of Fos immunoreactivity nuclei (per mm2) were averaged per rat and then per group. The effect of simvastatin versus DMSO on c-Fos immunoreactivity was established with ANOVA followed by the Fisher least significance test. Values were considered significantly different when $P < 0.05$. All statistical analysis was done using DeltaGraph software.

6.10 Results

6.10.1 Experiment one: Effect of Simvastatin on deprivation induced chow intake

The group of animals administered with 10nmol simvastatin ate less than the control group in every time bracket, while the group of animals administered with 30nmol of simvastatin ate more than controls in every time bracket (Figure 10). The group of animals administered with 100nmol simvastatin had varied results, eating less than the control group during the 0-1 hour time bracket but eating more than the control group in the two following time brackets.

There was a significant difference in the amount of chow eaten between the control group and the animals administered 30nmol simvastatin two hours after injection. There was also a significant difference in the amount of chow eaten between the control group and both the animals groups administered 30nmol and 100nmol simvastatin 4 hours after injection.

Injecting simvastatin at three different doses revealed the lowest effective dose of 30nmol, which was used in future experiments.

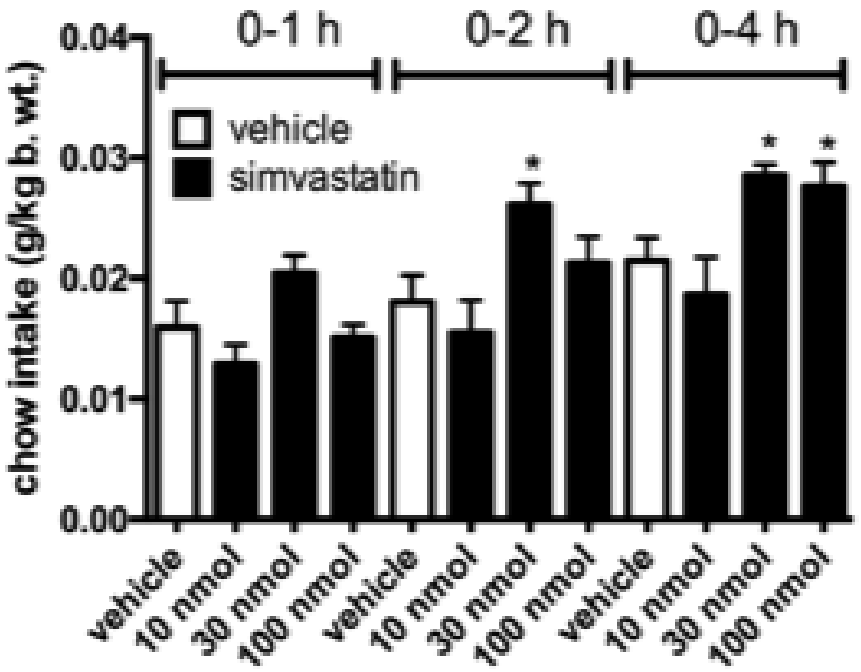

Figure 10. Deprivation induced chow intake at three different doses of simvastatin over a four-hour time period. Error bars indicate standard error of the mean (SEM). * indicates 5% significance.

6.10.2 Experiment two: Effect of Simvastatin on overnight chow intake

Without food deprivation, meal times are less profound than in experiment one.

The animals administered with simvastatin ate more chow than the control group up to four hours after injection, with a significant difference between the simvastatin and control group at three hours. After four hours, the animals administered with simvastatin eat less than the control group 6 and 12 hours after injection (Figure 11).

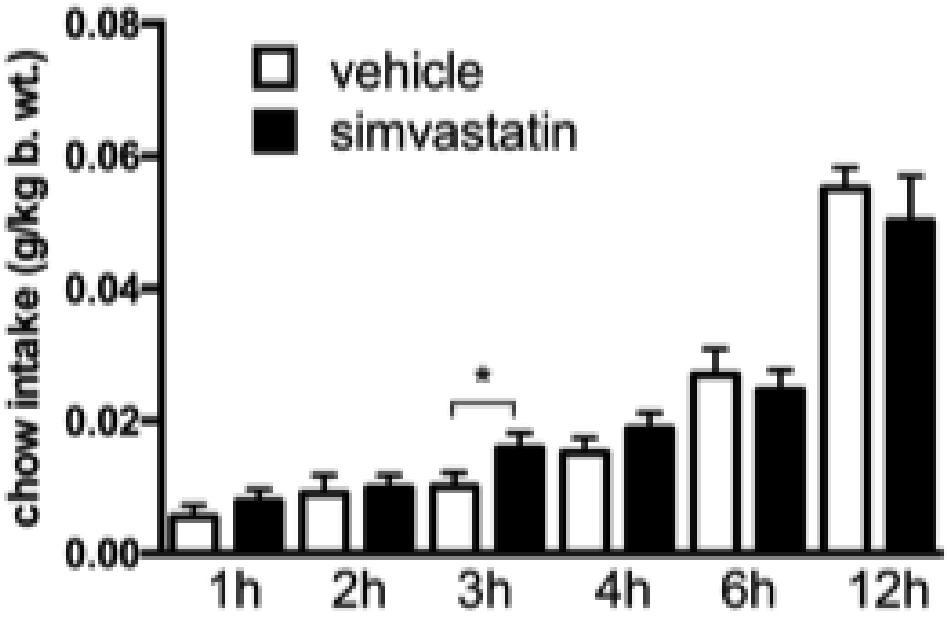

Figure 11. Overnight food intake after 30nmol simvastatin vs control over a 12-hour time period. Error bars indicate standard error of the mean (SEM).
* indicates 5% significance.

6.10.3 Experiment three: Effect of Simvastatin on deprivation induced palatable food intake

The palatable food solutions of sucrose, saccharin and sweetened milk were selected because sucrose was used as a high calorie food item, while saccharin contains the sweet taste without the calories. Sweetened milk contains less calories than sucrose, but includes other flavour compounds such as fat which adds complexity to the taste (Swiergiel, Smagin, & Dunn, 1997).

Animals administered with simvastatin drank a similar amount of all three palatable foods (sucrose, saccharin and sweetened milk) two hours after injecting when compared to the control animals (Figure 12).

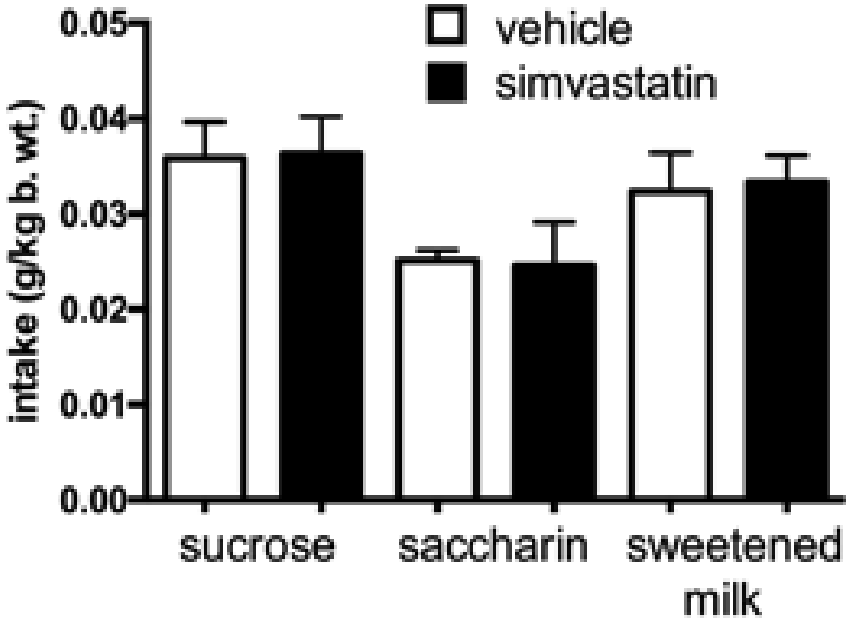

Figure 12. Palatability-driven food intake, two hours after injection of 30nmol simvastatin vs control. Error bars indicate standard error of the mean (SEM).

6.10.4 Experiment four: Effect of Simvastatin on deprivation induced water consumption

The group of animals administered with simvastatin drank a significantly (15%) more water than the control group two hours after injecting (Figure 13).

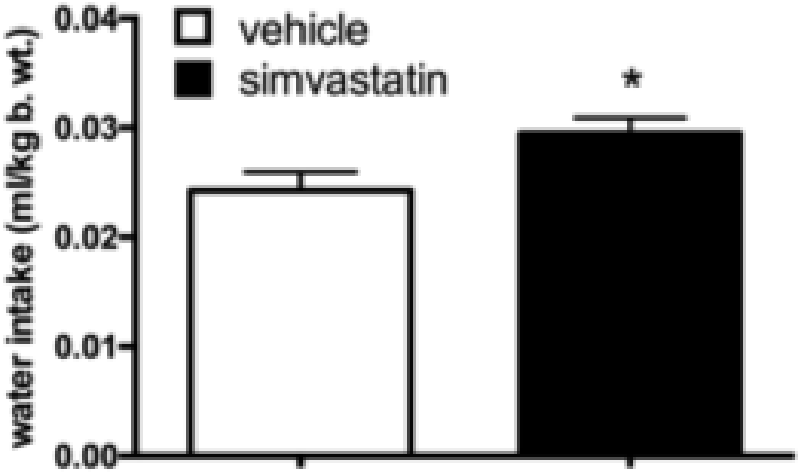

Figure 13. Water intake after deprivation, two hours after injection of 30nmol simvastatin vs control. Error bars indicate standard error of the mean (SEM). * indicates 5% significance.

6.10.5 Experiment five: Effect of Simvastatin on c-Fos immunoreactivity

Animals administered with simvastatin had a significant increase in c-Fos immunoreactivity when compared to controls in the PVN and ARC (Figure 14). There was an increase in activation of approximately 50% and 29% in the PVN and ARC respectively.

All other areas has similar levels of immunoreactivity, with no significant differences between control and treatment groups.

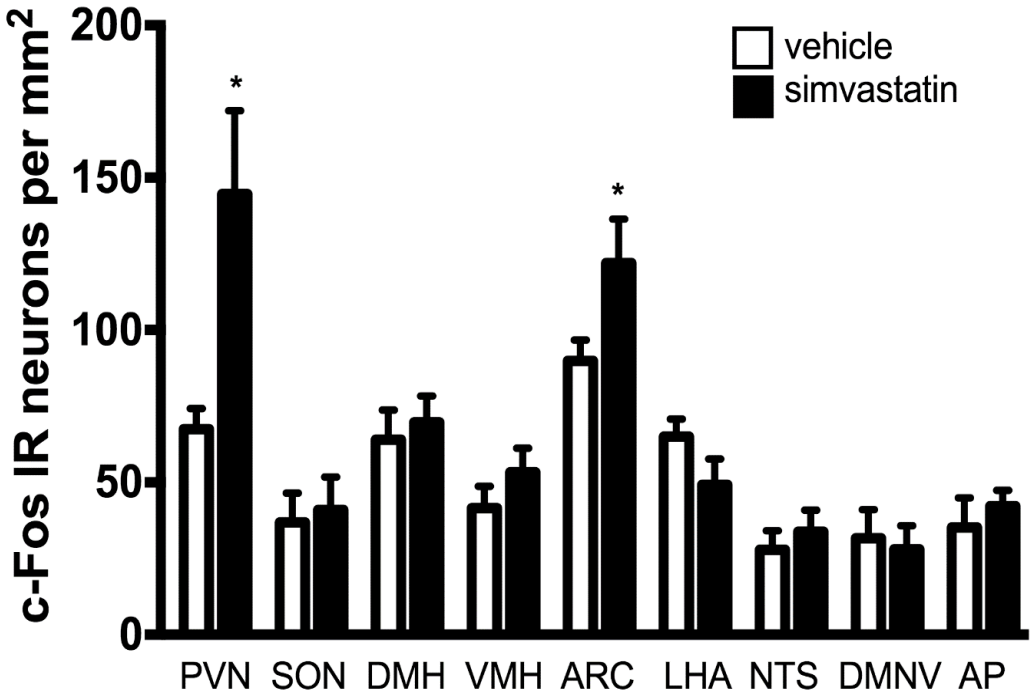

Figure 14. c-Fos immunoreactivity in different brain regions following injection of 30nmol simvastatin vs control. Error bars indicate standard error of the mean (SEM).
* indicates 5% significance.

PVN – paraventricular nucleus, **SON** – supraoptic nucleus, **DMH** - dorsomedial hypothalamic nucleus, **VMH** - ventromedial nucleus of hypothalamus, **ARC** – arcuate nucleus, **LHA** – lateral hypothalamic area, **NTS** – nucleus of solitary tract, **DMNV** - dorsal motor nucleus of the vagus nerve, **AP** – area postrema.

6.11 Discussion

The key findings of this research showed that simvastatin is able to increase FI when delivered centrally. This increase is related to FI for energy, with no influence from food palatability, and surprisingly also increased water consumption.

The results showed an increase in chow intake with and without deprivation by administering simvastatin centrally (experiments one and two). The idea that simvastatin could have an impact on the periphery causing a change in FI, in the context of this study, was dismissed. Peripheral influences may have some impact with different types of administration of simvastatin, such as intraperitoneal or oral. However, these results were able to show that regardless of peripheral interaction, FI increase still occurred due to the engagement of central mechanisms.

With further investigation into these central mechanisms, it is strongly suggested that simvastatin influences central pathways controlling food intake for energy and not for pleasure. Evidence for this was provided by food intake studies (experiment three) demonstrating in animals that were not water deprived, the same amount of palatable solutions were consumed between simvastatin and control groups. This finding was further supported by c-Fos immunoreactivity studies showing key areas of the brain involved in eating for energy, PVN and ARC, (experiment five) were 50% and 29% more active in animals that have been administered simvastatin. There are many possible mechanisms which may be responsible for the FI differences seen between simvastatin and control groups. One possible explanation is the melanocortin system may be increasing FI related signalling.

This system comprises of two neuronal pathways; NPY/AgRP and POMC/CART neurons, within the ARC which project to the PVN and have downstream impacts effecting FI for energy. The increase in FI may be explained by simvastatin interacting with NPY/AgRP neurons, eliciting an increase in FI, or perhaps simvastatin inhibits POMC/CART neuron impeding their ability to cause a reduction in FI.

Another possible explanation involves the ARC and PVNs influence on brown adipose tissue (BAT) metabolism. BAT has a crucial role in body heat production helping maintain thermal homeostasis, as well as having an influence on body weight regulation (Rothwell & Stock, 1979). In a cold environment, BAT produces heat that is used to keep the internal body temperature constant and prevent hypothermia (Rothwell & Stock, 1979). This is called cold-induced thermogenesis. However, during over eating BAT also produces heat, and this is thought to occur as a protection mechanism from obesity (Cannon & Nedergaard, 2004). This is called diet-induced thermogenesis, where the increase in EE attempts to compensate for the extra calories that are consumed (Cannon & Nedergaard, 2004). POMC/CART neurons in the ARC increase FI, but are also able to increase BAT thermogenesis (Münzberg, Qualls-Creekmore, Berthoud, Morrison, & Yu, 2016). When α-MSH is secreted from POMC/CART neurons, MC4Rs are activated which causes an increase in EE by activating BAT thermogenesis. While there are MC4Rs in the PVN, it is critical to note that these receptors so not have an influence on EE. There are MC4Rs elsewhere which contribute to EE, but the exact sites of MC4R mediated BA thermogenesis are still unknown (Münzberg et al., 2016). There are also non-NPY/AgRP and non-POMC/CART neurons within the ARC which effect FI and EE (Kong et al.,

2012). A portion of these neurons are called RIP-cre neurons which are distributed amongst NPY/AgRP and POMC/CART neurons, but are distinctly different. Manipulation of these neurons has shown that the release of GABA from RIP-cre neurons has an effect on energy balance. GABA released from these RIP-cre neurons in the ARC is suggested to provide input to the PVN, which sends projections to the NTS, causing an increase in EE by stimulating BAT. However, the increase of EE by RIP-cre neurons cannot be explained by the increase BAT thermogenesis alone, suggesting these neurons may also effect other areas of metabolism. Because the c-Fos immunoreactivity data did not show an increase in c-Fos activation (between control and simvastatin groups) in the NTS, it is unlikely this is causing the observed increase in FI. However, RIP-cre neurons are found in many regions throughout the brain suggesting a different population of RIP-cre neurons may be causing the observed increase in FI. It is still unknown which RIP-cre neurons are solely responsible for the regulation of EE, and the key neurotransmitters and downstream systems are also unknown (Kong et al., 2012). Therefore, simvastatin may be causing an increase in FI by increasing the activation of POMC/CART neurons to produce more α-MSH. This may activate MC4Rs outside of the PVN which increases BAT thermogenesis induced EE. Alternatively, perhaps simvastatin increases activation of RIP-cre neurons in the ARC which releases GABA to affect some undetermined brain region, causing an increase in BAT thermogenesis induced EE. Without additional research, it is difficult to narrow down which central mechanism is causing the increase in FI simvastatin causes. However, these two suggested mechanisms are only possible explanations. There are many other systems within the brain which may be having an influence on FI and EE.

Surprisingly, when animals were deprived of water the simvastatin group drank 15% more than controls (experiment four). Water balance is controlled by three systems; arterial baroreflex system, renin-angiotensin pathway and osmoreceptors.

Baroreceptors are sensitive to changes in pressure and are found throughout the cardiovascular system. They are present in the arteries which can detect both high and low blood pressure (Stauss, 2002; Terry N. Thrasher, 1994). When blood pressure is normal, baroreceptors send excitatory signals to the brain which inhibits the release of vasopressin, and during low blood pressure the inhibitory signal causes an increase in vasopressin release (Stauss, 2002; Terry N. Thrasher, 1994). Baroreceptors are also located in the carotid sinus and aortic arch. These baroreceptors are suggested to have a key role in the regulation of blood volume (Gabrielsen et al., 2000). When blood pressure drops, carotid and aortic baroreceptors signal to the brain to release vasopressin and in order to increase water reabsorption. Carotid baroreceptors signal communicate with the brain through the glossopharyngeal nerve, while aortic baroreceptors communicate through the vagus nerve (Wehrwein & Joyner, 2013). Both of these nerves end in the NTS where signals can be carried through to the hypothalamus (Wehrwein & Joyner, 2013).

The renin-angiotensin-aldosterone system (RAAS) is another way osmolarity and blood volume is regulated. A decrease in blood salt levels or blood pressure can be detected by receptors on juxtaglomerular cells in the kidney (Castrop et al., 2010). When these receptors are activated, they cause the release of a secondary messenger, cAMP, to stimulate the production of pre-pro-renin which is cleaved to form pro-renin. Pro-renin can be secreted into the bloodstream through the

constitutive pathway and is converted into active renin in the blood stream. However, when salt concentrations are significantly low, both pro-renin and renin will be secreted from the cell through the regulated pathway into the blood stream (Castrop et al., 2010). Once active, renin within the blood stream can cleave hepatically produced angiotensinogen to form angiotensin 1 (Lu, Cassis, Kooi, & Daugherty, 2016). This is the rate limiting step of the renin-angiotensin system (Lu et al., 2016). β1-adrenergic receptors within cardiac tissue are also able to cause the release of renin from juxtaglomerular cells through the activation of the fight or flight response. Angiotensin-converting enzyme in the blood is then able to cleave angiotensin I into angiotensin II. Angiotensin II is involved in several functions such as vasoconstriction, aldosterone release and vasopressin release, in order to increase blood pressure and normalise osmolarity. This is triggered by the binding of angiotensin II to AT1 receptors (Lu et al., 2016). Aldosterone secretion from adrenal glands is able to increase sodium retention from the distal tubule and collecting duct of the nephron through active transport using a Na^+/K^+ pump (Horton, 1973). This also passively transports water from the distal tubule and collecting duct (Horton, 1973). The binding of aldosterone creates a negative feedback loop inhibiting the secretion of renin (Lu et al., 2016). The release of vasopressin from the pituitary also initiates thirst (Lu et al., 2016). Vasopressin release causes vasoconstriction through binding to the V1 receptor (Holmes, Landry, & Granton, 2003). The binding of vasopressin to V2 receptors in the kidney increases the expression of aquaporin two on the apical membrane of the collecting duct. This increases the flow of water into the blood stream through permanent aquaporins three and four (Holmes et al., 2003). However, when blood

volume is normal, baroreceptors are able to inhibit renin and vasopressin (T. N. Thrasher, 1994).

Osmoreceptors in the hypothalamus react to changes in osmolarity in CSF which mirrors the osmolarity of the blood (Salata, Jarrett, Verbalis, & Robinson, 1988). When CSF becomes less dilute it sends signals through the magnocellular neurons from either the SOP or PVN, which then causes the release of vasopressin from the posterior pituitary. Vasopressin release also increases ACTH release from anterior pituitary to stimulate the HPA axis. The downstream release of cortisol, like aldosterone, also causes the conservation of sodium (Salata et al., 1988).

These three mechanisms maintain water balance by restoring osmolarity, however the mechanisms behind the sensation of thirst are largely unknown (Verbalis, 2007). Neural circuits from the magnocellular neurons project into the forebrain. Therefore, secretion of vasopressin may also trigger a parallel pathway to initiate thirst (Verbalis, 2007).

Because baroreceptors and the renin-angiotensin system are controlled in the periphery, it is unlikely simvastatin is influencing these mechanisms because it was delivered centrally. The interaction of simvastatin with osmoreceptors causing their activation or dysfunction, which subsequently releases vasopressin is a possible reason for this increase in water intake. This is further supported by the c-Fos results which showed increased activation in the PVN, which could potentially be caused by the release of vasopressin. Simvastatin could also be inducing the increase in water intake due to an increase FI altering the osmolarity of the blood, causing the release of vasopressin to correct this.

The finding that simvastatin is able to effect brain regions related to water balance supports the hypothesis that simvastatin has an effect on eating for energy due to a change in EE. Both the ARC and PVN are involved in food and water intake regulation, which supports the notion that simvastatin is effecting central mechanisms within the ARC and PVN, causing an increase in food and water intake, potentially due to a change in EE.

In conclusion, central infusion of simvastatin causes an increase in food and water consumption due to a change in FI or EE regulation. The influence of simvastatin on food and water consumption is likely occurring within the ARC and/or PVN of the hypothalamus. Although the exact mechanisms of how this occurs are unknown, it could be an interaction with either FI pathways in the ARC, such as the melanocortin pathway or RIP-cre neuronal pathway. A change in EE is supported by the unexpected increase in water intake, which central simvastatin elicited. Although the exact mechanisms of how this occurs is also unknown, it could be an interaction of simvastatin with osmoreceptor regulation in the PVN.

6.12 Possible clinical implications and perspectives

Simvastatin targets central pathways involved in FI and/or EE to create an increase in food and water consumption. This information gives some potential clinical implications that should be addressed. The fact that simvastatin is having a central effect causing these changes suggests that simvastatin crossing the BBB is inducing adverse effects counterproductive for the reduction of LDL-C. With this information, it may be essential to modify lipophilic statins so they cannot cross the BBB. This could either be done by modifying the molecular structure of the statin to make it more hydrophilic, or by administering a separate drug which can prevent simvastatin from crossing over the BBB.

Perhaps the use of stains needs to be reviewed. It has been almost 50 years since statins were first introduced as a drug to prevent CVD, and although their efficiency at doing this is satisfactory, with more research and information available on the progression of atherosclerosis and CVD susceptibility it may be time to revise our understanding of a variety of effects induced by these drugs. More information is now available on the functional mechanisms of how statins decrease LDL-C levels, which can be used for their improvement. However, new components involved in cholesterol production such as CETP, LDL receptor and ACAT activity are being uncovered that could be explored as potential targets for therapy as well as HMGR.

Although statins do have some adverse effects, they do decrease LDL-C levels to a point where patients' CVD risk is drastically reduced, increasing their quality of life as well as prolonging it. Statins are shown to be safe for long-term use, and there are several drugs of this class to choose from to fit an individual patient's responsiveness to the treatment. It is imperative to understand that although statins

come with some risk of adverse effects, the benefits of statin use greatly outweigh their side effects. They are an essential drug for our current obesogenic environment.

Certainly, double staining immunohistochemistry for the different hypothalamic neurons of interest (NYP/AgRP, POMC/CART, RIP-cre) to see whether there is increased activation in these neurons should be done in the future. This would shed more light on the central mechanisms causing the increase in food and water consumption. Also, including an analysis of gene expression in the hypothalamus to give a clearer idea of the central influence simvastatin would be helpful.

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