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Notice 1

Notice 2

MONASH University

Thesis accepted in satisfaction of the requirements
for the degree of Doctor of Philosophy on
8 July 2014

[redacted]

Graduate Research Committee

Page	Para	Line	ERRATA	Page	Para	Line	ERRATA
iv	3	2	'a single exercise bout' for 'a single exercise bouts'	28	2	1	'potent, selective' for 'potent selective'
19	2	5	'trials' for 'trails'				
20	1	4	'obesity' for 'obese'	30	2	5	'hormone reduces' for 'hormone exhibits reduced'
21	1	8	'lesion' for 'lessoning'	31	1	1	'projects α-MSH fibers' for 'projects the α-MSH fibre'
21	2	7	'these' for 'this'	31	2	6	delete 'appear to'
21	2	9	'nuclei' for 'nuclie'	31	3	1	'leptin-expressing' for 'leptin expressing'
21	2	11	'DMH' for 'DMN'	32	1	1	'to' for 'onto'
22	1	3	'eminence. The ARC' for 'eminence ARC'	32	2	3	'results' for 'resulting'
22	1	5	'the ARC is a site which can' for 'the ARC is a unique site, which can'	32	2	5	'results in protection' for 'results is the protection'
24	1	2	'hypothalamic regions' for 'hypothalamus'	32	2	9	'physiologically' for 'physiological'
25	1	4	'to' for 'with'	32	3	4	'in many brain' for 'in the brain'
25	1	6	'to the melanocortin' for 'to melanocortin'	33	1	1	'mice are lean and hypophagic, with increased energy expenditure, reduced' for 'mice lean, hypophagic with increased energy expenditure with'
25	1	9	'including' for 'to'	33	1	3	'diet-induced' for 'diet induced'
25	2	1	'peptide-containing' for 'peptide containing'	33	2	6	delete repeated comma after reference
25	2	3	'POMC, or the down-stream melanocortin receptors,' for 'POMC or the down stream melanocortin receptors'	33	2	10	reduced for reducing
25	3	1	'ARC' for 'arcuate'	33	2	12	'orexin in mice' for 'orexin mice'
25	3	2	'ARC' for 'arcuate'	33	3	5	'this sensing.' for 'this.'
26	1	5	're-feeding' for 'refeeding'	33	3	6	delete reference 319
26	2	4	'the actions of NPY in' for 'their actions in'	34	3	title	'affecting' for 'effecting'
26	2	6	'They are' for 'It is'	37	1	11	'obesity-related' for 'obesity related'
26	3	2	'sufficient' for 'suffiecient'	38	2	3	'The' for 'Whilst the'
26	3	3	down-regulation for down regulation	38	2	8	'whereas over-expression' for 'whereas expression'
26	3	4	'regulation, using antisense cRNA, in' for 'regulation using antisense cRNA in'	39	2	4	'leptin and causes' for 'leptin, causes'
26	3	5	'decreased' for 'decreases'	39	3	4	'effect on body weight' for 'effect of weight'
27	1	4	'of Ostuka Long Evans Tokushima Fatty (OLEFT) rats' for 'of OLEFT rats'	42	1	2	'AgRP' for 'AgPR'
28	1	1	'suppresses' for 'suppress'	42	1	3	'shows' for 'showed'

Page	Para	Line	ERRATA
42	1	8	'observed with complete' for 'observed complete'
43	2	6	'enhanced' for 'enhance'
45	1	4	'mechanisms' for 'mechanism'
45	2	4	'classes' for 'classes'
46	1	3	'POMC neurons has' for 'POMC has'
46	1	4	delete the sentence starting with 'However, glucose sensing...'
47	2	2	'On the other' for 'One the other'
47	3	1	'acids' for 'acid'
47	3	4	'to the brain' for 'to brain'
48	2	6	'LC-CoA' for 'LCFA-AC'
50	3	4	'today's' for 'todays'
51	1	3	'culminating' for 'cumulating'
51	3	1	'studied' for 'studies'
51	3	9	'with, or even caused by, a' for 'with or even caused by a'
52	1	3	'are' for 'is'
52	3	1	'tissues' for 'tissue'
52	3	5	'signaling in both' for 'signaling both'
52	3	7	'action against' for 'action again'
53	1	1	delete 'develops'
53	1	2	'resistant' for 'resistance'
53	1	4	delete 'not only'
53	1	7	'responses' for 'signalling'
56	2	1	'It is' for 'it is'
56	3	3	'tricarboxylic acid cycle' for 'tricarboxulic cycle'
59	1	2	delete 'gluconeogenesis'
60	1	5	'by an inflammatory' for 'by and inflammatory'
60	2	8	'they are used' for 'they used'
61	2	2	'disrupts' for 'disrupting'
62	1	3	'long-chain' for 'long-chai'
62	1	5	delete *'fa/fa'*
64	1	1	delete 'the ability of'
66	1	3	'affects' for 'effects'
68	2	9	'increased' for 'higher'
69	1	1	delete 'discussed'
69	1	6	'training in humans.' for 'training.'
71	2	3	'ratio' for 'ration'
73	1	3	'neurotrophin, acts' for 'neutrotrophin acts'
73	2	8	'affect' for 'effect'
74	2	4	'against' for 'again'
75	1	7	explosion of research for explosion research
75	1	8	'contribute to, and control, adult' for 'contribute and control adult'
80	1	4	'decreased' for 'decreases'
81	3	2	'cAMP response element-binding protein (CREB)' for 'cyclic Amp-CREB'
82	1	1	'influenced' for 'influence'
83	2	2	'(263)' for '(ref)'
84	1	3	'(815)' for '(ref)'
85	1	2	delete '(ref)'
85	2	4	'sustained reductions on' for 'reduced and sustained'
85	2	4	'humans' for 'humans''
101	2	7	'form' for 'from'
101	3	1	'TAG' for 'triglycerides'
101	3	2	'TAG' for 'triglycerides'
103	2	2	'10 s' for '10sec'
104	3	3	delete 'They'
105	1	17	'corrected' for 'corrects'
107	1	5	'then' for 'thwn'
108	1	3	'HFD exercise (Ex) mice' for 'HFD Ex mice'
138	2	5	'mice were simply' for 'mice simply'
142	1	3	'frozen and sectioned' for 'and sectioned'
142	2	6	'Sections' for 'Section'
143	3	3	'minipumps' for 'minipump'
149	1	4	'upregulated' for 'unregulated'
168	1	4	insert '(877)' after 'memory'
169	2	10	delete repeated '.'
171	2	1	'aimed' for 'sort'
179	2	8	add 'to increase protein stability' before reference
199	1	2	delete sentence 'For ghrelin...'
242	ref 117		add '521(10):2208-34'

Page	Para	Line	ADDENDUM
xi			Add OLETF- Ostuka Long Evans Tokushima Fatty
19	3	3	Add: high 'circulating levels of' low density lipoproteins
28	2	3	delete ' increase nocturnal food intake' and read 'increases food intake at the beginning of the dark phase'
32	2	2	'delete which is' and read 'a characteristic'
33	2	4	after 'in' read 'many regions including'
34	3	3	after reference insert (Table 1.1)
35	2	12	at the end of the sentence add '(discussed in section 1.5)'
38	2	14	after 'db/db' add '(global Ob-R knockout)'
175	After paragraph 1		Insert this paragraph: While the assessment of BrdU positive cells does not indicate neurogenesis but cell division, the results of the PCR array suggest that newly generated cells after exercise will adopt a neuronal phenotype. Furthermore, CNTF treatment induces neurogenesis, with cells responding to leptin and expressing both POMC and NPY neuropeptides (although this was not quantified) (387). It is well established that exercise as a stimulus can induce neurogenesis in the hippocampus that is functionally relevant for learning and memory (814, 815, 817). These previous studies highlight that exercise is a potent stimulator of neurogenesis in the hippocampus, and that chemical stimulation of proliferation in the hypothalamus leads to neurogenesis, therefore it is not a stretch to assume that the majority of BrdU cells in the current study will adopt a neuronal fate, although this is not yet tested. Whether or not they become cells relevant to food intake and body weight regulation is another question. Results of the current study, where blocking cell division during exercise training had no effect on food intake or body weight, suggest that they do not become orexigenic or anorexigenic. This will also need to be assessed in future studies.
174	After paragraph 1		Insert this paragraph: Of note, during four weeks of exercise training neurogenesis was blocked in the entire brain due to the infusion of AraC into the ventricular system. Therefore, the results obtained in the study cannot be attributed solely to hypothalamic neurogenesis, but to CNS neurogenesis in general. It is also assumed that neurogenesis will be blocked in the entire brain, not just the hypothalamus. In addition, one month of voluntary wheel running increases learning and hippocampal neurogenesis (814, 815). It would be interesting to also assess learning and memory in these mice, although this was not the main focus of the study.
170	After paragraph 2		Insert this paragraph: In the current study, neurogenic genes and BrdU incorporation were assessed in the whole hypothalamus. However, it is clear that different regions of the hypothalamus respond differently to metabolic perturbations and have function outside of the control of food intake and body weight. For example, during high-fat feeding cells of the ARC become leptin-resistant, while others (such as the DMH) remain leptin sensitive (549). ARC cells also become ghrelin resistant during high-fat feeding while the PVN remains responsive (87). These data highlight the dynamic nature/role of different hypothalamic nuclei in the control of food intake and body weight. In addition, hypothalamic nuclei such as the medial preoptic area is important for thermoregulatory control during exercise (Hasegawa et al. 2005, PMID:15618320) whereas the supraoptic nucleus responds to exercise training in controlling plasma osmolality (Nunez et al. 2012, PMID: 22554903). In the current study we did not observe preferential neurogenesis in any one nucleus of the ARC, DMH, VMH, PVN and LH (data not shown), although non-food intake/body weight nuclei were not examined.

EXERCISE-INDUCED HYPOTHLAMIC STRUCTURE & FUNCTION: IMPLICATIONS FOR ENERGY BALANCE & OBESITY

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Presents a thesis submitted in fulfilment of the requirements for the award of the degree

Doctorate of Philosophy

2013

Table of Contents

Abstract

Obesity has reached epidemic proportions worldwide, and for the first time in human history more people in the world are overweight than undernourished. Understanding the route of metabolic dysfunction during obesity remains a major medical challenge. The hypothalamus is integral to the control of food intake and becomes dysfunctional during obesity. This is a key event in the pathogenesis of obesity. This thesis sort to identify possible routes of hypothalamic dysfunction during obesity, and to investigate the potential benefits of regular exercise training on hypothalamic function.

This thesis found that lipid accumulation occurs in the hypothalamus of mice fed a high fat diet. Specifically, diacylglycerol and ceramide content are increased, and these lipids are known to interfere with insulin signalling in peripheral tissues such as skeletal muscle and liver. Six weeks of exercise training was unable to decrease lipids in the hypothalamus, despite improving body weight and whole body glucose metabolism.

Neurogenesis in the hypothalamus has been proposed to play a role in the regulation of food intake and body weight. This thesis showed that a single exercise bouts upregulates genes in the hypothalamus that promote neurogenesis and stem cell activation; while just 7 days of exercise training was able to stimulate neurogenesis in the hypothalamus of both lean and obese mice. By using the mitotic blocker, cytosine-β-D-arabinofuranoside, to block neurogenesis it was found that neurogenesis during four weeks of exercise may play a role in insulin-stimulated metabolism in the adipose tissue of high fat fed mice. Furthermore, when hypothalamic neurogenesis was stimulated with the cytokine CNTF, which results in a 17-fold greater amount of neurogenesis than exercised mice, there were no sustained effects on body weight or peripheral insulin action. Therefore, while exercise training stimulates hypothalamic neurogenesis, its role in body weight and energy balance is yet to be fully elucidated.

Finally, this thesis examined the effect of six weeks of exercise training on hypothalamic sensitivity to peripheral hormones, which is impaired during obesity. Contrary to previously published work showing increased hypothalamic sensitivity to leptin directly after exercise in both lean and obese mice; this thesis showed that exercise training does not confer long term changes to hypothalamic sensitivity to leptin or ghrelin.

Collectively, this thesis highlights that while exercise training during obesity is able to alter the structure of the hypothalamus through the induction of neurogenesis, functional outcomes remain largely unaltered.

General Declaration

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research master's regulations the following declarations are made:

I hereby declare that this thesis contains no material that has previously been accepted for the award of any other degree or diploma at any university or equivalent institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due acknowledgement is made in the text of the thesis.

This thesis includes 1 original paper published in a peer review journal and 2 original paper which have been submitted to journals and are currently under review, and 1 unpublished manuscript. The core theme of this thesis is obesity and exercise. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Physiology, under the supervision of Prof. Matthew Watt. The inclusion of co-authors reflects the fact that the work came from collaborations between researchers and acknowledges input into team-based research.

My contribution to the work involved the following:

Thesis chapter	**Publication title**	**Publication status**	**Nature and extent of candidates contribution**
3	Consumption of a high-fat diet, but not regular endurance exercise training, regulates hypothalamic lipid accumulation in mice	Published	Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation (85%)
4	Hypothalamic neurogenesis does not play a role in the improved insulin action associated with exercise training	Submitted: In review	Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation (85%)
5	Cessation of cilliary neurotrophic factor treatment causes rebound weight gain and impaired insulin action	Manuscript in preparation	Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation (85%)
6	Exercise training does not produce long-term changes in hypothalamic sensitivity to leptin or ghrelin in mice	Submitted: In review	Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation (85%)
Candidate's Signature			**Date**
[redacted]			**16.12.2013**

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It took the support and assistance of many people for me to complete the studies presented in this thesis. These are the people I would like to thank.

Foremost, I would like to express my sincere gratitude to my primary supervisor; Prof. Matthew Watt for the continuous support of my PhD. Our journey together into the world of metabolic neuroscience was definitely a very interesting one. Thank-you for your constant guidance and my development into a young scientist. Your ability to be enthusiastic about my projects invaluable.

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To all my family and friends, thank you for reminding that there was a life outside of the lab. Thank you to my family, for putting up with all my late nights at work and the many dinners left cold in the microwave. Without you this would not have been possible.

Publications

Publications and presentations that have arisen as a direct result of this thesis

Publications:

M. L. Borg, S. Omram, J. Weir, P. J. Meikle, M. J. Watt (2012) Consumption of a high-fat diet, but not obesity or regular exercise training, regulates hypothalamic lipid accumulation in mice. *Journal of Physiology*, **590**, 4377-4389

Highlighted in the perspective:
B.S. Muhlhausler (2012) Fat on the brain. *Journal of Physiology*. **590**, 4121

M. L. Borg, Z. B. Andrews, M. J. Watt (2013) Exercise training does not produce long-term changes in hypothalamic sensitivity to leptin or ghrelin in mice. *In review, Journal of Neuroendocrinology*

M. L. Borg, A. Selathurai, B. Oldfield, Z. B. Andrews, M. J Watt (2013) Hypothalamic neurogenesis does not play a role in the improved insulin action associated with exercise training. *In review, Diabetes*

Abstracts:

M. L. Borg, S. Omran, P. J. Meikle, M. J. Watt (2011) Hypothalamic lipid accumulation with nutrient oversupply and exercise training in mice. *The 1st Frontiers in Obesity and Diabetes Research (MODI)*. Oral presentation

M. L. Borg, V. R. Haynes, S. Omran, P. J. Meikle, M. J. Watt. (2011) Evidence of metabolic inflexibility in cultured hypothalamic neurons and hypothalamic lipid accumulation with nutrient oversupply in mice. *Australian Diabetes Society Annual Scientific Meeting* 210. Perth. Poster

M. L. Borg, S. Omran, P. J. Meikle, M. J. Watt (2011) High fat feeding, but not obesity or exercise training, regulates hypothalamic lipid accumulation in mice. *Exercise, Muscle and Metabolism*. Melbourne. Poster

M. L. Borg, Z. B. Andrews, M. J. Watt (2012) Exercise-induced hypothalamic neurogenesis: implications for energy balance and obesity. *Genetic and Molecular Basis of Obesity and Body Weight Regulation* 114. Keystone Symposia, Santa Fe, New Mexico, USA. Poster

Publications and presentations that have arisen in conjunction with this thesis

Publications:

M. L. Borg, Z. B. Andrews, E. J. Duh, R. Zechner, P. Meikle, M. J. Watt. (2011) Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. *Diabetes*, doi:10.2337/db10-0845

Abstracts:

M. L. Borg, C. G. Stathis, Hayes A. (2008) The effect of continuous *vs* intermittent exercise on substrate utilization during exercise and recovery in healthy adults. *Proceedings of the Australian Physiological Society* 39:43P. Oral presentation

M. L. Borg, M. Matzaris, R. Zechner. P. Meikle, M. J. Watt. (2010) Pigment epithelium-derived factor regulates lipid metabolism via adipose triglyceride lipase. *Australian Diabetes Society Annual Scientific Meeting* 420. Sydney. Poster

V. R. Haynes, M. L. Borg, M. J. Watt (2011) Fatty acid metabolism can be modified in immortalized hypothalamic neurons. *The 1st Frontiers in Obesity and Diabetes Research (MODI)*. Poster

R. R. Mason, M. L. Borg, S. Omran, M. J. Watt (2011) Perilipin 2-5 protein contents are not modulated by high fat diet or endurance exercise in skeletal muscle. *The 1st Frontiers in Obesity and Diabetes Research (MODI)*. Poster

A. J. Hoy, A. Barnett, M. L. Borg, M. J. Watt. (2011) Hepatic serine palmitoyltransferase activity is unaltered in both acute and chronic models of increased lipid availability despite elevated circulating ceramide. *Australian Diabetes Society Annual Scientific Meeting* 99. Perth. Oral presentation

Abbreviations

α-MSH- α-melanocyte-stimulating hormone

2-DG- 2-deoxyglucose

5-HT4- serotonin

ADP- adenosine diphosphate

AGPAT- 1-acylglycerol-3-phosphate O-acyltranferase

AgRP- agouti-related peptide

AMPK- 5' adenosine monophosphate-activated protein kinase

AraC- cytosine-β-D-arabinofuranoside

ARC- arcuate nucleus

ASC- acyl-CoA synthetase

ATGL- adipose triacylglycerol lipase

ATP- adenosine triphosphate

BBB- blood brain barrier

BDNF- brain-derived neurotrophic factor

BMI- body mass index

BMP- bone morphogenetic protein

BrdU- bromodeoxyuridine

Ca^{2+}- calcium

CaMKII- Calcium/calmodulin-dependent protein kinase II

CPT carnitine palmitoyltransferase

CART- cocaine- and amphetamine-related transcript

CCK- cholecystokinin

CNS- central nervous system

CRH- corticotropin-releasing hormone

CSF- cerebrospinal fluid

CVD- cardiovascular disease

DAG- diacylglycerol

DGAT- diacylglycerol acyltransferase

DMN- dorsomedial hypothalamic nucleus

EGF- epidermal growth factor

ER- endoplasmic reticulum

ERK- extracellular-signal regulated kinase

ETC- electron transport chain

FABP- fatty acid binding protein

FAS- fatty acid synthase

FA-CoA- fatty acyl-coenzyme A

FATP- fatty acid transport protein

FFA- free fatty acid

FGF2- fibroblast growth factor 2

GABA- gamma-aminobutyric acid

GE- glucose excited

GH- growth hormone

GHSR- growth hormone secretagogue receptor

GI- glucose inhibited

GI- gastrointestinal

GK- glucokinase

GLP-1- glucagon like peptide-1

GLUT- glucose transporter type

GPAT- glycerol phosphate acyltransferase

HDL- high-density lipoproteins

HSL- hormone-sensitive lipase

ICV- intracerebro ventricular

IGF-1- insulin-like growth factor 1

IL- interleukin

IMTG- intramuscular triacylglyerides

IRS- insulin receptor substrate

JAK- janus kinase

JNK- c-Jun NH_2-terminal kinase

K- Potassium

K_{ATP}- ATP-sensitive K^+

LC-CoA- long-chain fatty acyl-CoAs

LDL- low density lipoproteins

LHA- lateral hypothalamic area

LPA- lysophosphatidic acid

LPL- lipoprotein lipase

LTP- long-term potentiation

MAG- monoacylglycerol

MAP- mitogen-activated protein

MAPK- mitogen-activated protein kinase

MCH- melanin-concentrating hormone

MCR- melanocortin receptor

MET- metabolic equivalent of task

MGL- monoacylglycerol lipase

MPOA- medial preoptic area

Na- Sodium

NAFLD- non-alcoholic fatty liver disease

NASH- non-alcoholic steatohepatitis

NFκB- nuclear factor-κB

NT-3- neurotrophin-3

NPY- neuropeptide Y

OXR- orexin receptor

PA- phosphatidic acid

PDX-1- pancreatic/duodenal honeobox-1

PEDF- pigment epithelium-derived factor

PFA- paraformaldyhyde

PGC-1α- peroxisome proliferator-activated receptor-γ coactivator-1α

PI- phosphatidylinositol

PI3K- phosphatidylinositol 3-kinase

PKC- protein kinase C

POMC- pro-opiomelanocortin

ppMCH- prepro-MCH

PVN- paraventricular nucleus

PYY- peptide YY

S6K- S6 kinase

SGZ- subgranular zone

SOCS-3- suppressor of cytokine signaling-3

STAT-3- signal transducer and activator of transcription 3

SVZ- subventricular zone

TAG- triacylglycerol

TCA- tricarboxylic cycle

TLR4- toll-like receptor 4

TRH- thyrotropin-releasing hormone

VEGF- vascular endothelial growth factor

VLDL- very low-density lipoproteins

VMH- ventromedial hypothalamic nucleus

WHO- World Health Organization

ZDF- zucker diabetic fatty

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Chapter One: Literature Review

1.1. The obesity epidemic

Obesity is defined as an abnormal or excessive accumulation of body fat that may impair health. The body mass index (BMI) is a World Health Organization (WHO) accepted index for classifying the degree of obesity and was adopted by an expert committee of the NHLBI (NHLBI Guidelines, 1998). BMI = (weight (kg))/(height (m^2)). Under this convention for adults, grade 1. overweight (commonly and simply called overweight) is a BMI of 25-29.9 kg/m^2. Grade 2. overweight (commonly called obesity) is a BMI of 30-39.9 kg/m^2. Grade 3. overweight (commonly called severe or morbid obesity) is a BMI greater than or equal to 40 kg/m^2 (848).

Obesity is considered pandemic, with over 1.4 billion adults overweight and more than half a billion obese world wide. At least 2.8 million people each year die as a result of being overweight or obese. Globally over 40 million preschool children were overweight in 2008. In Australia, 61% of the population is overweight or obese.

1.1.1. Energy balance, calorie intake and physical activity

By the law of conservation of energy, body fat increases when energy intake/calories consumed is consistently greater than energy expenditure/physical activity. Excess body fat and obesity are the result of sustained positive energy balance. Increasing the amount of calories consumed can lead to obesity (4, 380, 688). Whereas, restricting caloric intake can reduce body weight and improve insulin sensitivity (261, 364, 421).

Classically, there are three major components of daily energy expenditure. These include basal metabolic rate, the thermic effect of food, and activity thermogenesis. Basal

metabolic rate is the energy required for core bodily functions and accounts for approximately 60% of daily energy expenditure in a sedentary person. The thermic effect of food is the energy expended in the digestion, absorption and fuel storage in response to a meal, and accounts for 10% of daily energy expenditure. Activity thermogenesis is composed of all types of physical activity, including sports based and gym exercise, and all forms of movement (walking, shopping, dancing, cleaning, gardening etc.) and accounts for approximately 40% of daily energy expenditure. Physical activity is the easiest component of energy expenditure to manipulate.

Physical activity is a metabolic stress that acutely increases the demand for energy production. It is associated with a number of health benefits including diminished diabetes, heart disease and some cancers; it is also associated with an increased life span. A decrease in daily physical activity has contributed to the increased prevalence of obesity worldwide (74, 272, 520, 853). Short-term human exercise trails are positively associated with a reduction in total body fat mass in a dose dependent manner (653), whilst endurance exercise decreases total body fat and improves glucose tolerance (52, 375). This suggests that the effects of obesity and glucose intolerance/insulin resistance in skeletal muscle can be improved with physical exercise.

1.1.2. Obesity and it's complications

The medical consequences of obesity range from a higher risk of premature death to severe non-lethal diseases or comorbidities. Recently, 38 different medical conditions were identified as being caused by overweight, including type 2 diabetes, high low density lipoproteins (LDL), hypertension, low immunity, congestive heart failure, endothelial dysfunction, Alzheimer's disease, dementia, depression, stroke, erectile dysfunction,

osteoporosis, obstructive sleep apnoea, peripheral artery disease, reduced skin wound healing and cancers of the breast, endometrium, prostate and bowel (81, 559). Globally, 44% of diabetes, 23% of ischemic heart disease, and 7-41% of certain cancers are attributable to overweight and obese (848).

The impact of obesity is not solely upon an individual's health. It also has serious implications for the health sector in term of cost and the burden on services. In 2008 it was estimated that the overall cost of obesity to the Australian society and governments was $58.2 billion, which included a burden of disease cost of $49.9 billion (including the cost of disability, loss of well-being and premature death); and direct financial costs of $8.3 billion (including productivity costs due to short- and long-term employment impacts ($3.6 billion), health system costs ($2 billion) and carer costs ($1.9 billion)) (3). Obesity also effects productivity in the workplace. The average days off work for employees due to their own illness or injury was 3.0 days. Whereas, people who were overweight or obese have an increased average of 3.2 day off from work a year (2). These results highlight the impact of obesity upon society and the healthcare system.

Obesity can be viewed as a disease of not only defective lipid and glucose metabolism, but also defective hypothalamic metabolism, the regulation of these processes will be outlined below.

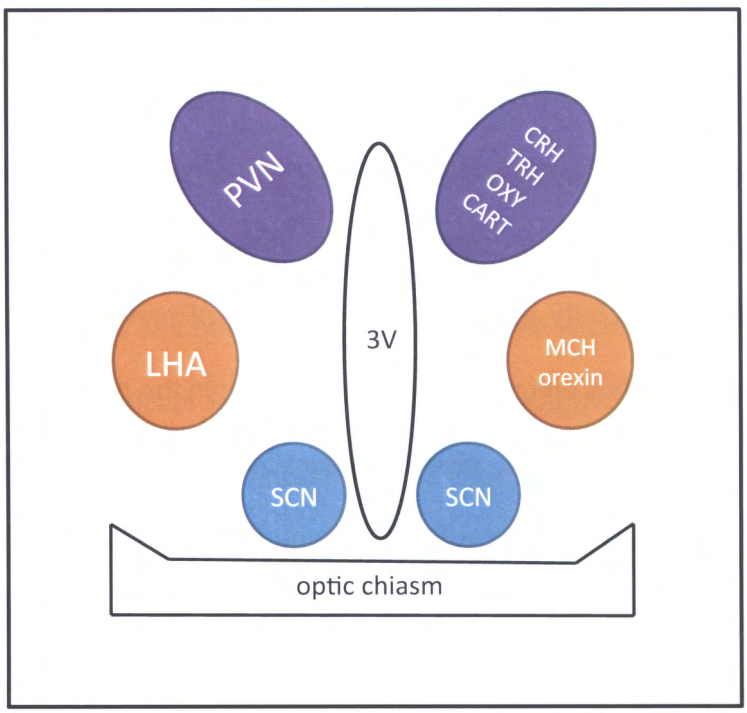
1.2. Role of the hypothalamus in energy balance: nuclei and neuropeptides

The capacity to adjust food intake in response to changing energy requirements is essential for survival. A series of complex systems maintain energy homeostasis in order that sufficient energy is available and body weight remains stable. Central circuits in the brain rely on peripheral signals that indicate satiety levels and energy stores. The hypothalamus controls feeding by integrating peripheral humoral signals that influence food intake and energy expenditure, with neural signals from the brainstem and higher cortical centres. The importance of the hypothalamus in energy homeostasis was first suggested by classical lessoning experiments in rodents (89, 301, 302).

Evidence obtained from both the clinical descriptions in tumour patients, and from lesions work in rats, showed that gross damage to the mediobasal hypothalamic areas, in particular the ventromedial hypothalamic nucleus (VMH), was associated with increased food intake, morbid obesity and insulin resistance, while damage to more lateral hypothalamic structures was associated with anorexia and adipsia (22). Electrical stimulation of the VMH resulted in decreased food intake, whereas stimulation of the lateral hypothalamic region increased appetite (141, 527, 801). As a whole, this data suggested that the mediobasal hypothalamus was a satiety centre, and that the lateral hypothalamus was an orexigenic centre. Subsequent studies have shown a role of hypothalamic nuclie, such as the arcuate nucleus (ARC), paraventricular nucleus (PVN), VMH, dorsomedial hypothalamic nucleus (DMN), and lateral hypothalamic area (LH) in energy homeostasis (Figure 1.1). These will be discussed in the current section.

1.2.1. Arcuate nucleus

The ARC acts as a feeding control centre and integrates hormonal signals for energy homeostasis (236). The ARC encloses the third ventricle and lies immediately above the median eminence, ARC-median eminence area is a 'circumventricular' organ where the blood brain barrier (BBB) is specially modified to allow entry of peripheral peptides and nutrients (88). Therefore, the ARC is a unique site, which can sample the peripheral circulation through semi – permeable capillaries of the median eminence and is ideally positioned to integrate hormonal signals for energy homeostasis. Certain plasma hormones, for example peptide YY (PYY) and glucagon like peptide-1 (GLP-1), cross the BBB via non-saturable mechanism (361, 565), whilst other signals, such as leptin, are actively transported from blood to brain via saturable mechanisms (50). Therefore, the BBB can play a dynamic role in regulating the passage of peripheral signals.


PVN
CRH
TRH
OXY
CART
3V
LHA
MCH
orexin
SCN
SCN
optic chiasm

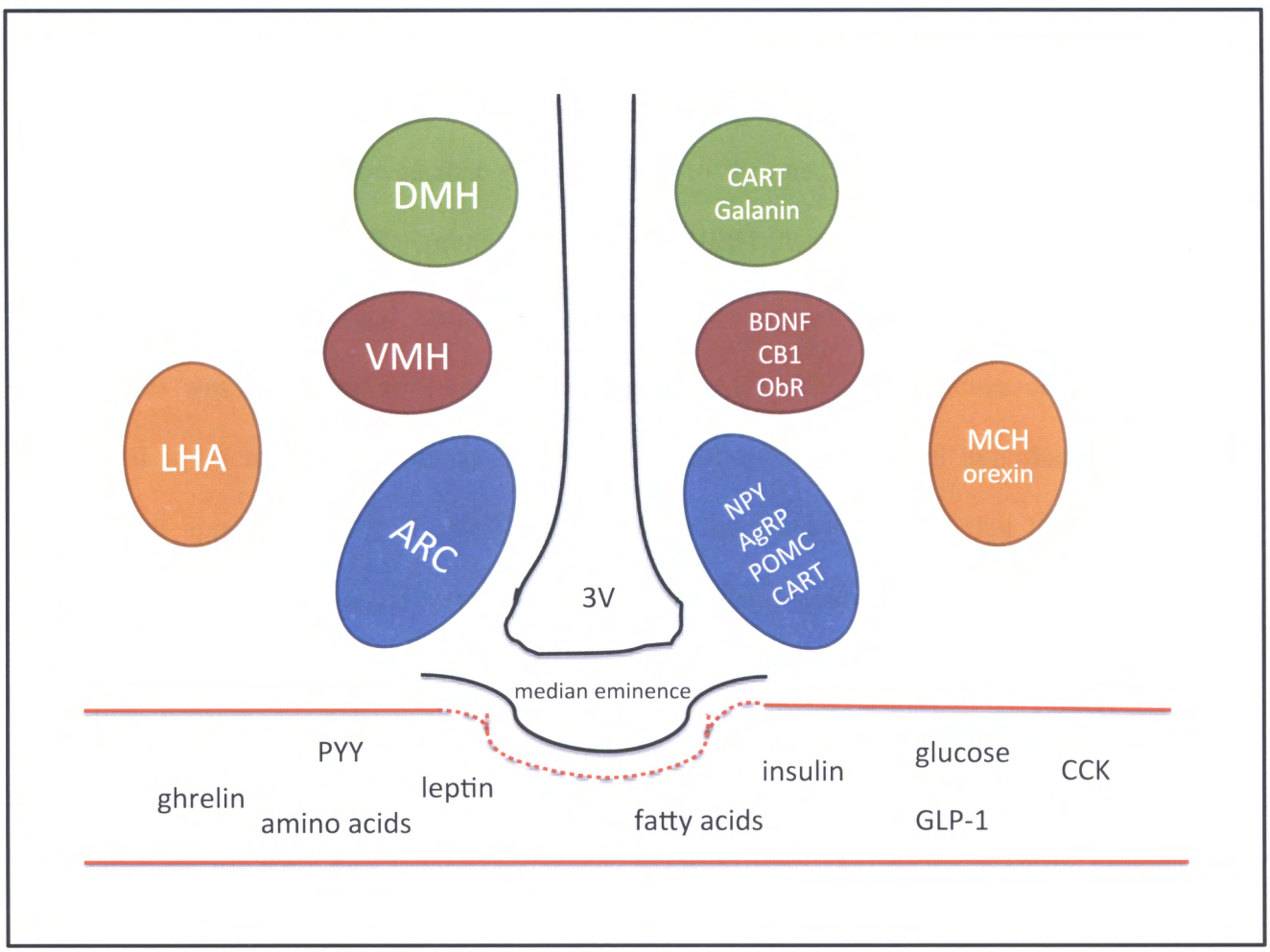

DMH
CART
Galanin
VMH
BDNF
CB1
ObR
LHA
MCH
orexin
ARC
NPY
AgRP
POMC
CART
3V
median eminence
PYY
ghrelin
leptin
amino acids
insulin
fatty acids
glucose
GLP-1
CCK

Figure 1.1: Neuroanatomy of the hypothalamus.

Schematic of the hypothalamus that express neuropeptides involved in energy homeostasis (coronal sections). Adiposity signals (leptin and insulin), satiety signals (ghrelin, PYY, CCK) and nutrients (glucose, amino acids, fatty acids) act on the neurons in the ARC via the blood stream due to the presence of an incomplete blood brain barrier (denoted by the dashed line). ArRP, agouti-related peptide; ARC, arcuate nucleus; BDNF, brain derived neurotrophic factor; CART, cocaine and amphetamine-regulated transcript; CB1 endocanabinoid receptor 1; CCK, cholecystokinin; CRH, corticotropin-releasing hormone' DMH, dorsomedial hypothalamus; GLP-1, glucagon-like peptide-1; LH, lateral hypothalamus; MCH, melanin concentrating hormone; NPY, neuropeptide Y; ObR, leptin receptor; OXY, oxytocin; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; PYY, peptide YY; SCN, suprachiasmic nucleus; TRH, thyrotropin-releasing hormone; VMH, ventromedial hypothalamus; 3V, third ventricle.

There are two major neuronal populations in the ARC implicated in the regulation of feeding (137). One population expresses the anorexigenic neuropeptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP) which increase food intake (91, 277). These neurons project primarily to the PVN (43), but also with the DMH and LH. The second population expresses the orexigenic neuropeptides melanocortin precursor pro-opiomelanocortin (POMC), a precursor to melanocortin agonist α-melanocyte-stimulating hormone (α-MSH), and cocaine- and amphetamine-related transcript (CART), which inhibit food intake (190, 397). These neurons project much more widely within the central nervous system (CNS), to hypothalamic nuclei such as the PVN, DMH, LHA, and the perifornical area (191, 196, 351).

The importance of these peptide containing neurons in the control of energy homeostasis is highlighted by the fact that ablation of AgRP neurons in adult mice leads to starvation and death (270, 476), while mice deficient in POMC or the down stream melanocortin receptors display hyperphagia and obesity (44, 98, 617, 766). Furthermore, direct stimulation of AgRP neurons through cell-type specific photostimulation evokes voracious feeding within minutes (28), through direct inhibition of oxytocin neurons in the PVN (37).

Overall, arcuate neurons act as the primary hypothalamic site of action of peripheral hormones, such as leptin and insulin. These modulate activity of arcuate neurons, which in turn project to secondary hypothalamic nuclei, for example the PVN or LHA. Here, the release of further anorexigenic or orexigenic peptides is modulated to adjust energy intake and expenditure to maintain stable body weight (696).

1.2.1.1. Neuropeptide Y

NPY is a 36 amino acid peptide homologue of the pancreatic peptide family. It is one of the most abundant neuropeptides in the CNS (16), but the ARC is the major hypothalamic site of NPY expression (541). Hypothalamic levels of NPY reflect the body's nutritional status with hypothalamic NPY mRNA and NPY release increasing with fasting and decreasing after refeeding (352, 668, 768). NPY is the most potent orexigenic neuropeptide known, and repeated third ventricle or PVN injection of NPY causes marked hyperphagia and obesity (744, 887). Central injection of NPY also inhibits brown fat thermogenesis (71), suppresses sympathetic nerve activity (184), and inhibits the thyroid axis (213) to reduce energy expenditure. In addition, NPY stimulates basal plasma insulin (531) (887) and morning plasma cortisol (887), effects which are independent of increased food intake.

NPY synthesis and secretion are all upregulated in models of energy deficiency or increased metabolic demand such as starvation, type 1 diabetes mellitus, obesity, lactation and physical exercise (334, 660, 668). Five G-protein coupled NPY recpetors have been identified-Y1, Y2, Y4, Y5, and Y6 that mediate their actions in the hypothalamus. Y5 receptors have been implicated as important receptors that mediate the feeding effects of NPY (485, 598). It is expressed at relatively high levels in the LHA, close to the site where NPY acts most potently to stimulate food intake (852).

Genetic models have also revealed the critical role that NPY plays in body weight regulation. Physiological overexpression of NPY in the ARC during adulthood is suffiecient to increase food intake and severe obesity, whilst a moderate down regulation resulted in a blunted response to food deprivation (739). NPY down regulation using antisense cRNA in the ARC reduced NPY expression and decreases food intake and body weight (246).

Overexpressing NPY in the PVN results in obesity with increased food intake and reduced locomotor activity (784). Furthermore, overexpression of NPY in the DMH increases food intake and body weight, and exacerbates high-fat diet induced obesity, whilst knockdown in the DMH ameliorates the hyperphagia, obesity and diabetes of OLETF rats (872).

1.2.1.2. Melanocortin system

The melanocortin system is comprised of the peptide products of POMC cleavage, their receptors, and the endogenous melanocortin antagonist AgRP. Hypothalamic POMC mRNA expression is regulated by nutritional status with low levels in fasting that are restored by exogenous leptin administration or 6h after refeeding (694, 768). Human POMC gene mutations or abnormal POMC peptide processing results in early-onset obesity and red hair secondary to lack of α-MSH, along with adrenal insufficiency due to loss of adrenocorticotropic hormone (401). Haploinsufficiency of the POMC gene is enough to render mice susceptible to diet-induced obesity (112).

Five melanocortin receptors have been identified, melanocortin receptor (MCR) MC1R-MC5R; however, MC3R and MC4R appear to play important roles in energy homeostasis. They are widely expressed in the hypothalamus and are found in the ARC, VMH, and PVN (290, 545). Absence of MC4R results in hyperphagia and obesity in rodents (208, 329), and abnormalities of this receptor have been implicated in 1-6% of severe early-onset human obesity (210, 470, 471). Although MC4R involvement in feeding is well established, the role for MC3R is less clear.

The main endogenous ligand for the MC3R/MC4R is α-MSH, which is expressed by POMC cells in the lateral portion of the ARC (834). Central administration of MC4R agonists

suppress food intake, while administration of the antagonist to MC4R produces hyperphagia (63). In addition, α-MSH also increases oxygen consumption (616), suggesting increased energy expenditure. α-MSH activates the thyroid axis (371), sympathetic nervous activity, and brown adipose tissue (876).

AgRP is a potent selective antagonist at MC3R and MC4R (577). It is expressed in the CNS, primarily in the medial portion of the ARC (719). Central administration of AgRP is able to block α-MSH-induced anorexia and increase nocturnal food intake (654). Moreover, this hyperphagia has been reported to persist for up to a week after a single injection (275, 654). AgRP and NPY are colocalised in 90% of ARC neurons (91, 277). Activity of ARC NPY/AgRP neurons potently stimulate feeding via a number of pathways: the orexigenic effect of NPY release in the PVN, AgRP antagonism of MC3R/MC4R in the PVN, and the local release of NPY and GABA within the ARC to inhibit POMC neurons via Y_1 and GABA receptors, respectively (239, 651). Furthermore, AgRP neurons target and inhibit oxytocin neurons within the PVN, which is critical for evoked feeding (37).

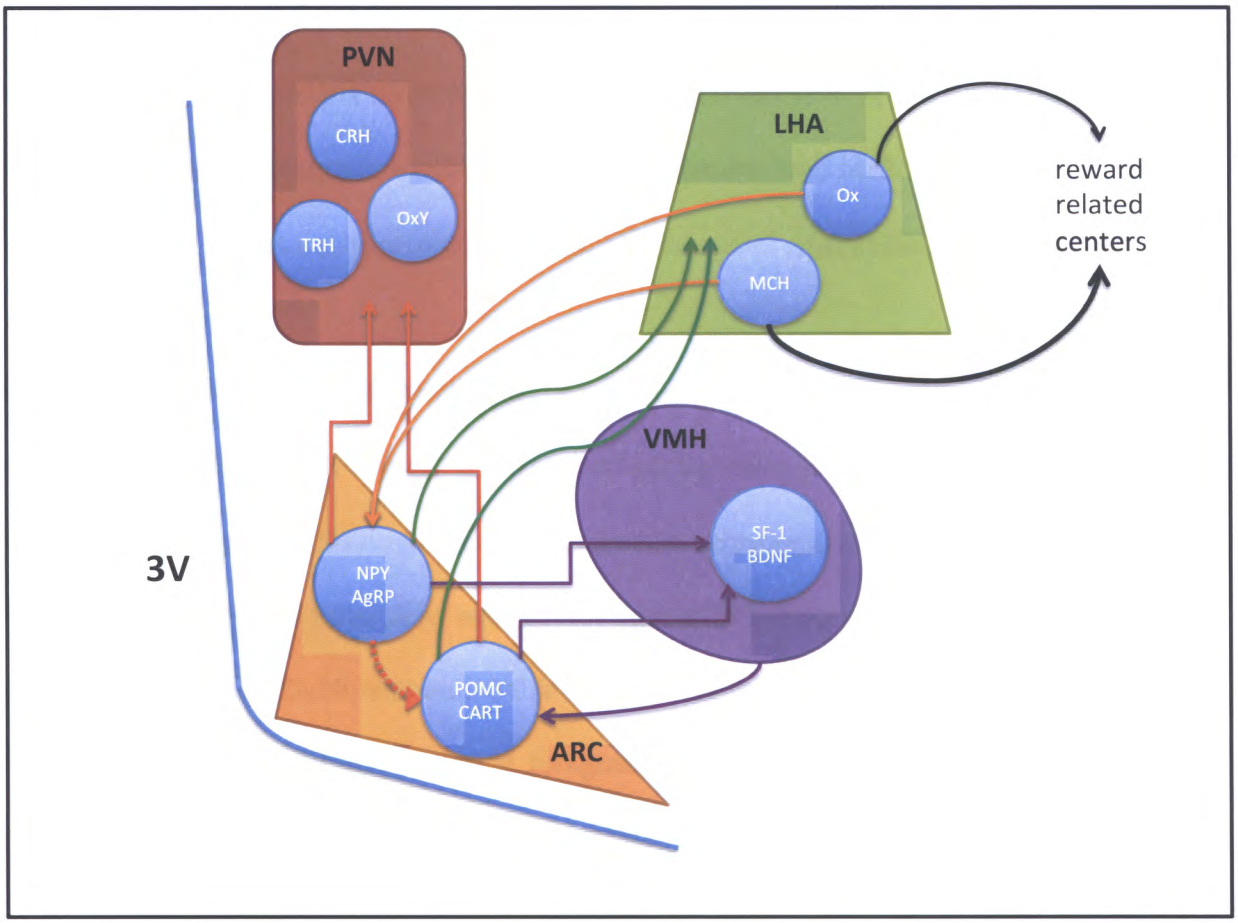


Figure 1.2: Neuroanatomy of energy homeostasis circuits in the hypothalamus

Schematic representation of the principal hypothalamic circuitry controlling food intake and body weight regulation. The ARC contains neurons that co-express NPY and AgRP, that act on downstream in the PVN to stimulate food intake. The ARC also contains neurons expressing POMC and CART, these also signal to the PVN to suppress food intake. Inhibitor inputs from the NPY/AgRP neurons synapse onto POMC neurons to suppress it's anorexigenic effects. NPY/AgRP neurons and POMC/CART neurons also synapse with neurons in the VMH and LHA. 3V-third ventricle, ARC-arcuate nucleus, VMH-ventromedial hypothalamus, PVN-paraventricular nucleus, LHA-lateral hypothalamic area, NPY-neuropeptide Y, AgRP- agouti-related peptide, POMC-pro-opiomelanocortin, , CART-cocaine- and amphetamine-regulated transcript, SF-1-steroidogenic factor 1, BDNF-brain derived neurotrophic factor, CRH-corticotrophin-releasing hormone, TRH-thyrotropin-releasing hormone, Ox-orexin, MCH- melanin concentrating hormone. Arrows indicates direction of synaptic inputs, broken arrow indicates inhibitory synapse.

The fundamental importance of both NPY/AgRP and POMC neurons in the control of food intake and energy balance are highlighted by conditional gene deletion experiments. The conditional deletion of AgRP neurons in adult mice resulted in a rapid reduction in food intake and body weight (270, 476), whereas deletion of POMC neurons in adult mice produced a gradual increase in food intake and bodyweight (476). Interestingly, AgRP-deleted mice without any intervention will starve to the point of death (476), whilst POMC-deleted mice will gradually become obese (270). These results highlight a greater evolutionary selection pressure to maintain NPY/AgRP neuronal function compared with POMC/CART.

1.2.2. Paraventricular nucleus

The PVN receives projections from of NPY and POMC neurons from the ARC (676) and contains neurons which express the anorexigenic factors, thyrotropin-releasing hormone (212, 439) and corticotrophin-releasing hormone (672). These second order neurons transmit the effects of NPY, α-MSH and AgRP, leading to profound changes in feeding and energy expenditure (10, 195). Overexpression of corticotrophin-releasing hormone exhibits reduced neuronal activation in the ARC and food intake in response to fasting (750) whilst thyrotropin-releasing hormone knockout results in glucose intolerance without affecting feeding or body weight. Microinjections of almost all known orexigenic and anorexigenic signals, such as NPY (417), ghrelin (424), orexin-A (183, 714), cholecystokinin (CCK) (281), leptin (193, 808), and GLP-1 (808) into the PVN alter food intake and body weight (745). Destruction of the PVN causes hyperphagia and obesity (440). Therefore, the PVN may have an inhibitory role in food intake and body weight.

1.2.3. Dorsomedial hypothalamus

The DMH receives NPY/AgRP projections from the ARC (865), and projects the α-MSH fibre to the PVN (338). Destruction of the DMH results in hyperphagia and obesity, although less dramatic than VMH lesions (67). Injections of orexigenic peptides, NPY, galanin, and gamma-aminobutyric acid (GABA) into the DMH increases food intake (367, 409, 743), and central NPY injection induces *c-fos* in the DMH (878). The DMH has extensive connections with other medial hypothalamic nuclei and the lateral hypothalamus and integrates and processes of information from these nuclei (192).

NPY expression in the DMH also serves as an important signalling peptide in the regulation of energy balance. Overexpression of NPY in the DMH leads to increased food intake and body weight, while exacerbating high-fat diet-induced obesity (872). Whilst NPY knockdown in the DMH resulted in reduced hyperphagia, obesity and diabetes of fatty rats (116, 872) as well as increasing energy expenditure through enhanced BAT thermogenesis and increase locomotor activity (116). These NPY cells of the DMH appear to have connections with other nuclei within the hypothalamus, including the PVN and LH (436).

Recent studies have also highlighted the importance of leptin expressing neurons in the DMH (890). These cells, in conjunction with those from the median preoptic area, mediate leptin's thermoregulatory action through sympathetic circuits to BAT.

1.2.4. Ventromedial hypothalamus

The VMH has been known to play a role in energy homeostasis for many years, since the finding that bilateral VMH lesions induce hyperphagia and obesity (673). The VMH receives NPY, AgRP, and α-MSH projections from the ARC and, in turn, VMH neurons

project onto both hypothalamic nuclei (DMH) and brainstem regions (nucleus tractus solitarius). Furthermore, POMC neurons receive strong excitatory inputs from the VMH(753), consistent with the known role of the VMH as a satiety centre. In the VMH, brain-derived neurotrophic factor (BDNF) is highly expressed, and VMH BDNF neurons supress food intake through MC4R signalling (867). Selective deletion of BDNF neurons in the VMH of adult mice results in hyperphagia and obesity (799).

The VMH also contains steroidogenic factor-1 (SF1) positive neurons. These neurons are leptin responsive, which is required for normal body weight homeostasis (172). The selective loss of leptin receptors from SF1 neurons resulting in obesity, hepatic steatosis, dyslipidaemia and hyperleptinaemia (72). In addition, the deletion of insulin receptors from SF1 neurons in the VMH results is the protection from diet-induced obesity, leptin resistance and impaired glucose tolerance (382). Furthermore, high-fat diet-induced, insulin-dependent PI3K activation in SF1 neurons of the VMH contributes to obesity development (382) and is required for the regulation of energy expenditure (868). Therefore, SF1 neurons in the VMH play a physiological relevant role in body weight and energy expenditure regulation.

1.2.5. Lateral hypothalamic area

The LH also receives projections from the ARC and contains two orexigenic neuropeptides, melanin-concentrating hormone (MCH) (334, 486). and orexin (664). The orexin cell population is distinct from the MCH producing neurons (166, 664). The MCH receptors are widely distributed in the brain areas, especially the hippocampus, amygdala, cerebral cortex (492), olfactory system, striatum and hypothalamus (117). Repeated ICV injection of MCH increases food intake and decreases energy expenditure (631). Transgenic mice overexpressing MCH are hyperphagic and develop obesity, glucose intolerance and

insulin resistance (473, 486, 603), whereas MCH null mice lean, hypophagic with increased energy expenditure with reduced plasma leptin and ARC POMC expression (486, 709). Conversely, the MCH- or MCH-1R deficient mice show resistance to high-fat diet induced obesity (473). In addition, the MCH system is thought to play a role in arousal in correlation with specific goal oriented behaviour such as feeding or reproduction (156).

Orexin A and B are produced by groups of neurons in the LH. These neurons project extensively to the olfactory bulb, cerebral cortex, thalamus, hypothalamus, brainstem, locus coeruleus, tuberomamillary nucleus, and raphe nucleus. The orexin receptors (1 and 2) are found in the ARC, PVN, VMH and suprachiasmatic nucleus. In addition to the orexigenic effects on food intake (664), orexins also play a role in other physiological functions such as the regulation of heart rate and blood pressure (667, 715),, the neuroendocrine system, body temperature and the sleep-waking cycle (613). Traditionally, orexin has been shown to increase food intake. However, in recent time genetic models have revealed interesting functions of orexin signalling. Over-expression of orexin results in resistance to high-fat diet-induced obesity and insulin sensitivity by promoting energy expenditure and reducing food intake, and is due to orexin receptor-2 signalling (238). Whereas, genetic ablation of orexin mice results in late-onset obesity, despite reduced food intake and motor activity (289).

NPY, AgRP and α-MSH terminals are extensive in the LH and are in contact with MCH and orexin-expressing cells (90, 191, 315). Central orexin neurons express both NPY receptors (103) and leptin receptors (315) and may therefore integrate their actions. A large number of glucose-sensing neurons are present in the LH (68) and orexin neurons may play a role in this. Hypoglycaemia increases orexin mRNA expression and *c-fos* in the LHA (101, 319). The mechanisms by which the MCH and orexin neurons influence energy homeostasis

remain to be fully elucidated. MCH receptors have been found on both NPY and POMC cells in the ARC, and furthermore, the majority of these POMC cells are also leptin sensitive, highlighting their importance for the orexigenic actions of MCH (117). Whilst orexins, made exclusively in the hypothalamus, are involved in motivation and rewards, may be responsible for the rewarding value of a high-fat diet (604).

In recent times, the importance of leptin signalling in the LH has been highlighted. Leinninger, et al. (441) has shown that leptin directly regulates a population of leptin receptor-expressing inhibitory neurons in the LH, and that leptin action via these neurons decreases feeding and body weight. These neurons are also involved in regulating the mesolimbic dopamine system (441), which is involved in motivation and the incentive to feed (363).

1.3. Circulating hormones effecting food intake and energy expenditure

The neurons within the ARC are located at the base of the hypothalamus and are in a prime position to respond to circulating satiety/hunger signals that are produced in peripheral tissues. These include hormones such as ghrelin, leptin and insulin (414). These hormones can function as substances for activating sensors for nutrient availability, energy requirements and energy stores within discrete hypothalamic regions. For example, adipose tissue secretes the hormone leptin; leptin is produced in proportion to fat stores, providing a mechanism for the CNS to 'sense' energy storage levels (452). Leptin directly activates anorexigenic POMC neurons and inhibits orexigenic NPY/AgRP neurons resulting in an overall reduction in food intake to correspond with increasing levels of adiposity (148). Adipose-secreted factors, or adipokines, are secreted by adipose tissue and include leptin, adiponectin and resistin. These

adipokines relay information to the hypothalamus on the levels on energy storage in the periphery. The gastrointestinal tract releases an array of hormones such as ghrelin, GLP-1 and PYY (552). These substances relay information to the hypothalamus on the contents of gut, and functions more on a meal-to-meal basis. These hormones can activate NPY neurons resulting in an overall increase in appetite and food intake. This section will discuss the three main hormones involved in energy balance in the hypothalamus (Table 1.1).

1.3.1. Leptin

Originally thought to be an inert tissue whose sole purpose was the storage of energy, it is now clear that adipose tissue is an active endocrine organ. One of its most important hormones is leptin, a peptide hormone with numerous actions in energy homeostasis, neuroendocrine and immune function. It is a product of the leptin gene (*ob*) and was discovered in 1994 by Friedman and colleagues (891). The *ob* gene is expressed predominantly in adipocytes (891), but also at lower levels in gastric epithelium (40) and placenta (487). Circulating leptin levels reflect both energy stores and acute energy balance. Plasma leptin levels are highly correlated with adipose tissue mass (478), whereas food restriction results in the suppression of circulating leptin (227, 478) which can be reversed by refeeding or insulin administration. Exogenous leptin administration, both centrally and peripherally, reduces spontaneous and fasting-induced hyperphagia (9) whilst chronic peripheral administration reduces food intake, resulting in a loss of fat mass and body weight (279). Furthermore, rodents and humans with mutations in leptin or its receptor are hyperphagic and severely obese (66, 118, 134, 432, 774).

Table 1.1 Peripheral hormones involved in energy balance

Substance	Origin	Effect on energy balance	Target	Reference
Leptin	Adipose tissue	anorexigenic	Inhibits NPY and activates POMC neurons	(189, 277, 891)
Adiponectin	Adipose tissue	↑ oxygen consumption	PVN	(64, 232, 681, 871)
Resistin	Adipose tissue	↑ insulin resistance	?	(752, 761)
Insulin	Pancreas	anorexigenic	NPY and POMC	(13, 333, 861)
Pancreatic polypeptide	Pancreas, distal GI tract	Peripheral administration is anorexigenic, central administration is orexigenic	Area postrema, brainstem pathways, modulates expression of other gut hormones	(34, 482, 500, 796)
PYY	L cells of GI tract	anorexigenic	NPY neurons	(54, 113, 537)
Ghrelin	Gastric oxyntic cells	orexigenic	NPY neurons, brain stem NTS	(557, 788, 864)
GLP-1	L cells of small intestine, pancreas, brainstem NTS	Anorexigenic	Brainstem NTS, PVN?	(506, 795, 870)
Oxyntomodulin	L cells of small intestine	Anorexigenic, ↑ energy expenditure	NTS, ARC	(158, 159)
Cholecystokinin (CCK)	GI tract, CNS	↓ meal size	NTC, DMH, and area postrema	(254, 377)
Bombesin	GI tract	anorexigenic	?	(253, 731)

Leptin signals via a single-transmembrane domain receptor of the cytokine receptor family (774). Alternative mRNA splicing and post-translational processing results in multiple isoforms of the receptor (Ob-R) (125, 773). The long form, Ob-R possesses a long intracellular domain that binds to janus kinase (JAK) (432) and to signal transducer and activator of transcription 3 (STAT-3) (800) resulting in signal transduction and leptin's effects on food intake (432). Activation of the JAK-STAT pathway induces expression of suppressor of cytokine signaling-3 (SOCS-3), one of a family of cytokine-inducible inhibitors of signalling. SOCS-3 expression is upregulated by leptin in hypothalamic nuclei expressing the Ob-R. Overexpression of SOCS-3 blocks leptin's actions *in vitro*, and neuron-specific conditional deletion of SOCS-3 in mice results in resistance to diet-induced obesity (539). These results suggest that obesity related leptin resistance might be a consequence of increased or excessive SOCS-3 expression.

Circulating leptin crosses the BBB via a saturable process (50), where the short forms of the Ob-R may play a role (186). The secreted (or soluble) form of the Ob-R is thought to bind to circulating leptin, and therefore play a role in its biological availability and activity (251).

The long form of the Ob-R is expressed widely within the hypothalamus, but is found predominantly in the ARC, VMH, DMH, LHA and medial preoptic area (194, 211, 278). Ob-R are also expressed in appetite-modulating pathways in the brain stem (512). Peripheral leptin administration alters neuronal activity in these hypothalamic and brain stem regions (193). In the ARC, Ob-R mRNA is expressed in the orexigenic NPY/AgRP neurons (511) and in the anorexigenic POMC/CART neurons (122). Leptin inhibits the activity of NPY neurons and reduces the expression of NPY and AgRP (189, 277, 690, 751), whilst activating POMC

neurons. Therefore, in conditions of low circulating leptin, such as food restriction, NPY/AgRP neurons are activated, and their neuropeptide expression upregulated, whereas in times of plenty with high plasma leptin, the anorexigenic pathways mediated by POMC and CART are switched on whilst the orexigenic pathways mediated by NPY and AgRP are supressed. Rising levels of leptin signal to the brain that excess energy is being stored, and this signal brings about adaptations of decreased appetite and increased energy expenditure that resist obesity.

Mice selectively deficient for Ob-R in POMC neurons exhibit a more modest obesity than mice globally deficient in Ob-R (44). This increase in adiposity is dependent on decreased energy expenditure and increased food intake. Whilst the combined removal of Ob-R from both the POMC and AgRP neurons also increases body weight (804). On the other hand, overexpression of Ob-R in POMC neurons increases the magnitude of high-fat diet induced obesity, but has no effect on chow fed mice (241). ARC specific reactivation of Ob-R in mice and rats with mutant Ob-R alleles results in modest improvements in body weight, whereas expression of OB-R in the ARC results in marked improvements in hyperinsulinaemia, and blood glucose levels (143, 543, 544). Furthermore, expression of Ob-R in POMC neurons alone results in modest improvements in body weight and complete normalisation of blood glucose (66, 328), which was caused by increased insulin sensitivity and decreased hepatic glucose production, independent of serum leptin (66). This suggests that direct action of leptin on POMC neurons regulates glucose homeostasis. Therefore, the fact that the removal of Ob-R from ARC neurons does not recapitulate the obesity of *db/db* mice shows that other populations of neurons expressing Ob-R are involved in energy balance. As such, removal of Ob-R from SF1 neurons of the VMH results in a greater obesity than that from Ob-R removal from POMC neurons, but is exacerbated with Ob-R deletion

from both POMC and SF1 neurons (44, 172). Furthermore, removal of Ob-R from neurotensin neurons of the LH promotes early-onset obesity, increased feeding and decreased locomotor activity (442).

The absence of leptin has profound effects on body weight. Lack of circulating leptin, due to a mutation in the *ob* gene, leads to hyperphagia, obesity, as well as neuroendocrine and immune disturbances in the *ob/ob* mouse, which can be normalized by leptin treatment (104, 279, 599). In humans, mutations in the *ob* gene results in the absence of leptin, causes severe obesity and hypogonadism (533, 757), which can be ameliorated with recombinant leptin treatment in children and adults (209, 457). Similarly, defective leptin receptor signalling also alters body weight and endocrine function. A point mutation in the intracellular domain of the Ob-R that prevents signalling results in obesity in the *db/db* mouse (118, 432).

A small proportion of obese human subjects have an absolute or relative leptin deficiency, but the majority of obese animals and humans have raised plasma leptin (138, 478). This suggests resistance to leptins' actions, and indeed, subcutaneous administration of recombinant leptin to obese humans had only a modest effect of weight (221, 303). Leptin resistance during obesity will be discussed in section 1.5.1.

1.3.2. Insulin

The pancreatic hormone insulin was one of the first adiposity signals to be described (692), and, like leptin, is positively correlated with long-term energy balance (42, 859). Plasma insulin concentrations depend on peripheral insulin sensitivity, which is related to both total fat stores, and fat distribution, with visceral fat being a key determinant (623).

However, unlike leptin levels, which are relatively insensitive to acute food intake, insulin secretion increases rapidly after a meal (621).

There is considerable evidence that insulin acts as an anorexigenic signal within the CNS. Centrally administered insulin or an insulin mimetic decreases food intake and body weight (13) and alters expression of hypothalamic genes known to regulate food intake. Insulin infusion into the third cerebral ventricle in rodents (861) or lateral ventricle in primates (333) dose-dependently decreases food intake, resulting in weight loss over a period weeks. Injection of insulin into the PVN also decreases food intake and weight gain in rats (510). Treatment with novel, orally available insulin mimetics also decreases weight gain, adiposity, and insulin resistance in mice on a high-fat diet (13). Conversely, antibodies to insulin injected into the VMH of rats stimulate food intake (758), and repeated administration of antiserum increases food intake and rate of weight gain (499). Neuron-specific deletion of the insulin receptor results in obesity, hyperinsulinemia, and dyslipidaemia in mice (95).

Insulin enters the CNS via saturable, receptor-mediated uptake across the BBB at levels proportional to circulating insulin concentrations (56). Little or no insulin in synthesized within the brain (47, 862). Therefore, peripheral insulin should have similar actions to central insulin treatment. Studies of systemic insulin administration are complicated by hypoglycaemia, which in itself potently stimulates food intake. However, hyperinsulineamic, euglyceamic clamps studies have demonstrated a reduction in food intake with both rodents and baboons (561).

Insulin signals via a cell-surface insulin receptor, which is composed of an extracellular, ligand binding α-subunit and an intracellular β-subunit with intrinsic tyrosine

kinase activity. Insulin receptors are widely distributed in the brain, particularly in hypothalamic nuclei such as ARC, DMH, PVN, suprachiasmatic and periventricular regions that are involved regulating food intake (145, 484). Insulin receptor activation is via several insulin receptor substrates (IRSs), which include IRS-1 and IRS-2 (53, 97). Although IRS-1 null mice show no difference in food intake or body weight from their wild-type littermates (29), IRS-2 null mice have increased food intake, increased fat stores and infertility (97). IRS-2 mRNA is highly expressed in the ARC, and may therefore mediate insulin's central actions (97). Insulin and leptin, along with other cytokines, appear to share common intracellular signalling pathways. Both hormones can signal via IRS and the enzyme phosphatidylinositol 3-kinase (PI3K) (564, 623), allowing intracellular integration of their appetite-regulating actions.

The pathways mediating insulin's effects on food intake remain to be fully elucidated. Both the NPY and POMC systems are important targets for the effects of insulin on food intake and body weight (61, 692, 695). ICV injection of insulin during fasting prevents the normal fasting – induced increase in NPY mRNA in the PVN and the ARC in rats (13, 695). NPY expression is also increased in insulin-deficient, streptozotocin-treated rats but restored by insulin replacement (846, 851). Insulin receptors have also been found on POMC cells in the ARC (61), and third ventricle administration of insulin increased POMC mRNA expression (61). POMC expression is greatly reduced in rats with untreated diabetes and partly restored by peripheral insulin treatment (728). Furthermore, reducing the insulin receptor proteins specifically in the ARC results in hyperphagia and increased adiposity (571).Therefore it seems likely that both the NYP and POMC neurons are important downstream mediators of insulin's actions on food intake and body weight.

The loss of insulin receptors selectively in POMC neurons does not influence energy and glucose homeostasis (305, 390) whilst insulin receptor deletion from AgPR neurons showed normal energy homeostasis but insulin fails to normally suppress hepatic glucose production during euglycemic-hyperinsulinemic clamps (390). However, mice deficient for insulin receptors selectively in SF1 neurons of the VMH are protected from diet-induced leptin resistance, weight gain, adiposity and impaired glucose tolerance (382). Furthermore, the acute effects of leptin and insulin in the VMH mimic those observed in ARC POMC neurons (382). However, this only accounts for a part of the phenotype observed complete loss of insulin receptors in the CNS (95, 348, 382).

1.3.3. Ghrelin

Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor (GHSR). Ghrelin comprises a chain of 28 amino acids with esterification of the hydroxyl group of the third serine residue by octanoic acid. It is the only known orexigenic gut hormone. Ghrelin is principally secreted from X/A-like cells within the gastric oxyntic glands (163, 662). Ghrelin also acts as a neurotransmitter, being expressed within the ARC and periventricular area of the hypothalamus (149, 385).

Plasma ghrelin levels are increased by fasting and decreased by refeeding or oral glucose administration (788). In rats, ghrelin levels show a diurnal pattern, with the bimodal peaks occurring before light and dark periods (551). In humans, ghrelin levels are in phase with the diurnal variation in leptin, which is high in the morning and low at night (153).

Levels of circulating ghrelin rise preprandially and fall rapidly in the postprandial period (153). Both central and peripheral administration of ghrelin increases food intake and

body weight along with a reduction in fat utilisation in rodents (557, 788, 864). Negative correlations between circulating ghrelin levels and body mass index are found in humans. Fasting plasma levels of ghrelin are reported to be high in patients with anorexia nervosa, which normalises after weight gain (583) and subjects with diet-induced weight loss (154, 288). In contrast, obese patients show a less marked drop in plasma ghrelin after meal ingestion (200, 427).

Ghrelin mediates its orexigenic action via stimulation of NPY/AgRP neurons within the ARC. Peripheral administration of ghrelin increases c-fos expression in the ARC NPY/AgRP neurons (829), and ablation of both AgRP and NPY neurons (119) or GHSR (764) completely abolishes the orexigenic effects of ghrelin. In addition to its potent orexigenic properties, ghrelin also increases gastric motility, stimulates the hypothalamo-pituitary-adrenal axis, and possesses cardiovascular effects such as vasodilation and enhance cardiac contractility (317).

Although ghrelin has potent actions on food intake in animals and humans, both ghrelin null mice and mice lacking GHS-R type 1a have normal appetite and body composition on a standard diet (119, 763, 764). This absence of a phenotype suggests that long-term ghrelin blockade may not alter body weight, and ghrelin receptor antagonism may not be an effective therapy for obesity.

1.4. Circulating nutrients effecting food intake and energy expenditure

Over 50 years ago, Mayer proposed the "glucostat hypothesis" for the regulation of feeding behaviour (493). Mayer suggested that the post-prandial rise in plasma glucose, sensed through glucose metabolism in key hypothalamic neurons, constituted a physiological signal for meal termination, It is now recognized that circulating carbohydrates, lipids and amino acids are involved in the control of energy homeostasis.

1.4.1. Glucose

The brain has evolved specialized glucose sensing neurons to monitor and respond to the availability of glucose, perhaps because the brain depends on a constant supply of glucose to fuel it's metabolic demands (733). Unlike most neurons, which use glucose to fuel their metabolic demands, these specialized neurons use the products of intracellular glucose metabolism to regulate their activity and neurotransmitter release. Glucose undergoes facilitated transport across the BBB, resulting in extracellular brain glucose levels that range from 10-30% of plasma levels during hypo- to hyperglycaemic conditions (171, 504, 505, 720). Extracellular brain glucose levels rapidly equilibrate with plasma levels (720), but vary considerably among brain areas. In the hypothalamus, basal levels are approximately 1.4 mmol/L (171) whereas they are 1.0 mmol/L in the hippocampus (504) and 0.5mmol/L in the striatum (504). Glucose sensing neurons have subsequently been identified in a number of discrete brain areas including the ARC (237), VMH (737), PVN (391), the substantia nigra (463), the area postrema and nucleus of the tractus solitarius (5, 160, 528).

In support of the glucostat hypothesis, insulin – induced hypoglycaemia powerfully stimulates food intake (271) and this effect is due to low glucose, not elevated insulin (529).

Inhibiting glycolysis through 2-deoxyglucose (2-DG) to produce cellular hypoglycaemia, can increase food intake as robustly as whole body hypoglycaemia (782). These effects are mediated by neurons in the brain (525). These early studies suggested that impairments in neuroendocrine glucose-sensing mechanism could lead to chronic positive energy balance, obesity and metabolic syndrome. Recent studies have begun to unravel the glucose sites of action and mechanisms mediating its effects.

Brain glucose sensing is dependent upon processes controlling glucose uptake and utilization. Glucose is transported across the BBB by facilitated transport (820). It is then taken up by both neurons and astrocytes, each using their own specific transporters (820). Glucose sensing neurons can be classes as glucose excited (GE), neurons that increase their firing rate in response to elevated extracellular glucose, and glucose inhibited (GI), neurons that increase their firing rate in response to a decrease in extracellular glucose (451, 655, 874).

There is considerable evidence that the mechanisms of glucose sensing by cells in the CNS may rely on different mechanisms, and one of which may be similar to that of the pancreatic β-cell (686, 873). Glucose signalling in these cells requires glucose uptake by the low-affinity glucose transporter type 2 (GLUT2), glucose phosphorylation by glucokinase, and the consequent metabolism of glucose to increase the intracellular ATP-to-ADP ratio.

Because GE neurons increase their firing activity when extracellular glucose rises, they may share similarity to the β-cell. The presence of GLUT2 in hypothalamic nuclei where glucose-sensing neurons are present has been reported (30, 353, 444, 454, 558). GLUT2 null mice show abnormal feeding behaviour (41) with increased *ad libitum* food intake and a blunted response to refeeding after a 24h fast. In this study ICV injection of glucose in fasted

mice decreased NPY and increased POMC expression in control, but not GLUT2 null mice (41). GLUT2 has been found in neurons, endothelial cells, and tanycytes of the third ventricle (244, 477, 560). Specific expression in NPY or POMC has, however, not been shown. However, glucose sensing by POMC neurons, as shown through ATP-mediated closure of ATP-sensitive potassium channels (590). Specifically, POMC neurons are involved in the whole-body response to a systemic glucose load, which is impaired during obesity (590).

A candidate for the mechanism of action of glucose sensing in the brain is the glycolytic enzyme, glucokinase (GK; hexokinase IV) that controls the rate of glucose utilization (249, 488). GK mRNA is highly localized in brain areas known to contain glucose sensing neurons, including both NPY and POMC neurons (547). Glucosamine, a GK inhibitor, stimulates food intake when injected into the third ventricle, whilst also blocking the effects of glucose on hypothalamic neuronal activity (235).

GE neurons utilize an adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channel to alter their firing rate in response to substrate availability (35, 463, 657, 737). The increase in extracellular glucose leads to an augmentation of the ATP-to-adenosine diphosphate (ADP) ratio and the closure of the K_{ATP} channels in these GE neurons (35, 160, 434, 514, 805), which leads to plasma membrane depolarization and Ca^{2+} entry though voltage-gated channels, thereby increasing neuronal activity (8, 450, 453, 540), and neurotransmitter secretion (21, 435, 449). For GI neurons, the mechanisms linking a decrease in glucose concentrations to increased firing activity is less clear. Suppression of firing activity may be controlled by the increase in ATP-to-ADP ratio, which leads to an increase in sodium (Na)-potassium (K)-ATPase activity (580, 721) and/or opening of ATP-regulated chloride channels (655, 738), which hyperpolarizes the plasma membrane.

Together, these data demonstrate that large decreases in glucose availability stimulate food intake and the associated neuroendocrine responses. However, it remains uncertain whether daily fluctuations in blood glucose play a physiological role in daily appetite regulation.

1.4.2. Fatty acids

As discussed previously, changes in lipid storage generate hormonal and nutritional signals that target CNS regions implicated in the control of energy homeostasis. One the other hand, the availability of circulating substrates controls various adipose tissue-related signals (665, 827), and may directly influence CNS energy centres. There is mounting evidence that fatty acid metabolism in the hypothalamus can effect food intake and body weight regulation.

Plasma free fatty acid (FFA) are mainly bound to albumin in the circulation and cross the BBB, predominantly by diffusion of the unbound form (282). Chylomicrons are likely to be a major circulating source of brain FFA after a meal, while a combination of unbound FFA and locally hydrolysed lipoproteins contribute to brain FFA pool during fasting. Overall the access of circulating FFA to the CNS is generally proportional to the plasma concentration of FFA (517, 637), although their concentration in the cerebrospinal fluid (CSF) is ~6% of the plasma concentration in anesthetized dogs (264). Furthermore, triglyceride rich lipoproteins from the circulation are sensed by the brain by a lipoprotein lipase-dependent mechanism and provide lipid signals for the central regulation of body weight and energy balance (825). However, dogma states that fatty acid oxidation in the brain is either low or non-existent (445).

Intravenous infusion of a lipid emulsion is sufficient to suppress food intake in baboons (863), independent of measurable changes in plasma insulin and does not require gastrointestinal nutrient absorption (491, 532, 689, 863). ICV administration of the monounsaturated FFA, oleic acid, inhibits food intake and hepatic glucose production (538, 570). ICV oleic acid administration also reduces the expression of NPY and AgRP in the hypothalamus and gucose-6-phosphate expression in the liver (538, 570). Furthermore, oleic acid can directly excite POMC neurons, but does not effect AgRP neurons, therefore, β-oxidation in POMC neurons may mediate the appetite supressing effects of oleic acid (346).

Upon entry into the neuron, FFAs are rapidly esterified to a fatty acyl-coenzyme A (FA-CoA). The transfer of long-chain fatty acyl-CoAs (LC-CoA) to the mitochondria where they undergo β-oxidation, requires two-membrane bound carnitine-dependent long-chain acyltransferases, carnitine palmitoyltransferase (CPT)1 and CPT2. Peripheral infusion of FFA causes a rapid increase in hypothalamic LC-CoA. Co-administration of FFAs and an inhibitor of LCFA-AC prevent the anorectic effects of peripherally administered FFA, suggesting that the anorexigenic effects of FFA is mediated via increased hypothalamic LC-CoA levels (414). Furthermore, elevating hypothalamic FA-CoA levels by blocking their oxidation also leads to reduced ARC NPY and AgRP expression and reduced food intake (568). Under genetic or pharmacological inhibition of CPT1, the concentration of hypothalamic LC-CoA increase roughly two-fold, where as the expression of NPY and AgRP are reduced (568). This also leads to a marked reduction in food intake and liver glucose production (568). This data signifies a paradox in the literature, where during fasting, circulating FFA are elevated, but increasing FFA should reduce food intake through hypothalamic signalling. Should it not make more sense for appetite to be increased during times of fasting? Therefore, the

importance of FFA in the hypothalamus to physiological conditions, both normal and pathogenic (i.e. obesity), remains undefined.

Fatty acid synthase (FAS) catalyses the production of the FFA palmitate form acetyl-CoA and malonyl-CoA. Two inhibitors of FAS activity, cerulenin and C57, reduce food intake and body weight in rodent models (131, 242, 443, 467, 480). FAS inhibitors also have anorectic effects in the *ob/ob* mouse and in rats lacking the leptin receptor (Zucker rats), suggesting that FAS inhibitors mediate their effects via a leptin – independent pathway (131, 242, 467). In addition, rodents treated with FAS inhibitors lose more weight than non-treated rodents in which food intake is limited to the FAS inhibitor treated group, suggesting that FAS inhibitors increase energy expenditure in addition to reducing food intake (467). The anorectic effects of FAS inhibitors are associated with decreased NPY and AgRP expression and increased POMC and CART expression in the ARC (713).

The specific hypothalamic nuclei involved in FFA sensing remains to be fully elucidated. Both genetic and pharmacological inhibition of CPT1 increases LC-CoA in the ARC, but not in other hypothalamic nuclei (568). Furthermore, inhibition of fatty acid oxidation in the LH does not alter food intake (69).

1.4.3. Amino acids

Amino acids have also been implicated in the regulation of food intake and body weight. A relationship between fluctuations in serum amino acids and appetite in humans was originally suggested by Mellinkoff, et al. (508). Dietary protein induces satiety in the short term (622), and consumption of protein-deficient diets leads to increased appetite for protein containing foods (255). Furthermore, deficiencies of certain essential amino acids in the diet

leads to a rapid reduction in food intake in rats, although these were not attributed to the ARC (256). Amino acids may influence food intake via direct actions in the CNS or via receptors located in the liver or portal vein (563). ICV administration of the branched-chain amino acid L-leucine reduces food intake and body weight in rats (75). L-leucine is thought to induce its anorexigenic effects by increasing hypothalamic mTOR activity, which reduces ARC NPY expression (147). Furthermore, ICV coadministration of rapamycin (mTOR inhibitor) with L-leucine abolished the anorexigenic effects of L-leucine. This finding shows that direct CNS administration of L-leucine inhibits food intake and body weight. However, it remains undetermined whether normal physiological changes in amino acid levels can alter daily food intake via mTOR-mediated pathways.

1.4.4. Metabolites

In addition, a number of other metabolic products have effects on food intake. Lactate (556), pyruvate (556), and ketones (217) inhibit feeding in animals. Postprandial circulating lactate concentrations are increased in proportion to the carbohydrate content of a meal (292), and may therefore contribute to the acute inhibition of food intake during carbohydrate consumption.

1.5. Hypothalamic resistance to hormone/nutrient cues in obesity

The majority of obesity cases do not result from single genetic mutations, but a combination of genetic, behavioural and environmental factors. The improvements in food availability and alterations in dietary patterns with an increase in energy dense fat and sweet foods are crucial environmental factors in todays obesity epidemic. Distinguished from 'homeostatic feeding' where food intake is restricted to satisfy ones biological needs, this

kind of 'non-homeostatic feeding' or 'feeding for pleasure' has gained a special place in our society and overeating, food craving and compulsive eating are important deleterious factors cumulating in obesity. The increased attraction towards pleasurable feeding and the loss of control over food intake have been compared to addictive behaviour.

It has been well established that the hypothalamus is implicated in the development of obesity in experimental animals. As discussed previously, peripheral hormones such as leptin, insulin, and ghrelin signal to the brain to regulate food intake and maintain energy balance. Most cases of rodent (226) and human (138) obesity are characterized by high circulating levels of these hormones, however they fail to elicit the appropriate hypothalamic response (59). This is known as central resistance and will be discussed in the following section.

1.5.1. Central leptin resistance

Leptin is perhaps the most widely studies biological factor controlling food intake. The amount of circulating leptin is proportional to the degree of peripheral adiposity. In the genetically obese *ob/ob* mice that lack functional leptin, recombinant leptin treatment is highly effective at reversing obesity (104, 279, 599). However, most cases of rodent (226) and human (138) obesity are characterized by high circulating leptin levels as a consequence of their large fat mass, but they don't adequately respond to these increased leptin levels with reduced food intake, (59, 188, 201, 548, 549). This under-responsiveness to endogenous and exogenous leptin in most forms of obesity has given rise to the idea that obesity is associated with or even caused by a state of relative leptin resistance, similar to that of insulin resistance in type 2 diabetes. While the mechanisms behind central leptin resistance are not fully understood, a number of mechanisms have been proposed, including compromised transport across the BBB, and defects in the leptin receptor signalling pathways.

Decreased transport of leptin into the brain has been suggested as one mechanism causing central leptin resistance in obesity (49, 810). Concentrations of leptin in the CSF of obese humans is not increased in proportion to their circulating levels (107, 693), and during the development of obesity, mice are still responsive to ICV leptin administration after 4 weeks on a high-fat diet, but not IP leptin as shown through phosphorylation of $STAT_3$ (186). However, the extent to which leptin transport across the BBB contributes to leptin action is not clear, especially in the ARC, which is close to the median eminence and the portal circulation, and may not be as protected by the BBB (394, 610).

It is also clear that the ability of leptin to activate hypothalamic ARC signalling is decreased during obesity (186, 549, 810), yet other areas such as the DMH, PVN and LH remain leptin sensitive (202, 549). Increased SOCS3, a known inhibitor of leptin signalling may contribute to defective leptin signalling in the ARC (549). As such, decreased SOCS3 seems to cause increased leptin sensitivity and resistance to diet-induced obesity (320, 550). Another inhibitor of leptin signalling, protein tyrosine phosphatase 1B, may also contribute to leptin resistance during obesity (120).

Other pathways also limit cellular leptin action. In peripheral tissue, obesity promotes both endoplasmic reticulum (ER) stress, and a state of chronic low-level inflammation that contributes to insulin resistance, both of which may also contribute to leptin resistance during obesity (573, 585, 889). Increased activity of inflammatory signalling pathways in the hypothalamus of obese mice can impair leptin signalling both *in vivo* and *in vitro* models, whereas genetic or pharmacological blockade of inflammatory signals in the brain of obese rodents promotes leptin action again diet-induced obesity (573, 585, 889).

1.5.2. Central insulin resistance

Just like insulin resistance develops in peripheral tissues such as skeletal muscle and liver (718, 756, 828), the CNS also becomes insulin resistance during obesity. High circulating insulin levels that fail to promote the appropriate anorexigenic hypothalamic response characterize obesity (59). Obesity not only impairs the transport of insulin through the BBB (335, 350), while direct administration of insulin into the hypothalamus or the ventricles fails to reduce food intake (6, 130) and glucose production (579) in high-fat feeding. This data demonstrates a direct impairment of hypothalamic insulin signalling in obesity (59).

An inability of central insulin to lower food intake during obesity is associated with an impairment of hypothalamic AKT activation (130) that is partly due to an activation of hypothalamic PKC-θ (62). Concomitantly, high-fat feeding impairs the ability of hypothalamic insulin to inhibit glucose production through activation of p70 S6 kinase (S6K) (579). Therefore, high-fat feeding disrupts hypothalamic insulin-AKT signalling in rodents, which could lead to hyperphagia and hyperglycaemia.

The development of ER stress and inflammation leads to peripheral insulin resistance during obesity (318). Correspondingly, high-fat feeding activates inflammatory processes in the rodent hypothalamus (169), and IKKβ/NF-κB signalling via elevated ER stress to negatively impact hypothalamic insulin signalling to reduce food intake (889). High-fat feeding induces hypothalamic inflammation and insulin resistance through the activation of the toll-like receptors (381), and once again, this process is paralleled during peripheral insulin resistance (706).

1.5.3. Central ghrelin resistance

Continuing with the current theme, during obesity, the hypothalamus also becomes resistant to the hyperphagic actions of the peripheral hormone ghrelin (87, 245). Ghrelin during obesity fail to increase food intake (607). A number of mechanisms have been proposed to explain hypothalamic ghrelin resistance during obesity. These include a down regulation of circulating ghrelin (789, 850), an impairment of ghrelin to cross the BBB (48) and an inability for ghrelin to stimulate the NPY from ARC neurons (87). However, not much is known about the disruption to signalling cascade of the GHSR after ghrelin is bound, which disturbs neuropeptide release, but may be related to 5' adenosine monophosphate-activated protein kinase (AMPK) signalling.

1.6. Lipotoxicity during obesity

1.6.1. Regulation of fatty acid metabolism

1.6.1.1. Lipid sources and transport

Cells can obtain FFA from three main sources; consumed food, intracellular lipid stores (lipid droplets) and the circulation (cholesterol particles). Dietary FFA are absorbed through the small intestine and incorporated into chylomicrons (844). Chylomicrons containing triacylglycerol (TAG), cholesterol and protein, are transported through the lymphatic system into the circulation. From the circulation, chylomicrons can be taken up by cells where they are either stored or oxidized for energy. The liver can process chylomicrons into various lipoproteins, such as very low-density lipoproteins (VLDL), LDL, and high-density lipoproteins (HDL), which then enter the circulation. In peripheral tissues, lipoprotein lipase (LPL) digests part of the VLDL into LDL and FFA, which are taken up for metabolism. LDLs can be absorbed into cells via the LDL receptors.

FFA can also be released into the blood stream directly from adipocytes through a process called lipolysis, which will be discussed in the following sections. FFA in the blood stream are bound by serum albumin and transported to the required tissue, the uptake of which will be discussed in the next section.

1.6.1.2. Lipid uptake

The precise mechanisms of fatty acid uptake into cells are still unclear. However passive diffusion via a "flip-flop" mechanism (283), or facilitated diffusion via the putative transporters fatty acid binding protein (FABP) (841), CD36/FA translocase (185), or fatty acid transport protein (FATP) (78), have all been suggested.

1.6.1.3. Lipid esterification

Two major pathways, the glycerol phosphate pathway and the monoacylglycerol (MAG) pathway can synthesize TAG. The MAG pathway is found mainly in the small intestine to generate TAG from MAG derived from dietary fats. The glycerol phosphate pathway is the major pathway used by most cells. Acylation of glycerol 3-phposphate occurs through the stepwise addition of fatty acyl groups, each of which is catalysed by a specific enzyme.

In the body, FFA can be directed towards either oxidative processes in skeletal muscle or brown adipose tissue, or to TAG storage. Once FFAs enter a cell they are esterified by acyl-CoA synthetase (ASC) to form the metabolically active LC-CoA. LC-CoA can then be esterified for storage as TAGs or undergo β-oxidation (273). The first step in the synthesis of TAG is the esterification of LCA-CoA to glycerol-3-phosphate by the enzyme glycerol phosphate acyltransferase (GPAT) (842) resulting in the formation of lysophosphatidic acid

(LPA). It is then converted to phosphatidic acid (PA) by 1-acylglycerol-3-phosphate O-acyltranferase (AGPAT). PA is dephosphorylated by lipins to become diacylglycerol (DAG), which is then catalysed by diacylglycerol acyltransferase (DGAT) to produce TAG (135).

1.6.1.4. Lipid lipolysis

Lipolysis is the process of TAG breakdown. it is the catabolic branch of the FFA cycle that provides FFA in times of metabolic need and removes them when they are present in excess. The hydrolysis of TAG to FFA and glycerol requires 3 consecutive steps that involve at least 3 different enzymes. Adipose triacylglycerol lipase (ATGL), and to a lesser extent hormone-sensitive lipase (HSL), catalyses the initial steps of lipolysis, converting TAG to DAG (342, 830, 899), HSL is mainly responsible for the hydrolysis of DAG to MAG; and monoacylglycerol lipase (MGL) hydrolyses MAG to produce glycerol and FFA (228). In adipose tissue, ATGL and HSL are responsible for more than 90% of TAG hydrolysis (697). Although most non adipose tissue also expresses ATGL and HSL, expression levels are low in some tissue, raising the question of whether other lipases are additionally required for efficient lipolysis.

1.6.1.5. Fatty acid oxidation

The mitochondria function within the cell to provide ATP for cellular function. Cells can generate ATP from FFA through two major processes, β-oxidation, which produces acetyl CoA and reducing equivalents, and flux through the tricarboxylic cycle (TCA), which also produces reducing equivalents for the electron transport chain (ETC). Before entering the mitochondria to undergo β-oxidation, LCA-CoA is converted to acyl carnitine by CPT1 to enable its transport across the mitochondrial membranes (273, 496). Once inside the mitochondria, CPT2 converts acyl carnitine back to LCA-CoA to then undergo β-oxidation,

the sequential degradation to acyl-CoA by very-long, long and medium chain dehydrogenases. Acyl-CoA then enters the TCA cycle to release electrons for the ETC to produce ATP (312).

1.6.2. Lipotoxicity and obesity

Under normal physiological conditions, insulin concentrations control, within a narrow range, the balance between postprandial FFA storage as TAG and their release (lipolysis) into the circulation during the fasting state. Adipose tissue is extremely sensitive to insulin concentrations, inhibiting lipolysis at insulin concentrations that are much lower than those needed to inhibit hepatic glucose production or stimulate muscle glucose uptake. In obesity there is marked adipocyte resistance to the anti-lipolytic effect of insulin, and plasma FFA concentrations are typically elevated. This leads to an increased uptake of FFA into non-adipose tissue, with limited 'storage space' for lipids. When the storage capacity of these non-adipose cells is exceeded, the resulting cellular dysfunction and/or cell death is termed lipotoxicity.

The term "lipotoxicity" was coined by Unger and colleagues almost two decades ago and refers to the excessive accumulation of lipids in non-adipose tissue that causes cellular dysfunction and, in severe cases, apoptotic cell death (438, 797). The exact mechanisms by which lipotoxicity cause death and dysfunction in tissue is not fully elucidated. The cause of apoptosis and the extent of cellular dysfunction in lipotoxic states are related to the cell effects, as well as the type and quantity of excess lipids. The accumulation of TAG in cells is unlikely to contribute directly to lipotoxicity because it is stored within discrete lipid droplets, and is likely to arise from FFA and their products such ceramide and DAG. For example, ceramides can inhibit the production and release of insulin, and induce programmed cell death

in pancreatic β-cells (620, 628, 707), decrease in cardiac muscle content (769), induce insulin resistance in skeletal muscle myotubes (794) and induce hepatic steatosis resulting in the over production of glucose (121, 175). The following sections will discuss lipotoxicity in peripheral tissues.

1.6.2.1. Disease states associated with lipid overload

High plasma FFA and TAG levels lead to increased import of FFA into non-adipose tissues, contributing to intracellular lipid accumulation. In addition to primary hyperlipidaemia, serum TAG (280, 410) and FFA (224, 902) are elevated in type 1 and type 2 diabetes, and plasma FFA are elevated in obese individuals (102). More rare are congenital or acquired lipodystrophies in which the absence of functioning adipose tissue leads to high serum TAG and FFA levels that promote excess lipid accumulation in the liver and skeletal muscle (247). In each of these cases, excess FFA can be taken up directly by cardiac myocytes, hepatocytes, jejunal enterocytes and adipocytes, since increased substrate concentration leads to increased transport into these cells (759).

Another mechanism for lipid accumulation is seen in tissues with a high metabolism of FFA, such as the heart, when utilization of FFA is impaired during increased FFA uptake or production. Pharmacological inhibition of FFA β-oxidation in a rat model leads to intramyocellular lipid accumulation, which is exacerbated in the setting of a high fat diet (173).

1.6.2.2. Lipotoxicity in the liver

The liver is a vital organ, which functions to clean and remove harmful substances from the circulation, and synthesize proteins and enzymes for digestion. It is essential for

normal metabolic function including glycogen storage, plasma protein synthesis, and hormone production, whilst regulating gluconeogenesis, gluconeogenesis, glycogenesis, cholesterol, TAG and albumin synthesis (121).

During obesity and diseases of lipid oversupply, elevated levels of circulating FFA cause TAGs to accumulate in the liver, a condition known as steatosis (175). Accumulation of fat in the liver, in the absence of excessive alcohol ingestion is referred to as non-alcoholic fatty liver disease (NAFLD). There is a strong correlation between NAFLD and obesity, almost 90% of obese patients present with some form of fatty liver change (23). Although the majority of hepatic lipids during steatosis are stored in the form of TAGs (93), several other lipid metabolites such as different FFAs, DAG, free cholesterol, cholesterol esters, ceramide, and phospholipids also accumulate (123). Isotope-labelling studies show that circulating FFAs (derived from adipose tissue lipolysis) can account for up to 60% of hepatic TAGs in NAFLD patients (175) Impaired peripheral insulin action leads to uninhibited adipose tissue lipolysis, resulting in an increased flux of FFA to the liver, and to a compensatory hyperinsulinemia, which in turn increases *de novo* lipogenesis (24, 625). These two factors are crucial to the hepatic accumulation of TAG.

Hepatocytes develop steatosis during prolonged high fat feeding, which can lead to increased caspase 3 activity, a key activator of apoptosis, and oxidative stress (831). Cultured hepatocytes incubated with a mixture of the saturated FFA palmitate, and the monounsaturated FFA oleate, have increased expression of proinflammatory markers NF-κB with activation of TNF-α (215). Hepatocyte cell lines incubated with palmitate have increased caspase 3 activity, DNA fragmentation and increases in ER stress markers eIF2α and XBP1 (839).

In a mouse model of impaired β-oxidation due to lack of mitochondrial trifunctional protein, moderate to severe lipid accumulation in the liver can lead to cell dysfunction, manifesting as a failure to appropriately catty out gluconeogenesis (331). In humans, TAG and FFA accumulation in the liver is associated with non-alcoholic steatohepatitis (NASH), which is characterised by and inflammatory response with evidence of hepatocyte damage and fibrosis that can progress to cirrhosis (248). NASH has been described in obese individuals, in diabetics and in patients with lipodystrophy.

1.6.2.3. Lipotoxicity in skeletal muscle

FFA and glucose are the main substrate for aerobic ATP synthesis in skeletal muscle (634). Most endogenous FFAs are stored as TAG in subcutaneous and deep visceral adipose tissue, with less in ectopic tissue such as skeletal muscle. Increased plasma FFA lead to intramyocellular lipid accumulation in humans that has been proposed to play a critical role in the development of type 2 diabetes (718). With lipid oversupply in the form of a high caloric diet or lipid infusion, more TAG is stored within the skeletal muscle in the form of intramuscular triacylglyerides (IMTG). IMTG are predominantly located close to the mitochondria, suggesting that they used as a fuel source for β-oxidation (313). Obesity is characterized by impaired fatty acid oxidation (36, 724) and ectopic lipid accumulation in non-adipose tissue (479). Increased skeletal muscle IMGT is associated with lower basal ATP production (365, 644).

Intracellular FFA or their metabolites activate a serine/threonine kinase cascade that ultimately results in reduced insulin receptor substrate-1 tyrosine phosphorylation, reduced insulin receptor substrate-1 associated phosphatidylinositol 3-kinase activity and failure to promote translocation of the GLUT4 glucose transporter to the plasma membrane in response

to insulin stimulation (268). Intramyocellular lipid accumulation is associated with activation of protein kinase C (PKC)-θ (268), PKC-ε (684) and activation/translocation of PKC-β and –δ isoforms from the cytosol to the cell membrane (336). Decreased activation of atypical PCK isoforms (ζ/λ/ι) has been observed (742). Changes in PKC activation can not only interfere with normal insulin signalling, but may also contribute to activation of the nuclear factor-κB (NFκB) pathway (327, 336). While IMTG, DAG and ceramides all induce insulin resistance and disrupt cellular function in skeletal muscle, they also induce toxic effects leading to cell death (611, 685, 793, 794) (see Figure 1.3).

1.6.2.4. Lipotoxicity in the pancreas

Type 2 diabetes is associated with a decreased β-cell mass (99, 663, 879), which diminishes the amount of circulating insulin and thereby disrupting glucose homeostasis (99, 666). Lipid overload in pancreatic β-cells leads to dysregulated insulin secretion, with short-term increases, and chronic decreases (129, 629, 895). FFAs have effects on the expression of PPARα, glucokinase, the GLUT2 glucose transporter, prepro-insulin, and pancreatic/duodenal honeobox-1 (PDX-1) (880). FFAs also serve as a ligand for PPARα, which may modulate insulin secretion (340, 497).

In addition to FFA-induced β-cell dysfunction, accumulation of excess FFA also causes β-cell apoptosis. In Zucker diabetic fatty (ZDF) rats, TAG accumulation in islets is associated with decreased β-cell mass and decline in insulin production with evidence for DNA laddering (798). *In vitro*, excess FFA leads to apoptosis in primary rat pancreatic β-cells and β-cell lines (628, 708) and in isolated human islets (475).

1.6.2.5. Lipotoxicity in the heart

In humans, the cardiac lipid overload that occurs with inherited defects in the mitochondrial fatty acid oxidation pathway is associated with heart failure and sudden death (366). Lipid accumulation in cardiomyocytes of mice that are null for long-chai acyl-coenzyme A dehydrogenase leads to replacement fibrosis pathologically, which may provide foci for initiation of arrhythmias (407). Cardiac dysfunction is also observed in obese *fa/fa* ZDF rats, which presents with lipid accumulation in the cardiac myocytes that is followed by evidence of apoptotic cardiomyocyte death and a modest decrease in systolic function (896). Apoptosis can occur in cell culture systems (593) and is induced by palmitate (170, 304, 741).

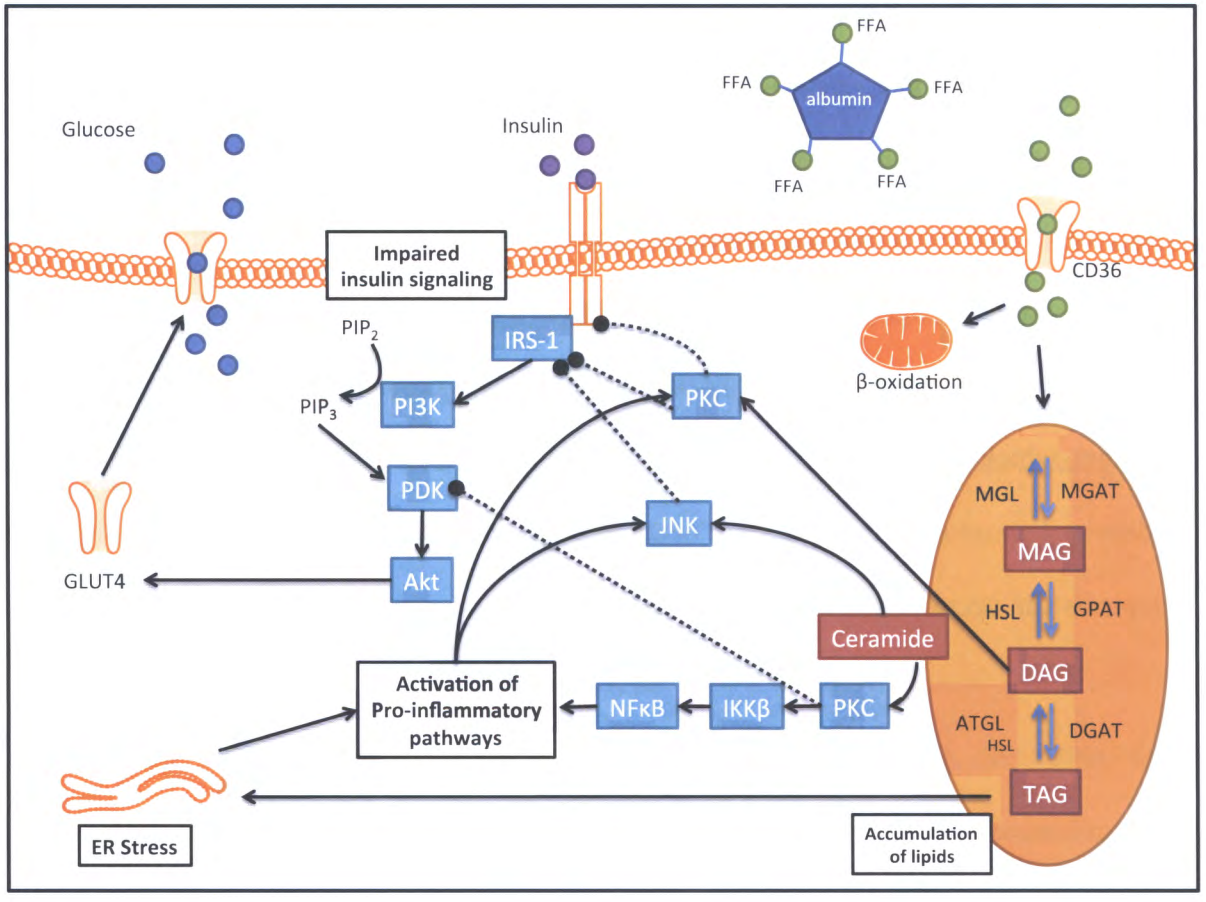


Figure 1.3: Generalised pathway for insulin-stimulated glucose uptake and lipotoxicity

Simplified overview of intracellular signalling cascades involved in skeletal muscle insulin signalling, and insulin resistance induced by lipid metabolites. Free fatty acids (FFA) are bound to albumin in the circulation; they enter the cell through transporters, where they are directed to either β-oxidation or incorporation into the lipid pool. The process of esterification involves the addition of a FFA at each step until triacylglycerol (TAG) is created, monoacylglycerol (MGAT) joins a glycerol and a FFA, glycerol phosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) add the remaining FFA to produce TAG. TAG is hydrolysed primarily by adipose tissue lipase (ATGL) to liberate a FFA and generate diacylglycerol (DAG). Hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL) liberate the remaining FFA from glycerol. In obesity, lipid oversupply leads to an accumulation of lipids within the cell, increasing the amount of lipid metabolites such as DAG and ceramide. They two metabolites lead to the activation of protein kinase C (PKC) and pro-inflammatory pathways such as c-Jun N-terminal kinase (JNK), IκB kinase-beta (IKKβ) and nuclear factor kappa-B (NFκB). These impair the normal insulin-signalling cascade, leading to impaired glucose uptake. The lipid accumulation also leads to ER stress, further activating proinflammatory pathways. CD36: cluster of differentiation 36, IRS-1: insulin receptor substrate 1, PI3-K: phosphatidylinositol 3 kinase, PDK: phosphatidylinositide-dependent kinase, PIP_2: phosphatidylinositol 4,5-bisphosphate, PIP_3: phosphatidylinositol 3,4,5-triphosphate, ER: endoplasmic reticulum, GLUT4: glucose transporter type 4.

1.6.2.6. Lipotoxicity in the central nervous system

There is increasing evidence for the ability of lipid storage in neurons. Two-thirds of the seventy or so inborn errors of lysosomal function affect the nervous system. Tay-Sachs disease (659, 777) and Sandhoff disease (669) arise from a deficiency of the lyosomal acid hydrolase, β-N-acetylhexosaminidase; that are characterized by neuronal accumulation of GM2 ganglioside and related glycconjugates (428, 481, 767). Niemann-pick disease results in unesterified cholesterol and other lipids become sequestered in late endosomes and/or lysosomes in neurons (359) and glial cells (358, 595). Metachromatic leukodystrophy is characterized by C18:0 fatty acid containing 3-O-sulfogalactosylceramide accumulation in neurons, which contributes to the hyperexcitability and axonal degeneration phenotype seen during the lysosomal storage disorder (182). Therefore, neurons are capable of lipid storage, which contribute to disease pathologies.

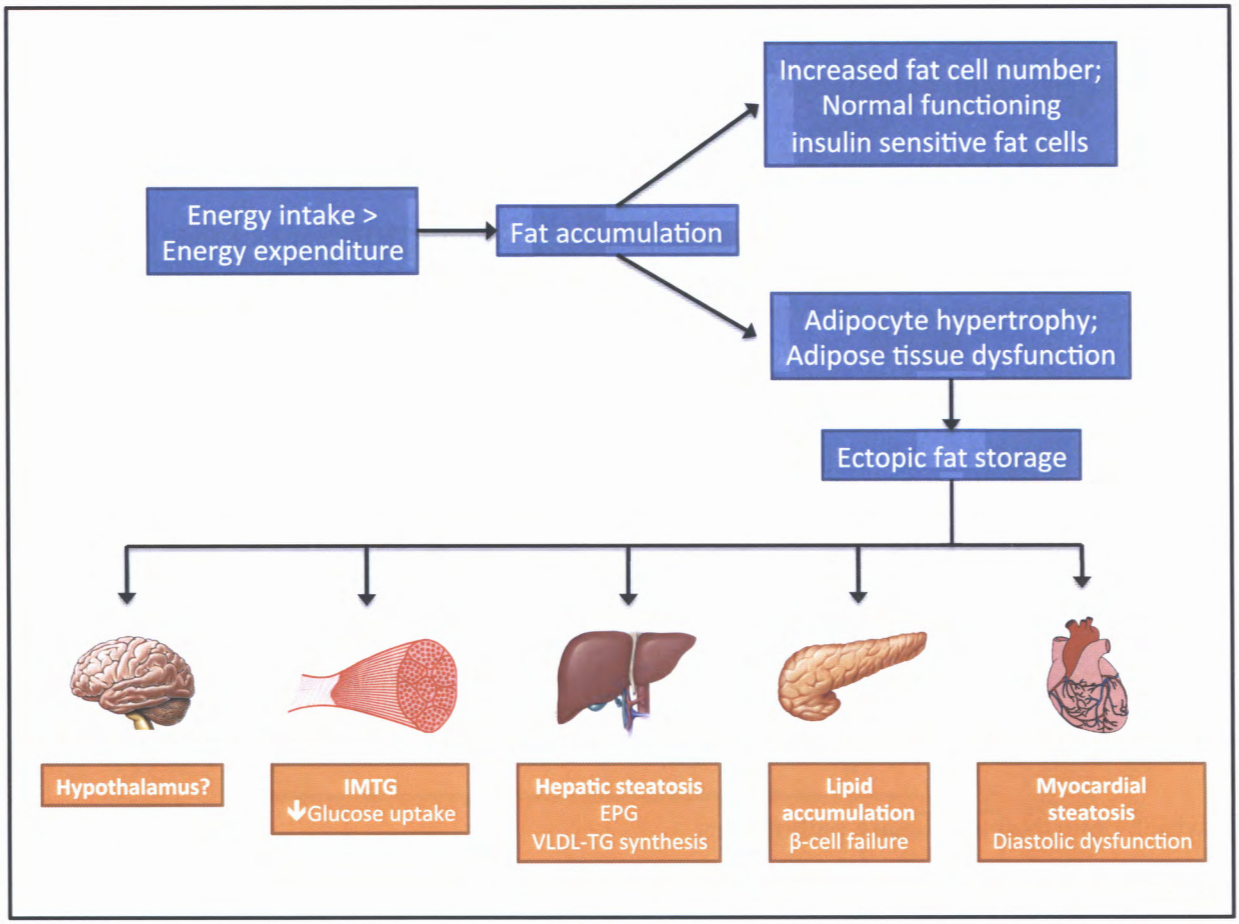


Figure 1.4: Summary of the consequences of lipid accumulation in non-adipose tissue

Once the balance between energy intake and expenditure is disrupted, lipids begin to accumulate in adipose tissue. This can lead to a 'healthy' increase in adipocyte number, or an 'unhealthy' expansion of the adipose tissue. The latter ultimately leads to lipid accumulation in non-adipose tissue, the consequences of which are organ specific. EGP: endogenous glucose production, VLDL-TG: very low-density lipoprotein-triglyceride, IMTG: intramuscular triacylglyceride.

Obesity can have deleterious effects on the CNS, and recent evidence suggests that obesity rates are higher in children that develop epilepsy (161). Sustained excess caloric intake adversely effects cognitive function, and a sedentary lifestyle exacerbates these effects (490). Several neurological disorders are characterized by defective lipid metabolism, and their prevalence is increased with obesity. For example, long chain ceramides (C_{18-24}) and cholesterol are increased in the brain during Alzheimer's disease (155, 285, 286), HIV-dementia (291), stroke and ageing (155, 675), and ceramide accumulation is detrimental to neuronal cell function via the induction of apoptosis (287). Dysregulation of cholesterol homeostasis in the brain has been linked to chronic neurodegenerative disorders, including Alzheimer's disease (386, 725), Huntington's disease (355, 802, 803), Parkinson's disease (165, 323, 819), as well as to acute neuronal injuries such as stroke and brain trauma (819).

As discussed previously, lipid metabolism and lipid sensing is a vital process in the hypothalamus. Surprisingly, few studies have investigated whether lipotoxicity occurs in the hypothalamus during obesity, instead focusing on lipid sensing in the hypothalamus in obesity (62, 415, 568, 570, 630). Holland, et al. (307) showed that DAGs and ceramides accumulated in the hypothalamus during a 6 hour intravenous lard infusion in a toll-like receptor 4 (TLR4) dependent manner. These results were recapitulated in a high fat diet model (307). While Posey, et al. (624) showed increased hypothalamic palmitoyl-CoA with a 4 hour palmitate ICV infusion, and elevated hypothalamic palmitoyl- and stearoyl-CoA with high-fat feeding. Furthermore, ceramide metabolism in the ARC appears to play a role in leptin's central control of feeding (243). Therefore, there is evidence to suggest that lipids can accumulate in the hypothalamus with high-fat feeding, however the types of lipids that accumulate, and their functional consequences remain largely unknown.

1.7. Exercise training as a tool for obesity treatment

Individuals with obesity and type 2 diabetes have at least twice the risk of premature death, heart disease and stroke compared with the general population. Diabetes prevalence is 3- to 5- fold higher in obese than in normal weight individuals (530). Many of the complications associated with type 2 diabetes can be prevented through regular exercise training, healthy diet and weight-loss (383, 411, 791), with improvements exceeding the benefits of current diabetes medications (383). At least 150-minutes of moderate-intensity physical activity per week is associated with a lower prevalence of the metabolic syndrome (325). This relationship also holds true for increased fitness level and a lower risk of developing metabolic syndrome (223). The metabolic syndrome is the name for a group of risk factors that occur together and increase the risk for coronary artery disease, stroke and type 2 diabetes (900).

There is strong epidemiologic data showing that both regular exercise training and maintaining a healthy body weight greatly reduce the risk of developing type 2 diabetes (674, 703, 840). Additionally, maintaining a higher level of fitness in patients with type 2 diabetes greatly reduces the risk of developing cardiovascular disease (CVD) (126, 838). Church, et al. (126) demonstrated that although there is a strong inverse trend for the risk of CVD mortality across different levels of fitness; in the lower fitness levels, a hazard ratio of 1.2 for CVD mortality was associated with each incremental 1-MET (metabolic equivalent of task; a measure expressing the energy cost of physical activity) difference in fitness. On the other hand, in moderate to high levels of fitness, the large differences in risk factors associated with 1 MET differences largely diminishes. Therefore, unfit individuals with type 2 diabetes stand to benefit substantially from small increases in fitness (126).

1.7.1. Exercise training, lipids and insulin resistance

As discussed previously, the accumulation of IMTG is associated with insulin resistance (337, 400, 588, 614). However, it is not synonymous with obesity given the fact that endurance-trained athletes, who are highly insulin sensitive, also have high IMTG content (260, 314, 780, 812). Rather, the capacity to oxidize fatty acid-derived IMTG appears to determine whether they play a physiological or pathological role. In endurance-trained athletes, IMTG are an adaptive response serving as a readily available energy source. This is supported by the fact that they are located in close proximity to the mitochondria (772). In these athletes, IMTGs are not deleterious because of the increased capacity to oxidize lipids (20). In insulin resistant/type 2 diabetics, the increased IMTG are mainly a result of increased FA availability (364, 722, 723), and the suggested impaired FFA oxidation in morbidly obese patients (326, 364, 747), leading to accumulation of lipid intermediates, interfering with insulin signalling, as discussed previously.

Diet and exercise has long been prescribed for the management of obesity and type 2 diabetes. Despite minimal weight-loss with exercise only interventions (1-3kg), studies consistently show increased insulin-stimulated glucose disposal in both obese normal glucose tolerant (179) and intolerant subjects after training (180, 734). These studies also showed increased IMTG with a decrease in intramuscular ceramide and DAG levels along with increased lipid oxidation rates (179, 180). Others have shown improved glucose tolerance with reduced muscle DAG and ceramides, with no change in TAG in obese individuals after 8 weeks of training (94). However, a recent study showed muscle DAGs to be higher in highly trained athletes, which corresponded with higher insulin sensitivity (19), suggesting a more complex role of DAG in insulin action.

As discussed previously discussed, obesity leads to hepatic steatosis, lipid accumulation in the liver, which leads to impaired hepatic insulin signalling and abnormal glucose production. Six weeks of exercise training has been shown to improve peripheral and hepatic insulin sensitivity, despite no changes in hepatic lipid content (717). This is in contrast with another study showing a 21% reduction in hepatic TAG with one month of aerobic exercise training (347). Therefore, these studies highlight the profound effects that exercise training during obesity and type 2 diabetes, can have on whole body, and organ specific insulin sensitivity, despite limited changes lipid accumulation.

1.7.2. Metabolic response to exercise

The increase in contractile activity associated with physical exercise is a profound regulator of several metabolic functions in skeletal muscle, including glucose transport and glycogen metabolism. Exercise can also induce transient changes in the gene expression and can alter the rates of protein metabolism, both of which may be responsible for the chronic adaptations in skeletal muscle to repeated bouts of exercise. These will be discussed in the following section.

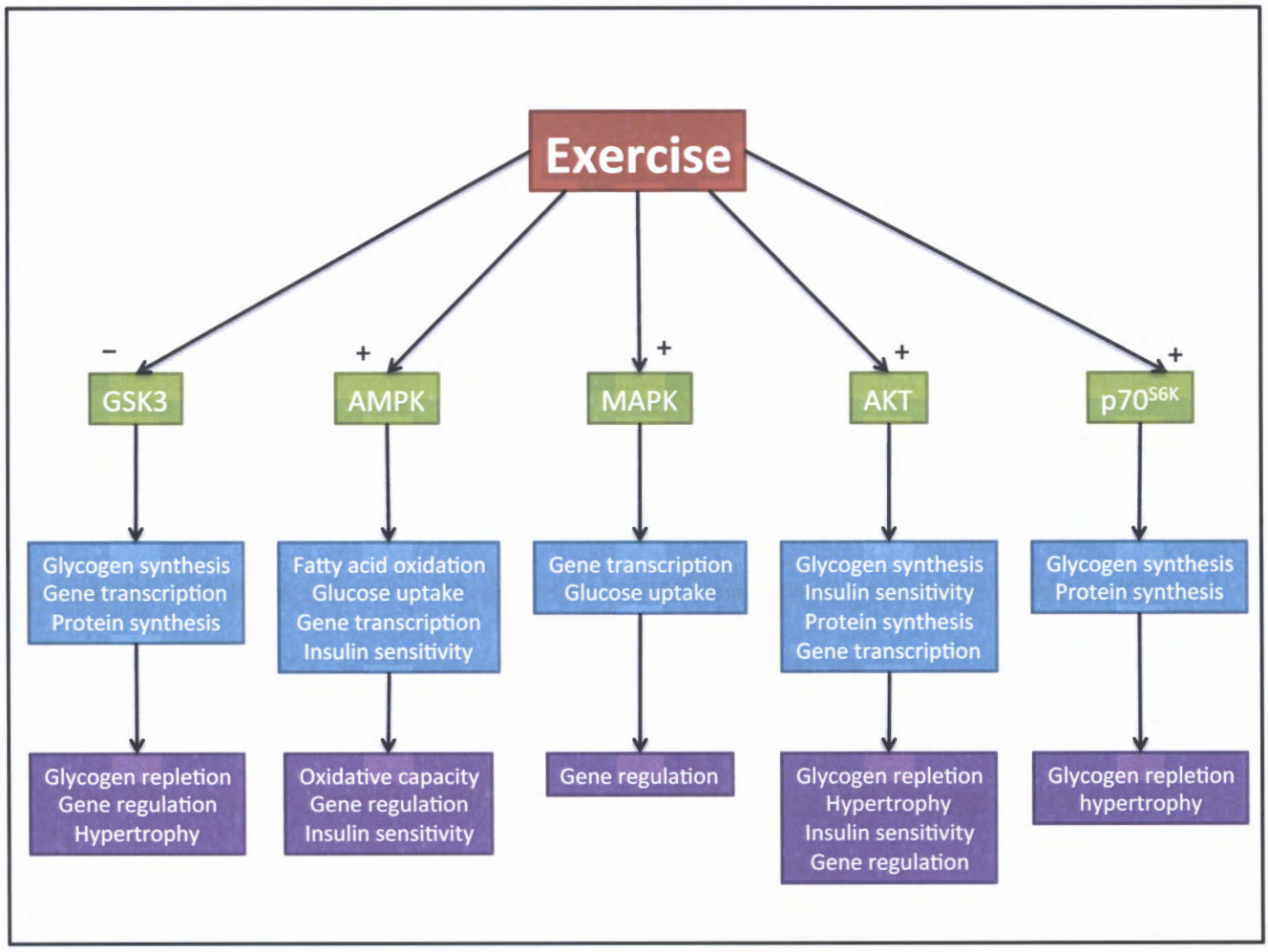


Figure 1.5 Exercise regulates multiple signalling molecules in skeletal muscle

Putative intracellular functions that may be regulated by each molecule are shown. The blue row includes functions that may utilise these signalling proteins in response to an acute bout of exercise in skeletal muscle. The purple row list functions regulated by these proteins from chronic adaptations to exercise. GSK3, glycogen synthase linase-3; AMPK, 5' adenosine monophosphate-activated protein kinase; MAPK, Mitogen-activated protein kinase; p70S6K, 70-kDA S6 protein kinase, +, positive regulation; −, negative regulation

Exercise and muscle contraction *per se* leads to alterations in skeletal muscle metabolism and alters the metabolic capacity of muscle. Acute exercise leads to an immediate increase in glucose transport in skeletal muscle, mediated via an insulin-independent translocation of GLUT4 to the cell surface (176, 398, 897).

Muscle contractions alter the energy state of the cell, increasing energy expenditure. AMPK is activated and its nuclear abundance increases during exercise when the AMP:ATP ration rises (295, 468, 498). APMK acts as a metabolic master switch, increasing cellular energy levels by inhibiting energy consuming pathways (FFA synthesis, protein synthesis etc.) and stimulating energy producing pathways (FFA oxidation, glucose transport). In addition, many biochemical adaptations of skeletal muscle during acute and chronic exercise are thought to be mediated in part by AMPK activation.

These include increased mitochondrial biogenesis and capacity (65, 309, 901), increased muscle glycogen (311), and increased GLUT4 and hexokinase II expression (576, 755), involved in glucose uptake.

Muscle contractions are initiated by depolarisation of the plasma membrane, triggering the release of calcium from the sarcoplasmic reticulum. This spike in intracellular calcium leads to the interaction of actin and myosin filaments, resulting in the development of tension in the muscle fibres. PKC is a calcium dependent signalling intermediary that is activated during muscle contractions (132, 609, 643, 649). PKC is involved in contraction- but not insulin-stimulated glucose uptake (332, 857). Calcium/calmodulin-dependent protein kinase II (CaMKII) is also activated in response to elevated levels of cytosolic calcium during muscle contractions (124, 649, 650). CaMKII is an important regulator of exercise-induced

upregulation of GLUT4 to enhance glucose transport capacity and insulin sensitivity (575). It is also involved in the upregulation of FFA uptake and oxidation in contracting rat muscle (635)

Skeletal muscle contractions stimulate numerous mitogen- and stress-activated protein kinases. Exercise in both rodents and humans, as well as contraction of isolated rodent muscle *in vitro*, activate the extracellular-signal regulated kinase (ERK) (582, 849), the p38 mitogen-activated protein (MAP) kinase (262) (33, 294, 888), and c-Jun NH_2-terminal kinase (JNK) (32, 262, 888) and NF-κB (306, 771, 821) signalling cascades. These signalling pathways couple cellular stress to changes in transcriptional activity, and therefore, exercise training induced changed in gene expression can be regulated through the activation of these kinases. They have been implicated in growth and cell proliferation (656), muscle protein turnover (100), apoptosis, inflammation and DNA repair (356), and may confer adaptive alterations in glucose and lipid metabolism through the activation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (14, 636, 656, 792)

1.7.3. Exercise and the role of contraction-induced myokines

Exercise results in a complex range of physiological responses. Contracting skeletal muscle expresses a number of cytokines and other peptides referred to as myokines, which can be secreted into the circulation. These muscle-derived signals may be involved in 'metabolic cross talk' and influence functions in other tissues. 635 secreted proteins have been identified from skeletal muscle (298). Muscle contractions lead to the release of the cytokine interleukin-6 (IL-6) (596), which play a role in inducing lipolysis and gene transcription in adipose tissue (809, 858), improving whole-body insulin sensitivity and reducing inflammation (596, 746). Another cytokine released by the muscle during exercise is

interleukin-8 (IL-8) (114, 562), which may have a role on endolethial cells to stimulate angiogenesis (233). Contracting skeletal muscle also release BDNF (489, 597) (216, 698), a neurotrophin acts in the brain to reduce food intake and promote neurogenesis (601) and regulate blood glucose and lipid metabolism (790). Irisin, a recently discovered myokine, is also released from contracting muscle (82), and plays a role in the browning of white adipose tissue to increase energy expenditure (82). Another recently discovered myokine, myonectin (700), is secreted during voluntary wheel running in mice, and promotes FFA uptake into adipose tissue and hepatocytes by upregulating the expression of FFA transporters (CD36, FATP1, FABP1 and FABP4) (700). Therefore, contracting skeletal muscle secret a number of factors that can impact other organs.

1.7.4. Exercise training, satiety and central hormone sensing

The first studies looking at the relationship between exercise and food intake were performed in the early 1950s and showed that treadmill running of varying intensities had different effects on food intake, such that 1h or less per day reduced food intake, more than 1h per day increased food intake (494). It was also shown that humans employed in jobs requiring physical labour were able to match their energy intake with energy expenditure compared to those with sedentary jobs (495). Since then it has been show that voluntary wheel running prevents obesity-induced hyperphagia (70, 373). In the short term, exercise does not appear to effect hunger and food intake in humans, but during vigorous exercise (>60% VO_2 max) a phenomenon termed exercise-induced anorexia occurs where there is reduced appetite for a short time following the exercise (376, 466, 640, 783, 845).

Exercise training can also have profound effects on hormone signalling in the hypothalamus. An acute swimming exercise bout increases hypothalamic sensitivity to ICV

leptin and insulin, as seen through reduced food intake and enhanced signalling via STAT3 and PI3K compared with sedentary controls (219). While obese rodents are unable to reduce their food intake in response to ICV insulin (central insulin resistance)(95), exercise restores the central effects of insulin on reduced food intake in obesity (648). The authors suggested that this was mediated through reduced inflammation in the hypothalamus (IKKβ/IκBα) and reduced markers of ER stress (648). Two weeks of voluntary wheel running acutely increases leptin signalling in the hypothalamus in aged obese rats (705). Six weeks of voluntary wheel running improved the food intake response to leptin in both and high-fat fed mice (393). Eight weeks of treadmill running improved both leptin and insulin signalling in the hypothalamus of dexamethasone-induced diabetic rats (589). Of particular note, in all of the above-mentioned studies, hypothalamic sensitivity was measured acutely after an exercise bout, and therefore the effect of exercise training on chronic changes in the hypothalamus is yet to be determined.

Interestingly, one group suggests that exercise training in young mice induces stable changes in the hypothalamus, which enhance leptin sensitivity into adulthood. Just 3 weeks of voluntary wheel running in the early post weaning age in rat was enough to improve leptin signalling in the hypothalamus and protect again diet induced obesity into adulthood, in a cohort selectively bred to develop obesity (592). This was ascribed to prolonged increases in central leptin sensitivity and signalling in the hypothalamus (591). These results lead to the possibility of neuronal reprogramming with exercise training.

1.8. Neurogenesis and Exercise

Neurogenesis is defined as the process by which neurons are generated from neural stem cells and progenitor cells, and is an essential component of CNS development. Stem

cells in the developing embryo undergo asymmetric division to form the embryonic notochord, neural tube, and neural crest; they must also migrate to their ultimate destinations. Once there, the process of developmental neurogenesis continues with the differentiation of stem cells into mature postmitotic neurons, aggregation, synaptogenesis, and synaptic pruning (310). From the beginnings of modern neuroscience in the late 19th century, it was assumed that the mammalian CNS became structurally stable soon after birth and remained that way throughout life. However, the last decade has seen an explosion research examining the mechanisms that contribute and control adult neurogenesis. The finding of neural stem cells in adult brain largely fuelled this interest (203). Subsequent studies showed that adult neuronal stem cells are capable of proliferation and differentiation into mature neuronal phenotype (735). Stem cells in the adult brain appear to be to be limited in number and isolated to specific brain regions, most prominently in the subventricular zone (SVZ) that surrounds the rostral end of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus. Proliferating cells can be identified using immunohistochemistry to track the uptake of the thymidine analogue bromodeoxyuridine (BrdU) or the expression of Ki67, a protein expressed in cells that are in all active phases of the cell cycle but not expressed in cells in G_0.

It is known that a variety of factors, including age, stress physical activity, antidepressant drugs, brain injury, stroke, seizure, and energy intake regulate adult neuronal stem cell proliferation, survival, and differentiation.

1.8.1. Adult neurogenesis in the hippocampus and olfactory bulb

The SVZ and the SGZ are important brain regions with significant neurogenesis potential that are critical for normal CNS function. The SVZ supplies neuroblasts that migrate

along the rostral migratory stream toward the human and rat olfactory bulb, where they differentiate into interneurons (847) and participate in olfaction. Stem cells originally proliferating in the SGZ of the dentate gyrus migrate into the granular cell layer and differentiate into neurons that are integrated into the hippocampal circuitry (Figure 1.6) (459, 847), a region of the brain known to participate in learning and memory. As part of the limbic system, both the olfactory bulb and the hippocampus regulate emotion.

1.8.2. Adult neural stem cells and progenitors

Neural stem cells within the adult nervous system can self renew and differentiate into several cell types, including neurons, astrocytes, and oligodendrocytes (240). Neurons are functional components of the nervous system and are responsible for information processing and transmission. Astrocytes and oligodendrocytes are collectively known as glial cells, providing supporting roles that are essential for the proper function of the nervous system. The term "neural progenitor' has been used to loosely describe all dividing cells with some capacity for differentiation.

1.8.3. The neurogenic niche

For a long time, neural stem cells were only isolated from the SVZ and SGZ. It was hypothesized that the microenvironments of the SVZ and SGZ, known as the neurogenic niche (refer to Figure 1.6), may have specific factors that are permissive for the differentiation and integration of new neurons (542). In the SGZ, adult hippocampal progenitors are closely opposed to a dense layer of granule cells that includes both mature and newborn immature neurons, astrocytes, oligodendrocytes and other types of neurons. Hippocampal astrocytes promote the neuronal differentiation of adult hippocampal progenitor cells and their integration (736) possibly through Wnt signaling (458).

The SVZ progenitor cells are adjacent to the ependymal cell layer of the lateral ventricles, which express the protein Noggin that promotes SVZ neurogenesis by antagonizing signalling of the bone morphogenetic proteins (BMPs) (460). They also promote the self-renewal of adult neural stem cells through pigment epithelium-derived factor (PEDF) (633).

Proliferating cells and putative neural progenitors in both SGZ and SVZ are closely associated with the vasculature, indicating that factors released from the blood vessels may have a direct impact on adult neural progenitors (18, 587). Infusion of vascular endothelial growth factor (VEGF) promotes cell proliferation in the SGZ and SVZ, which can be blocked by a dominant-negative VEGF receptor 2 (105). VEGF is also required for increased neurogenesis in adult mice exposed to an enriched environment (205), a manipulation of the rearing environment that includes enhanced physical activity and sensory, cognitive, and social stimulation (816).

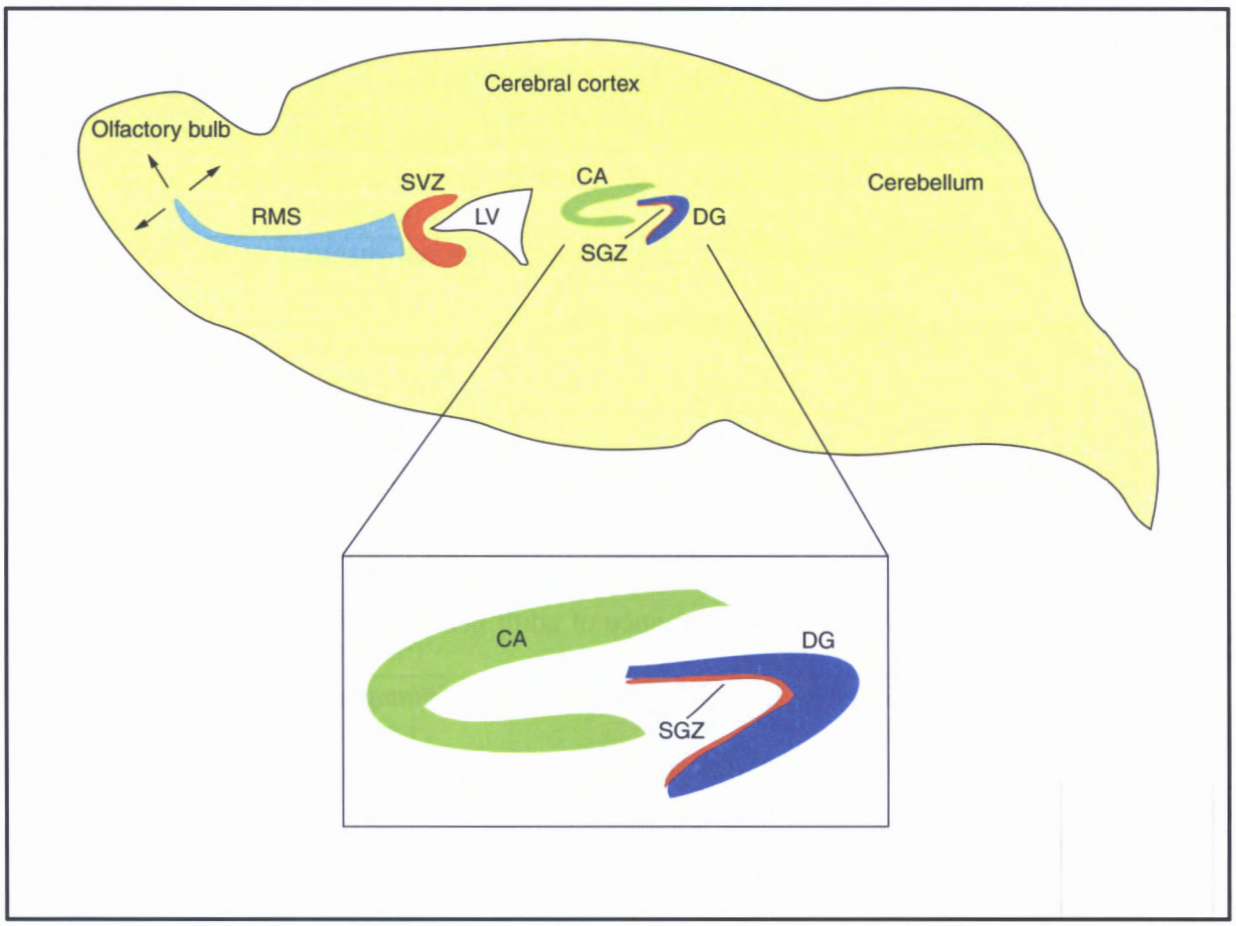


Figure 1.6: Neurogenic zones in the adult mouse brain

Adult neurogenesis is best characterised in two zones in the adult brain: the subventricular zone (SVZ) adjacent to the lateral ventricle (LV), where neurons are produced that migrate to the olfactory bulb via the rostral migratory stream (RMS); and the dentate gyrus (DG) of the hippocampus. The hippocampus (shown enlarged in the insert) consists of two interleaved layers of cells- the pyramidal cell layer (CA) and the DG. Proliferating neural precursors and quiescent neural stem cells are found in the zone immediately adjacent to the DG celled the subgranular zone (SGZ). Reproduced from (627).

Studies of neurogenesis in animal models of depression have revealed the importance of the BDNF-TrkB pathway in regulating neurogenesis. Infusion of BDNF into the hippocampus increased neurogenesis and elicited antidepressant responses in several behavioural tests (678, 716). BNDF also promotes neuronal differentiation and survival in cultured NSCs derived from adult SVZ (374, 448).

1.8.4. The influence of growth factors on neurogenesis

Growth factors such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) are potent factors for the maintenance of adult neural stem cells *in vitro*. *In vivo*, both factors promote proliferation in the SVZ, but only FGF2 increases the number of neurons in the olfactory bulb (405). Adult neural progenitors can be regulated by a variety of extrinsic factors. Signalling through the sonic hedgehog pathway may regulate adult neurogenesis (12, 46). The neurotrophin BDNF is a key positive regulator of adult neurogenesis (299, 661, 678). Mice deficient in p75, one of the BDNF receptors, have smaller olfactory bulbs and decreased neurogenesis in the SVZ (see Table 1.2)

1.8.5. Function of adult neurogenesis

SVZ neurogenesis is regulated by the olfactory experience of animals (464, 465). Deprivation of olfactory sensory inputs hinders maturation and survival of newborn neurons in the olfactory bulb. Enriched odour exposure increases the survival of newborn neurons and transiently improves odour memory.

Table 1.2 Growth factors and trophic factors involved in neurogenesis

Growth factor	Reference
Epidermal growth factor (EFG)	(405, 757)
Fibroblast growth factor 2 (FGF2)	(104, 209, 405)
Brain derived neurotrophic factor (BDNF)	(58, 299, 661, 678, 679, 882)
Neurotrophin 3 (NT3)	(711).
Vascular endothelial growth factor (VEGF)	(105, 205)
Nerve growth factor (NGF)	(110, 111)
Insulin-like growth factor 1 (IGF-1)	(324)

Many genetic and environmental factors that affect hippocampal neurogenesis cause corresponding changes in cognitive performance. FGF receptor deficient mice have decreased proliferation in the hippocampus and manifest with defective memory consolidation (894). Likewise, neurotrophin-3 (NT-3) deficient mice also show decreases neuronal differentiation and survival, and manifest with defective learning (711). Exposing mice to an enriched environment increases neuronal survival and improves learning and memory (368). Conversely, stress (psychological; or unexpected chronic mild stress) decreases neurogenesis and impairs spatial memory (574). Ageing is also associated with decreased neurogenesis and impaired learning and memory (177, 178), which may be related to the decline in insulin-like growth factor 1 (IGF-1) with ageing. Therefore, hippocampal neurogenesis plays a crucial role in the function of the CNS. However, the requirement for neurogenesis for other CNS functions is unknown.

1.8.6. Enhancing neurogenesis to treat disease

It is well established that neurogenesis can occur throughout adult life and that it plays a crucial role in CNS function. The question arises of how neurogenesis can play a role in neurological/neurodegenerative disorders, such as depression or Alzheimer's disease, and if neurogenesis could represent a novel target for therapeutics. This will be discussed in the following section.

1.8.6.1. Depression

Major depression is frequently associated with significant atrophy within the hippocampus, which can persist for several years after remission from depression episodes (586). Both a reduction in hippocampal volume and a decrease in neurogenesis have been reported in subordinate tree shrews subjected to social interaction stress (157, 234, 586). This leads to the hypothesis that depression and declining neurogenesis in the hippocampus is causally linked (214).

Antidepressant treatment can increase neural plasticity, promote adult neurogenesis, block stress-induced decrease of neurogenesis and upregulate cyclic Amp-CREB cascade with proliferative effects (106). Chronic antidepressant treatments increase cell proliferation in the SGZ. (833). Chronic fluoxetine administration increases the proliferation of hippocampal progenitors and the survival of newborn neurons in mice (197). Acute treatments with serotonin receptor agonists, which are putative antidepressants, also increase neurogenesis (472). Furthermore, antidepressants are able to prevent or reverse stress-induced decreases in neurogenesis (833). Furthermore, ablation of hippocampal neurogenesis renders antidepressants inactive in behavioural paradigms for antidepressant responses and anxiety-like behaviours in mice (250, 670). However, the dependence of behavioural effects of

antidepressants on neurogenesis is influence by factors such as species, the genetic background of the animal, the nature of the antidepressant, and the type of behavioural paradigms (516).

1.8.6.2. Neurodegenerative diseases

Neurodegenerative diseases are characterised by a net loss of neurons from specific regions of the CNS. Until recently, research has focused on identifying mechanisms that lead to neurodegeneration, while therapeutic approaches have been primarily targeted to prevent neuronal loss. Recent studies suggest that in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, neural stem and progenitor cell proliferation and neuronal differentiation is altered (174, 252, 344, 702, 881, 884). Stimulating neurogenesis in models of Alzheimer's disease reduces the appearance of amyloid plaques, and appears to contribute to functional recovery with improvements of memory and learning capabilities (146, 345, 425).

1.8.7. Exercise and cognitive function

A sedentary lifestyle is accompanied by increased risk for cardiovascular, metabolic and metastatic diseases (17, 626). Reducing energy intake (dieting) and exercise is the first line treatment for inducing weight loss. (222), and the incidence of cancer, diabetes, and heart disease can be reduced by physical activity (80, 749). The beneficial effects of physical exercise extend beyond the periphery to the CNS, having significant effects on human brain function and structure.

In both humans and rodents physical activity enhances cognition (645, 765, 856), counteracts age-related memory decline (392, 422, 818), delays onset of neurodegenerative

diseases (7, 231, 360, 785), enhances recovery from brain injury (77, 258, 267) and depression (39, 422).

Older adults frequently experience cognitive defects accompanied by deterioration of brain tissue and function in a number of cortical and subcortical regions (ref). The results of epidemiological studies are remarkably consistent in revealing that high physical activity is associated with improved cognitive function in older adults (11, 133, 732). There is a strong positive correlation between physical fitness, cognitive performance and brain volume in humans (263, 658). Indeed five months of regular exercise training increases cognitive function and vascular volume in the cortex in middle aged cynomologus monkeys (642).

1.8.8. Exercise training increases hippocampal neurogenesis

Kempermann and colleagues (369) carried out the first study that showed a positive effect of environmental enrichment on neurogenesis and enhanced cognition. Environmental enrichment has many aspects, including increased opportunity for learning, socialization and physical activity. It was not known which one of these variables contributed to the beneficial effects of enrichment on neurogenesis. Similar to environmental enrichment, voluntary wheel running enhanced the survival of newborn neurons in the dentate gyrus (814). The neurogenesis also occurs in an exercise 'dose dependent' manner (15, 641). Exercise induced hippocampal neurogenesis peaks after 3 days of wheel running, and continues over a 32-day period (399). The enhancement of hippocampal neurogenesis by running is well documented and substantiated (205, 378, 584, 787, 806).

The exercise-induced increase in neurogenesis is associated with enhanced hippocampal synaptic plasticity. In particular, long-term potentiation is enhanced by physical

activity (815). The beneficial effects of exercise on learning and memory in humans and animals may be mediated by enhanced synaptic plasticity of individual newborn neurons, and exercise associated increases in dentate granule cell number (ref).

Exercise training has the potential to reverse age and Alzheimer's disease related hippocampal decline (639). Physical activity can reinstate hippocampal function by enhancing the expression of BDNF and other growth factors that promote neurogenesis, angiogenesis and synaptic plasticity. For example, physical activity has been identified as a protective factor against cognitive impairment and dementia (420, 677). Wheel running in rodents results in a 3-4 fold or even greater increase in the production and survival of new neurons in the dentate gyrus of the hippocampus (813).

1.8.9. Hypothalamic neurogenesis and body weight regulation

For a long time, adult neurogenesis was thought to be confined to the SGZ in the hippocampus and the SVZ. In recent years, other potential neurogenic areas have been discovered, the hypothalamus being one of them. The administration of trophic factors such as BDNF, CNTF and IGF-1 into the brain can induce or promote adult neurogenesis in the hypothalamus. These will be discussed further.

Neuronal stem cells have been identified in the hypothalamus, and hypothalamic neurogenesis has been described to occur at a low rate the SVG (388). Several neurotrophic factors are able to enhance the generation of new neurons in the adult hypothalamus when administered ICV. BDNF infusion into the lateral ventricle enhances the generation of new cells in the hypothalamus two fold (601) while CNTF produces a 4 to 5 fold increase of new

cells, depending on the mode of BrdU administration (387, 388). The latter effect was also evident in cultured neurons from adult mouse brain (ref).

CNTF promotes neuronal survival (419, 701) and the maintenance of neuronal stem cells (710). It also activates signalling pathways in the hypothalamus involved in feeding and energy homeostasis (73, 416). Axokine (an analogue of CNTF developed as a drug candidate for the treatment of obesity) induces reduced and sustained body weights in humans' weeks to months after the cessation of treatment in obese mice and humans (204, 416). This profound effect of CNTF on weight loss was recapitulated in obese mice, was ascribed to neurogenesis in the hypothalamus (387), because coadministration of the mitotic blocker cytosine-β-D-arabinofuranoside (AraC) eliminated the proliferation of neural cell and abolished the long-term, but not short-term effect of CNTF on body weight. These new neurons expressed POMC and NPY, and were leptin responsive. Similarly, infusion of BDNF into the lateral ventricle also resulted in a pronounced increase in the number of new-born neurons in the hypothalamus (601). The ability for BDNF treatment to reduce bodyweight has been well documented (266, 370, 418, 600). IGF-1 infusion into the lateral ventricle increases adult neurogenesis in hypothalamus (605), as does EFG and bFGF (605), although the functional significance is unknown. This highlights the functional significance of hypothalamic neurogenesis in regulating food intake and energy metabolism.

1.9. General Hypothesis

This thesis seeks to investigate the role that obesity and exercise training have on the structure and function of the hypothalamus.

The specific aims of this thesis are:

Chapter 3.

To determine whether lipid accumulation extends to the hypothalamus in the setting of rodent obesity during high-fat feeding and leptin deficiency, and whether regular exercise training can alter this accumulation.

Chapter 4.

To examine and characterize whether exercise training can induce neurogenesis in the hypothalamus of lean, obese and aged mice; and to determine whether exercise-induced hypothalamic neurogenesis plays a functional role in whole body insulin sensitivity.

Chapter 5.

To determine to role of centrally administered CNTF in whole body insulin action and glucose metabolism

Chapter 6.

To examine whether exercise training can improve the hypothalamic response to peripheral hormones in lean and obese mice.

Chapter Two: Methodological considerations for assessing neurogenesis

For decades, tritiated ([^{3}H]) thymidine autoradiography has been used to study cell proliferation *in situ*, and to determine the time of origin, migration lineage and fate of neuronal cells in the developing central nervous system (42, 56, 621). [^{3}H] thymidine autoradiography also provided the first evidence that neurogenesis occurs in discrete areas of the rodent adult mammalian brain, the SVZ and the DG of the hippocampus (145, 484). The use of a radiolabelled substrate and the time-consuming process involved in [^{3}H] thymidine autoradiography mandated the development of new strategies to study cell proliferation and neurogenesis.

5-Bromo-2'-deoxyuridine (BrdU) is a halopyrimidine used therapeutically as an antiviral and antineoplastic agent (57, 230) (Figure 2.1). It is a thymidine analogue that incorporates into DNA of dividing cells during the S-phase of the cell cycle. As such, BrdU is used for the birth dating and monitoring of cell proliferation. BrdU can be detected by immunohistochemistry using an antibody directed against single-stranded DNA containing BrdU. BrdU offers several advantages over [^{3}H] thymidine autoradiography. It allows faster studies without handling radioactive materials, and the detection of labelled cells throughout the relatively thick tissue sections (20-40μM) required for stereological studies of the brain. This has lead to the wide use of BrdU for studying adult neurogenesis and is currently the most widely used technique. However, it is not without pitfalls and limitations. BrdU is a toxic and mutagenic substance that induces many side effects, however, these limitations can be overcome and will be discussed below.

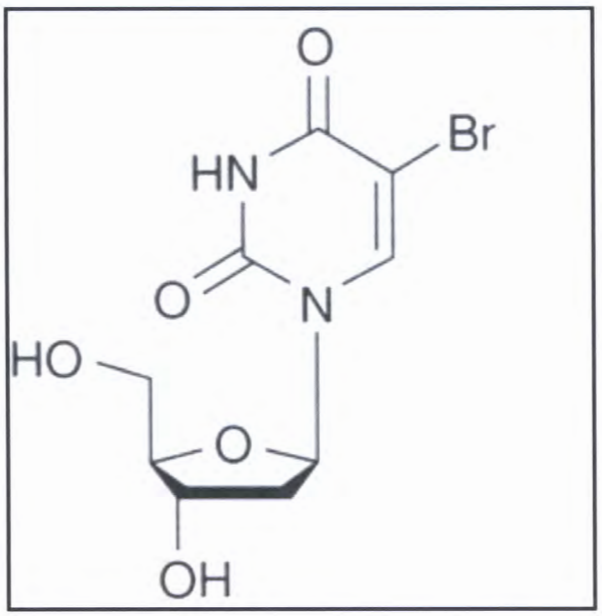


Figure 2.1 Structure of 5-Bromo-2'-deoxyuridine (BrdU)
BrdU is a thymidine analogue that incorporates DNA of dividing cells and allows for the birth dating and monitoring of cell proliferation.

2.1. Mode of administration

BrdU crosses the BBB. It can be delivered by intracerebroventricular, intraperitoneal, intravenous injection, or orally for studying neurogenesis. It is metabolised through dehalogenation when integrated in the DNA. BrdU is also metabolised rapidly through dehalogenation in the plasma, with the half-life of BrdU in the plasma reported to be around 8-11 minutes (396). The concentration of BrdU that reaches the brain is therefore only a fraction of the administered intraperitoneal dose. Hence, ICV injection may lead to a high concentration of BrdU in the brain compared with those concentrations attained in the brain with peripheral delivery due to direct infusion into the CSF (354, 388, 893).

2.1.1. BrdU delivery

In the past, research on adult neurogenesis has focused on two brain regions, the SVZ of the lateral ventricles, and the SGZ of the hippocampus. In contrast, other brain regions have received much less attention in the context of adult neurogenesis. These studies have relied on intra-peritoneal delivery of BrdU, and this mode of delivery has been proven to be insufficiently sensitive to detect newborn cells outside of the SVZ or SGZ. Kokoeva, et al. (388) compared CNTF-induced BrdU incorporation into the hypothalamus using two different delivery systems. BrdU was either infused into the cerebroventricular system by implanted osmotic mini pumps, or injected intraperitoneally over the course of a week. They showed that ICV BrdU delivery labelled ~350% more BrdU positive cells than IP delivery, indicating that IP BrdU delivery is not adequate to detect adult neurogenesis in brain regions other than the SVZ and SGZ. Therefore, in this thesis, we have used ICV BrdU administration to investigate cell proliferation in the hypothalamus.

2.2. Other IHC markers of cell proliferation

The expression of markers of the cell cycle, such as Ki-67 can be used to assess cell proliferation *in situ*. Ki-67 is expressed in all phases of the cell cycle except the resting phase and a short period at the beginning of the phase G1 (167, 198, 469, 886). Ki-67 has a very short half-life between 60 and 90 minutes (469), it is not detectable during DNA repair processes and is strongly down regulated or absent in quiescent cells. However, the use of markers of the cell cycle for studying adult neurogenesis is limited by the temporal expression of cell cycle proteins that are only expressed during the phase(s) of the cell cycle, but not anymore when newly generated neuronal cells exit the cell cycle and begin their maturation process.

Proliferating cell nuclear antigen (PCNA) is a protein that acts as a processivity factor for DNA polymerase δ. PCNA staining is most noticeable during late G_1 and early S phases, but PCNA labelling also occurs in the G_2 phase of the cell cycle, and the half life of PCNA has been reported to be 20 hours (86, 406, 807). Therefore it is possible that cells remain PCNA-positive even after leaving the S phase.

The major down fall to all the aforementioned markers of cell proliferation is that they only offer a single snap shot to the neurogenesis occurring at any one moment, whereas BrdU provides a complete over-view of proliferation occurring over a set period of time.

2.3. Limitations to BrdU

BrdU labelling is currently the most used method for studying adult neurogenesis. There are however pitfalls and limitations to the use of BrdU IHC for studying cell proliferation, and neurogenesis. These will be discussed in the following section.

2.3.1. BrdU IHC and multiple labelling

Protocols to detect BrdU by immunohistochemistry are devised using antibodies directed against single-stranded DNA containing BrdU (265). The use of primary antibodies directed against BrdU on single-stranded DNA required denaturing DNA. The standard protocol for denaturing the DNA, and the method employed in this thesis, involves partial hydrolysis of the tissue with HCl treatment (265, 536). This treatment can affect the cell morphology and antigenicity recognition in multiple labelling studies, thereby limiting the morphological and phenotypical identification of the newly generated cells. As such, the study of 'neurogenesis' requires the use of co-labelling BrdU cells with a neuronal marker.

2.3.2. BrdU is a toxic substance

With all of its advantages, the BrdU technique has its pitfalls. BrdU is a toxic substance. When administered to pregnant rodents, it can lead to exencephaly, cleft palate, limb abnormalities, and can lead to teratogenic malformations and behavioural changes in the progenies (51, 389, 408, 555). When administered postnatal, BrdU may cause lung changes in mature rodents, and alter the development of the cerebellum (553, 883). It can lead to morphological and behavioural abnormalities, trigger cell death and leads to the formation of teratomas (389, 699, 775). These effects are observed when the peripheral BrdU administration ranges between 60-600 mg/kg of body weight. However, using a single dose of 50 mg/kg of body weight shows no evidence of toxicity (518, 770). In the present thesis we will be using ICV administration of 12 μg of BrdU per day, which equates to approximately 0.48 mg/kg body weight, well below the reported toxic concentrations. Therefore, in the current experimental paradigm, BrdU is an appropriate and safe substance for the detection of proliferating cells in the CNS.

2.3.3. DNA repair

BrdU may also affect DNA synthesis events not directly related to cell proliferation, such as DNA repair and/or abortive re-entry into the cell cycle (142, 567, 775, 776). Therefore, BrdU is not a marker of cell proliferation, but of DNA synthesis. DNA repair is a normal occurring process in the life of a cell and is carried out by cellular enzymes, which secure genomic stability (509). As such, there is concern that BrdU IHC may label cells undergoing DNA repair, and not just dividing cells (402, 632, 875). DNA repair occurs normally *in vivo* through a mechanism that replaces 1-2 nucleotides at each site, as opposed to cell division where the entire genome is replicated. Therefore the incorporation of BrdU into repairing DNA is likely to be low, and not sensitive enough for IHC detection. This hypothesis was

investigated by Bauer, et al. (55) who showed that BrdU incorporation is not detected in dying postmitotic neurons under three different models of injury-induced neuronal apoptosis *in vivo*. This suggests that the amount of BrdU that would incorporate during normal, ongoing neuronal cell death *in vivo* is below the detection limit in IHC.

Overall, BrdU IHC is an appropriate tool for the investigation of cell proliferation in the CNS. Although it has a number of limitations and pitfalls, every effort has been made to avoid these in the present thesis.

Declaration for Thesis Chapter Three

Declaration by Candidate

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation	85%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of Contribution	Extent of contribution (%) for student co-authors only
Simin Fallah Omran	Exercise trained the mice	
Jacquline Weir	Provided technical assistance for lipidomics analysis	
Peter J. Meikle	Provided the facilities and assistance for lipidomics analysis, review/edited manuscript	
Matthew J. Watt	Study design, data and statistical analysis, data interpretation, manuscript preparation	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contribution to this work

Candidate's Signature	Date
[redacted]	16.12.2013
Main Supervisor's Signature	**Date**
[redacted]	16.12.2013

Chapter Three: Consumption of a high-fat diet, but not regular endurance exercise training, regulates hypothalamic lipid accumulation in mice

3.1. Introduction

The term "lipotoxicity" was coined by Unger and colleagues almost two decades ago and refers to the excessive accumulation of lipids in non-adipose tissues that causes cellular dysfunction and, in severe cases, apoptotic cell death (437, 797). For example, the sphingolipid ceramide induces pancreatic β-cell failure and apoptosis (707), several lipid species cause insulin resistance in skeletal muscle and liver (837) and lipid accumulation is related to cardiomyopathy and heart failure (362). In light of the widely accepted association between excessive lipid accumulation and dysregulated cell function in obesity, it is surprising that few studies have examined whether lipotoxicity extends to the central nervous system (CNS).

Discrete regions of the CNS detect neural, endocrine and metabolic signals from the periphery to monitor whole-body nutrient availability. While not discounting the input of neuronal types located elsewhere in the brain, specialised neurons localised within the arcuate nucleus of the hypothalamus are critical regulators of feeding behaviour and body weight. This is largely attributable to the compromised blood brain barrier in this region (478). Plasma fatty acids cross the blood brain barrier and gain access into the cerebrospinal fluid (CSF) (229, 517) where CSF fatty acids generally reflect plasma fatty acid levels (517, 727). Hypothalamic fatty acid uptake is increased in patients with the metabolic syndrome (357); however, the rate of fatty acid β-oxidation is very low in the hypothalamus (445), indicating that fatty acids do not serve as an important metabolic substrate for ATP production. This apparent mismatch between fatty acid availability/uptake and oxidation suggests that fatty acids are stored in the hypothalamus; however, the capacity for hypothalamic cells to accumulate/store lipids, the resulting type of stored lipid and the effect of these lipids on

energy homeostasis remain undefined. In light of the apparent inability of the hypothalamus to modulate fatty acid oxidation when fatty acid delivery is increased, it is postulated that the various cell types located in the hypothalamus would also be susceptible to lipotoxic outcomes. Indeed, diets enriched with the fatty acid palmitate (C16:0) promote diacylglycerol accumulation (62) and apoptosis (535) in the brain. Therefore, the first aim of the present study was to examine the effects of high-fat feeding on hypothalamic lipid species accumulation. Understanding the fate of fatty acids bears relevance for understanding the bases of cellular processes, including energy metabolism, and the pathogenesis of lipid related disease.

Physical activity (exercise) reduces body weight and adiposity, increases daily energy expenditure and is used to enhance fatty acid oxidation, reduce tissue lipid content and enhance insulin action in peripheral tissues (259, 297, 322, 652). While these effects are well described in cardiac and skeletal muscle, liver and adipose tissue, the effects of exercise on hypothalamic lipid content remains unresolved. Thus, the second aim of this study was to examine the effect of regular endurance exercise training on lipid accumulation in the hypothalamus. It is hypothesised that a high-fat diet and obesity in *ob/ob* mice would cause lipid accumulation in the hypothalamus and that regular exercise would reduce hypothalamic lipid levels in the setting of rodent obesity. These questions were addressed by performing unbiased mass spectrometry analysis on hypothalamic lysates obtained from mice fed a low-fat or high-fat diet that were sedentary or subjected to regular endurance exercise training.

3.2. Methods

3.2.1. Animal experimental procedures

All animal protocols used in this study were approved by the School of Biomedical Sciences Animal Ethics Committee, Monash University, and conformed to National Health & Medical Research Council (Australia) guidelines regarding the care and use of experimental animals. All mice (C57Bl/6) used in this experiment were obtained from Monash Animal Services at six weeks of age and maintained on a 12 h light, 12 h dark cycle with lights on at 0700 h at Physiology Department, Large Animal Facility, Monash University. Mice were housed five to a cage and allowed to acclimatize for one week with standard rodent chow and water *ad libitum*.

Mice were randomly assigned their respective diets at eight weeks of age, which consisted of a low-fat diet (n=10) (5% energy from fat, LFD) or a high-fat micronutrient matched diet (n=20) (59% energy from fat, HFD) *ad libitum* for 12 weeks (Table 3.1) (Appendix Table 8.1). The LFD was composed of 15.6% saturated, 45.2% monounsaturated and 39.2% polyunsaturated fatty acids, while the HFD was composed of 60.3% saturated, 32.9% monounsaturated and 6.7% polyunsaturated fatty acids. Body mass was monitored weekly throughout the course of the study. *Ob/ob* mice were purchased from Monash Animal Services at eight weeks of age and were maintained on a standard rodent chow (9% fat) until 12 – 14 weeks of age (n=6).

Table 3.1 Details of diets

	Rodent chow	Low-fat diet	High-fat diet
Manufacturer	Barastoc Irradiated mice cubes	Specialty Feeds SF08-020	Specialty Feeds SF03-002
Total Crude Fiber	3.2%	4.7%	4.7%
Total Protein	20%	13.6%	19.5%
Total fat	6%	4%	36%
Total energy from fats	15%	9%	59%
Digestible energy (kJ/g)	13.2	15.5	22.8

3.2.2. Treadmill Running

Mice were randomised at eight weeks of age to four groups with the following diet and exercise interventions: chow sedentary (n=10), HFD sedentary (n=10), or HFD exercise (n=10). After 6 weeks on their respective diets, mice ran on the treadmill (Columbus Instrumentals, OH) once daily, five times a week for six weeks (Table 3.2). An endurance exercise test was performed on all mice before and after the exercise-training period to assess the efficacy of the endurance-training program.

3.2.3. Endurance exercise capacity test

Before and after exercise training/dietary intervention, treadmill testing was performed on all mice. Each mouse was placed on the treadmill, which was set at 17 m/min at a 5% slope. Mice ran until they reached exhaustion, which was defined as sitting at the base of the treadmill and not attempting to re-engage the treadmill with manual prompting from the experimenter. The change in the time taken to reach exhaustion between baseline and the end of the study was taken as a measure of the change in overall endurance capacity during the study course.

Table 3.2 Six week exercise training protocol

Week	Day	Exercise protocol	Week	Day	Exercise protocol
1	Mon	Endurance test	4	Mon	15m/min (55min)
	Tue	12m/min (30min)		Tue	15m/min (55min)
	Wed	13m/min (30min)		Wed	15m/min (60min)
	Thu	13m/min (30min)		Thu	16m/min (60min)
	Fri	13m/min (30min)		Fri	16m/min (60min)
	Sat			Sat	
	Sun			Sun	
2	Mon	14m/min (30min)	5	Mon	16m/min (60min)
	Tue	14m/min (30min)		Tue	16m/min (65min)
	Wed	14m/min (40min)		Wed	16m/min (65min)
	Thu	14m/min (40min)		Thu	16m/min (70min)
	Fri	15m/min (40min)		Fri	17m/min (70min)
	Sat			Sat	
	Sun			Sun	
3	Mon	15m/min (40min)	6	Mon	17m/min (70min)
	Tue	15m/min (45min)		Tue	17m/min (70min)
	Wed	15m/min (45min)		Wed	18m/min (70min)
	Thu	15m/min (50min)		Thu	18m/min (70min)
	Fri	15m/min (50min)		Fri	Endurance test
	Sat			Sat	
	Sun			Sun	

3.2.4. Glucose tolerance test

A glucose tolerance test (GTT) determines how quickly glucose is cleared from the blood after a bolus of glucose is administered. The GTT was conducted on the Monday of the last week of training, allowing two exercise free days before the test. Mice were fasted for 4 h and blood glucose was measured at 1100 before and after a bolus of glucose (2g/kg body weight, 50% D-glucose in water) was injected intraperitonealy. Blood glucose was measured from the tip of the tail at 0, 15, 30, 45, 60, 90 and 120 min, using a glucometer (Accu-Chek, Roche). As an index of glucose tolerance, the incremental area under the curve was calculated from the blood glucose profiles using the 0 min time-point as the baseline.

3.2.5. Analytical methods

Mice were killed three days after the last exercise bout to preclude acute effects of exercise on the measured variables. Mice were fasted for 4 h (1100), anaesthetized via isoflurane inhalation and killed by decapitation; trunk blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Whole blood was centrifuged for 2 min at 8000rpm and plasma collected for analysis.

3.2.5.1. Plasma fatty acids

Free fatty acids were measured in plasma using a commercially available kit (Wako, Wako Pure Chemical Industries, Osaka, Japan), which uses an enzymatic colorimetric method. The intensity of the coloured product is directly proportional to the concentration of FFA in the sample. The absorbance of the colour produced is measured spectrophotometrically at 550 nm and the concentration of FFA in each sample is calculated from a standard curve generated from the absorbance values of the standards. The kit utilises the following reactions to from a purple colour adduct:

1. $RCOOH + ATP + CoA \rightarrow Acyl\text{-}CoA + AMP + PPi$
2. $Acyl\text{-}CoA + O_2 \rightarrow 2,3\text{-trans-}Enoyl\text{-}CoA + H_2O_2$
3. $H_2O_2 + MEHA + 4\text{-aminoantipyrin} \rightarrow \text{Final reaction product} + 4H_2O$

Adenosine monophosphate (AMP), pyrophosphate (PPi), adenosine triphosphate (ATP), hydrogen peroxide (H_2O_2), 3-methyl-N-ethyl-N(β-hydroxyethyl)-aniline (MEHA).

3.2.5.2. Plasma triglycerides

Plasma triacylglycerides are hydrolysed to glycerol and FFA in a reaction catalyzed by lipoprotein lipase. Plasma triacylglycerides were determined by the Wako Triacylglycerol E

kit (#432-40201) according to the manufacturer's instructions. The absorbance of the colour produced is measured spectrophotometrically at 600 nm and the concentration of triglycerides in each sample is calculated from a standard curve generated from the absorbance values of the standards.

3.2.5.3. Plasma alanine aminotransferase

Alanine aminotransferase (ALT) is predominantly found in the liver, and appears in the plasma during hepatocellular injury, and is therefore used as a marker for liver damage. The ALT kit was obtained from ThermoElectron (GTP #1160-200) and used according to the manufacturer's instructions. The plasma sample was added to reagent A to initiate the following reactions.

1. L-alanine + 2-Oxoglutarate → Pyruvate + L-glutamate (via ALT)
2. Pyruvate + NADH → L-lactate + NADH (via lactate dehydrogenase)

The rate of decrease of absorbance at 340nm is due to the oxidation of NADH, and is proportional to the activity of ALT at 37°C.

Activity U/L = Δ Abs/min × [(total reaction vol × 1000)/ 6.3 × sample vol)]

3.2.5.4. Plasma aspartate aminotranferase

Aspartate aminotransferase (AST) is found predominantly in the heart and liver, and like ALT, appears in plasma during liver cellular damage. The AST kit was obtained from ThermoElectron (GOT #1180-200) and used according to the manufacturer's instructions. The plasma sample was added to reagent A to initiate the following reactions.

1. L-aspartate + 2-Oxoglutarate → Oxaloacetate + L-glutamate (via AST)
2. Oxaloacetate + NADH → Malate + NAD (via Malate dehydrogenase)

The rate of decrease of absorbance at 340nm is due to the oxidation of NADH, and is proportional to the activity of AST at 37°C.

Activity U/L = Δ Abs/min × [(total reaction vol × 1000)/ 6.3 × sample vol)]

3.2.5.5. Liver and muscle triglycerides

Approximately 30 mg of liver and skeletal muscle (mixed quadriceps) were placed in 1.5mL of 2:1 chloroform:methanol (v:v) and homogenized on ice for approximately 10sec followed by the addition of 0.5 mL of 100% chloroform. Samples were incubated at room temperature (RT) for 20 min, 1.5 mL of 4 mM $MgCl_2$ was added and centrifuged at 1000 g for 20 min. The upper phase was aspirated and 1.5 mL of the lower phase was dried under nitrogen. After re-suspension with 250 µL of KOH solution (5:95 80% KOH:100% ethanol) samples were incubated at 60°C for 1 hr, after-which 0.5 mL of 0.15 M $MgSO_4$ was added and centrifuged at 1400 g for 2 min. To determine the glycerol concentration, free glycerol reagent (Sigma-Aldrich, #F6428) was added with each sample and incubated at 37°C for 5 min. The triglyceride content was determined from the linear relationship of glycerol standards with the absorbance of the glycerol standards (GPO-PAP reagent, Roche Diagnostics).

3.2.6. Immunoblotting

3.2.6.1. Protein extraction and quantification

The Bichinchoninic (BCA) Protein Assay Kit™ (Quantum Scientific) was used to determine total protein content. Hypothalamic lysates were diluted (1:20) and added to a 96 well microtitre plate with BSA standards of 0, 25, 50, 100, 200, 300, and 500 μg/mL. 200 μL of the BCA buffer (98:2 Reagents A:B) were added to each well and then the plate was incubated for 30 min at 37°C. Absorbance was determined at 540 nm. The protein content was determined by the linear relationship of protein concentration with absorbance.

3.2.6.2. Western immunoblotting

Western blotting determines the relative amounts of a specific protein in samples. Briefly, proteins were separated by molecular weight via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a polyvinylidene fluoride membrane (PVDF, PALL Corporation, Port Washington, NY, USA) to which antibodies were applied to conjugate with the protein of interest allowing visualization after the incubation with a secondary antibody and chemiluminescent reagent.

Hypothalamic lysates normalised for protein concentration (BCA method, Pierce Kit, Progen Industries, Darra, QLD, Australia) were solubilised in Laemmeli sample buffer and boiled for 5 min. Proteins (20μg) They were then resolved by SDS-PAGE on 10% polyacrylamide gels, which consisted of a running gel (2.3 mL 40% acrylamide (BioRad), 3.5 mL 1M Tris, 93 μL 10% SDS, 1.5 mL 50% sucrose, 1.6 mL ddH_2O, 2.3 μL TEMED, 234 μL 10% ammonium persulphate) and a stacking gel (1.5 mL 0.375M Tris, 468 μL 40% acrylamide, 46.8 μL 10% SDS, 2.22 mL ddH_2O, 1.8 μL TEMED, 375 μL 10% ammonium persulphate). The gels were placed in an electrophoresis tank (X cell Sure Lock, Invitrogen)

and running buffer (19 mM Tris HCl, 192 mM glycine, 3.4 mM SDS, ddH_2O, pH 8.8) was poured into the tank. 5-10 μL of BioRad Precision Plus Protein Standard was used, and 20 μg of protein of prepared sample was loaded into the gels. The gels were run at 130 V (BioRad Power Pac 300) through the stacking gel and 160 V until the desired separation had occurred. The gels were then placed on top of a PVDF membrane placed in transfer buffer (191 mM Tris, 1.9 M glycine, ddH_2O) and tranfered for 90 min at 65 mAmps/gel (BioRad Trans-Blot SD cell transfer apparatus). Upon completion, membranes were washed in Tris Buffered Saline with Tween20 (TBST, 37.9M Tris HCl, 13.6 M NaCl, 500 μL Tween20, ddH_2O). The membranes were incubated with 5% milk powder in TBST for 1 hr to minimize non-specific binding. The membrane was washed in TBST and incubated with the primary (1°) antibody overnight at 4°C, and then washed in TBST to remove any unbound 1° antibody. It was incubated with a secondary (2°) antibody for 1 hour at room temperature, and then washed in TBST to remove any unbound 2° antibody (see Table 3.3 for specific antibodies used). The immunoreactive proteins were then detected and developed with enhanced chemiluminescence (Pierce) before being exposed for varying durations on Fuji X-ray film and developed. Images were scanned for densitometery analysis by ImageJ (NIH). Results were corrects for total protein or α-actin levels as described. Phospho JNK was corrected for total protein loaded using a Ponceau S stain (Sigma-Aldrich Pty, Ltd, Sydney, Australia).

Table 3.3 Primary and secondary antibodies used in chapter 3 analysis

Antibody	Supplier	Catalogue #	Concentration
rb anti-pJNK (Thr183/Tyr185)	Cell Signalling	4671	1:1000
rb anti-IκBα	Cell Signalling	4812	1:500
rb anti-α-actin	Sigma-Aldrich	A5060	1:4000
rb anti-HRP	Amersham Biosciences		1:5000

3.2.7. Electrospray ionisation-tandem mass spectrometry of hypothalamic lipids

3.2.7.1. Lipid extraction

The hypothalamus was removed (defined caudally by the mammillary bodies, rostrally by the optic chiasm, laterally by the optic tract, and superiorly by the apex of the hypothalamic third ventricle). The whole hypothalamus (approximately 10-15 mg) was homogenised in modified RIPA buffer (Tris-HCl 50 mM, NaCl 150 mM, EDTA 1 mM, NaF 1 mM, 1× protein inhibitor (Roche), pH 7.4) and 20-50 μg protein (15-25 μL) was extracted with chloroform/methanol (2:1; 20 vol) following the addition of internal standards (Appendix Table. 8.2) (507). Samples were spun on a rotary mixer, sonicated, centrifuged and the supernatant dried under N_2. They were then reconstituted in water saturated butanol (50 μL) and MeOH (50 μL) each containing 10 mM ammonium formate.

3.2.7.2. Electrospray ionisation-tandem mass spectrometry

Analysis was performed on the supernatant by electrospray ionisation-tandem mass spectrometry (ESI/MS). An Agilent 1220 liquid chromatography system was used with Zorbax C18, 1.8 μm, 50 × 2.1 mm column (Agilent Technologies). The columns were heated to 50°C and the autosampler to 25°C. Solvents A and B were composed of

tetrahydrofuran:methanol:water in the ratio (30:20:50) and (75:20:5) respectively, both containing 10 mM NH_4COOH. To determine DAG and TAG species, 1 µL of injection was sparated using isocratic flow (100 µL/min) of 85% solvent B over 6 min. For all other lipid species, 5 µL of injection was separated under gradient conditions (300 µL/min) 0% to 100% solvent B over 8 minutes, 2.5 min at 100% B, a return to 0% B over 0.5 min thwn 10.5 min at 0%. The mass spectrometer used was Applied Biosystems API 400 Q/TRAP, with a turbo-ionspray source (350°C) and Analyst 1.5 data system. Quantification of individual lipid species was performed using scheduled multiple-reaction monitoring in positive ion mode and Multiquant v1.2. Total lipid concentration of each class was calculated by summing the individual lipid species.

3.2.8. Statistical analysis

All Statistical analysis was conducted using the statistical package GraphPad Prism version 5.0d (GraphPad Software, San Diego, California, USA, www.graphpad.com). All results are expressed as the mean ± SEM. Statistical analysis was performed by employing an unpaired students t-test, with LFD or HFD mice as the control. Body weight and glucose tolerance test data were analysed using a repeated measures two-way ANOVA with Bonferroni post hoc test. Metabolic data was analysed using a one-way ANOVA with a Dunnart's *post hoc* test, with HFD as the control group. Significance was established at the $P<0.05$ level.

3.3. Results

3.3.1. Metabolic characterization of mice in response to high-fat feeding and exercise training

The body mass of HFD mice was increased compared with LFD and exercise training did not affect absolute body mass in the HFD mice (Table 3.4). When expressed as a percentage of starting body mass, HFD Ex mice weighed less than HFD sedentary mice after 4 weeks of training, and this was maintained until the cessation of experiments (Figure 3.1A). The epididymal fat mass was increased in HFD vs. LFD; however, exercise training did not significantly decrease epididymal fat mass (Figure 3.1B). Liver triacylglycerol was increased with HFD and unaffected by exercise training (Figure 3.1C). Exercise training increased running capacity in HFD mice as demonstrated by a 2.6-fold increase in the time to exhaustion during an endurance running capacity test (Figure 3.1D). There was no change in running endurance in the LFD and HFD groups. Fasting blood glucose was increased in HFD vs. LFD and was reduced by exercise training (Table 3.4). Glucose tolerance was impaired in HFD vs. LFD mice, and was improved with exercise training. (Figure 3.1E and F). Surprisingly, fasting plasma FFA and TAG concentrations were decreased in the HFD vs. LFD (Table 3.4). Thus, exercise training induced marked changes in aerobic capacity and glucose tolerance, but mild alterations in blood lipids and body mass. High-fat feeding is accompanied by obesity in mice (Figure 3.1A). Therefore, to differentiate the effects of obesity *per se* and high-fat feeding we examined lipids in the hypothalamus of *ob/ob* mice fed a low-fat diet. The *ob/ob* mice are obese, possess many characteristics of other murine obesity models including insulin resistance, hyperlipidemia and hypertension and are routinely used in studies examining obesity (794). It is noteworthy that the *ob/ob* mouse is leptin deficient

and does not faithfully recapitulate the HFD model, which is leptin resistant and hyperleptinemic.

Table 3.4 Metabolic characteristics of mice fed a low-fat or high-fat diet, with exercise training

	LFD	HFD	HFD Ex
Initial body weight (g)	23.0 ± 0.5	22.5 ± 0.6	23. 6 ± 0.7
Final body weight (g)	31.1 ± 0.7*	36.8 ± 1.2	34.2 ± 1.1
Δ body weight (g)	8.1 ± 0.6*	14.3 ± 1.0	10.8 ± 0.9*
Fasting blood glucose (mmol/L)	8.0 ± 0.3*	10.7 ± 0.4	9.1 ± 0.3*
Plasma FFA (mmol/L)	0.67 ± 0.06*	0.43 ± 0.05	0.38 ± 0.04
Plasma TAG (mmol/L)	3.27 ± 0.38*	2.31 ± 0.38	1.98 ± 0.24
Plasma ALT (activity U/L)	10.6 ± 1.3	11.5 ± 1.5	10.1 ± 0.8
Plasma AST (activity U/L)	44.9 ± 7.3	45.5 ± 3.3	42.5 ± 6.1

GTT iAUC = glucose tolerance test incremental area under the curve, FFA = free fatty acid, TAG = triacylglycerol, ALT = alanine aminotransferase, AST = aspartate aminotransferase. Data presented as mean ± standard deviation. *P<0.05 vs HFD. n=5-10 per group.

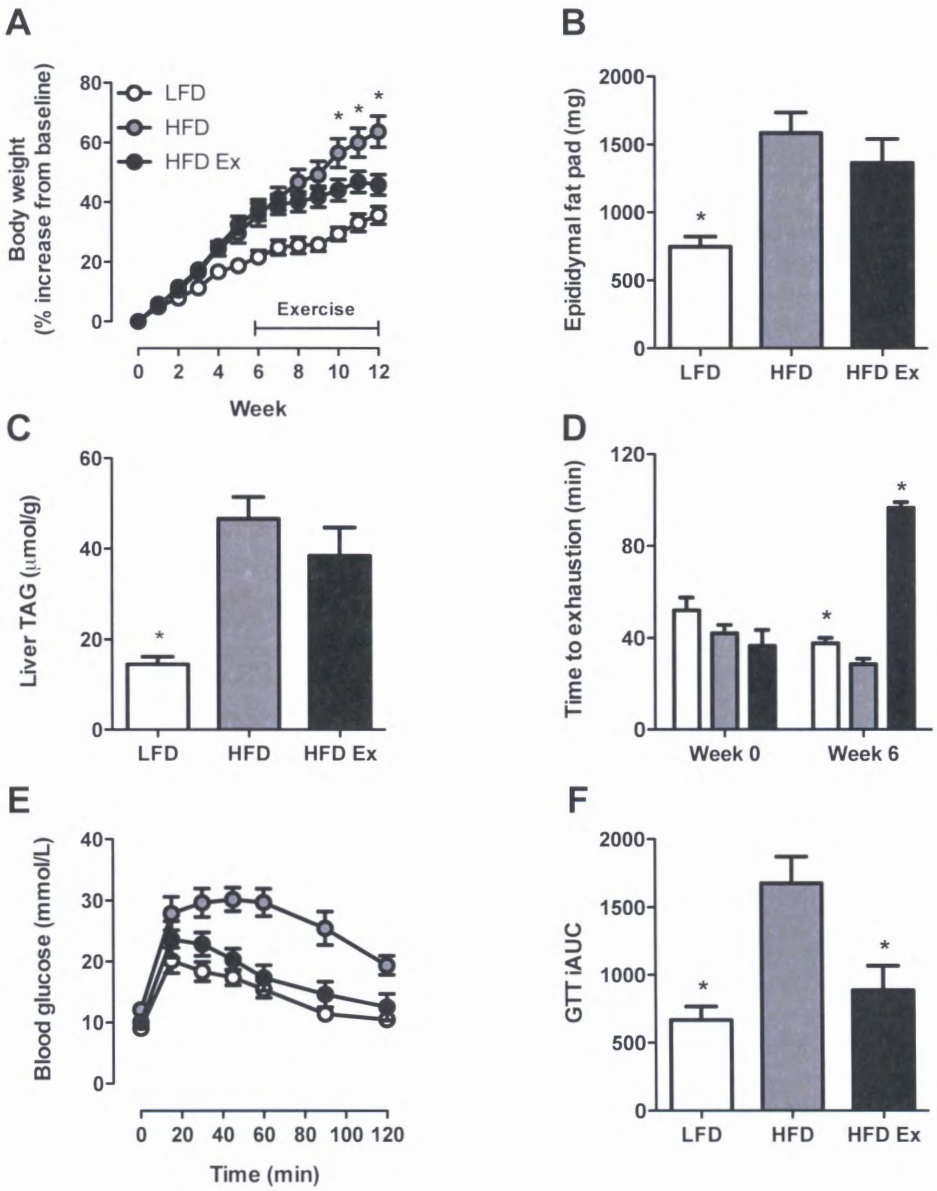


Figure 3.1 Metabolic characteristics of mice in response to high-fat feeding and exercise training.

(A) Changes in body weight presented as a percentage of starting body weight. n=10 per group, **P*<0.05 HFD vs. HFD Ex at specific time point. (B) Epididymal fat pads were excised and weighed at time of sacrifice *n*=10, per group **P*<0.05 vs. HFD. (C) Livers were excised from LFD, HFD and HFD Ex mice and TAG content analysed. *n*=8 per group, **P*<0.05 vs. HFD. (D) An endurance test was performed on all mice before (week 0) and after the 6 weeks of exercise training. LFD (*n*=10), HFD (*n*=10) HFD Ex (*n*=5), **P*<0.05 vs. HFD. (E) Glucose tolerance tests were performed on LFD, HFD and HFD Ex mice at the end of the exercise period *n*=5 per group. (F) The incremental area under the curve was calculated from the GTT data. *n*= 5 per group, **P*<0.05 vs. HFD

3.3.2. Hypothalamic lipids increase in response to high-fat feeding, but are not changed in *ob/ob* mice or with exercise training

A total of 335 molecular lipid species were identified in hypothalamic extracts. A summary of the lipid species and the most common molecular species within lipid types are listed in Table 3.5.

3.3.2.1. Phospholipids

Phospholipids constitute ~60% of the plasma membrane and >90% of some organelle membranes such as mitochondria (501). The most abundant phospholipid indentified was phosphatidylcholine (PC), followed by phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), alkenylphosphatidylethanolamine (PE(P)), lysophosphatidylcholine (LPC), alkylphosphatidylethanolamine (PE(O)), alkenylphosphatidylcholine (PC(P)), alkylphosphatidylcholine (PC(O)), odd chain phosphatidylcholine (odd PC), phosphatidylglycerol (PG) and bis(monoacylglycero)phosphate (BMP) in descending order. PC(O) (P=0.03, ↑13%) and BMP (P=0.04, ↑24.6%) content were increased in the hypothalamus of HFD vs. LFD mice, and LPC (P=0.07, ↑12.8%), PC (P=0.09,↑ 13.2%), PC(P) (P=0.08, ↑11.7%) and PG (P=0.09 ↑10.8%) tended to increase (Figure 3.2A). Phospholipids were largely unaltered in *ob/ob* vs. LFD (Figure 3.2B) or HFD ex vs. HFD mice with the exception of PG (P=0.06, ↓13.1%) (Figure 3.2C).

Table 3.5 Overview of lipid species in the hypothalamus

Species Name	Abbreviation	No. of species identified	Most abundant species	% of total lipid class
Phospholipids				
phosphatidylcholine	PC	31	34:1	17
phosphatidylserine	PS	7	40:6	43
phosphatidylinositol	PI	20	38:4	46
phosphatidylethanolamine	PE	31	18:0/22:6	21
alkenylphosphatidylethanolamine	PE(P)	12	18:0/22:5	19
lysophosphatidylcholine	LPC	21	16:0	45
alkylphosphatidylethanolamine	PE(O)	13	40:4	25
alkenylphosphatidylcholine	PC(P)	14	38:6	61
alkylphosphatidylcholine	PC(O)	23	34:1	35
odd chain phosphatidylcholine	odd PC	17	35:1	35
phosphatidylglycerol	PG	4	16:0 18:1	72
bis(monoacylglycero)phosphate	BMP	1	18:1 18:1	-
Sterol lipids				
Free cholesterol	COH	1	-	-
cholesterol ester	CE	21	16:0	24
Sphingolipids				
monohexosylceramide	MHC	6	24:1	58
sphingomyelin	SM	10	18:0	71
ceramide	Cer	6	18:0	81
hydroxysphingomyelin	SM(OH)	8	22:1	51
dihexosylceramide	DHC	6	18:0	53
G_{M3} ganglioside	GM3	6	18:0	84
dihydroceramide	dh Cer	6	18:0	74
trihexosylceramide	THC	2	18:0	91
Glycerolipids				
diacylglycerol	DAG	25	16:0 18:0	39
triacylglycerol	TAG	41	16:0 16:0 18:0	44

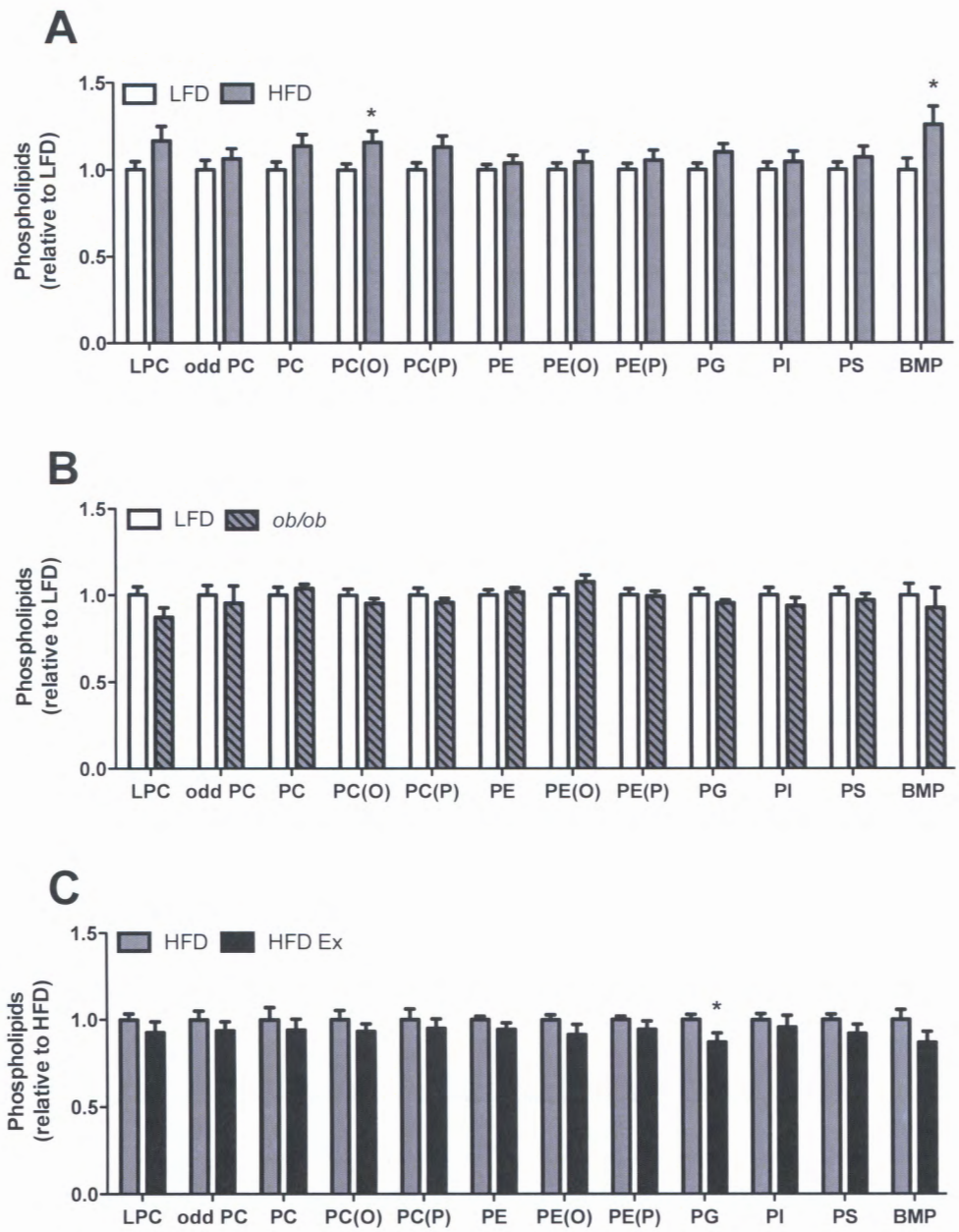


Figure 3.2 Hypothalamic phospholipid content

The hypothalamus was excised from LFD, HFD, *ob/ob*, and HFD-ex mice and analysed for total phospholipid content. (A) LFD vs. HFD (B) LFD vs. ob/ob and (C) HFD vs. HFD ex. lysophosphatidylcholine (LPC), odd chain phosphatidylcholine (odd PC), phosphatidylcholine (PC), alkylphosphatidylcholine (PC(O)), alkenylphosphatidylcholine (PC(P)), phosphatidylethanolamine (PE), alkylphosphatidylethanolamine (PE(O)), alkylphosphatidylethanolamine (PE(P)), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS). n = 10-14 per group. *P<0.05 vs. relevant control, analysed by an unpaired t-test.

3.3.2.2. Sterol lipids

Sterol lipids are important components of biological membranes and can act as hormones and signalling molecules. The most abundant sterol lipid quantified was free cholesterol (COH) followed by cholesterol esters (CE). CE tended to be increased in the hypothalamus of HFD vs. LFD mice (P=0.09, ↑82.4%) (Figure 3.3A). CE and COH were unchanged in the hypothalamus of *ob/ob* vs. LFD (Figure 3.3B) and HFD ex vs. HFD mice (Figure 3.3C).

3.3.2.3. Sphingolipids

Sphingolipids play important roles in signal transmission and cell recognition, while sphingolipid metabolites, such as ceramide, participate in numerous signalling cascades that result in apoptosis, proliferation, and inflammation. The most abundant sphingolipid identified was monohexosylceramide (MHC), followed by sphingomyelin (SM), ceramide (Cer), hydroxysphingomyelin (SM(OH)) dihexosylceramide (DHC), G_{M3} ganglioside (GM3), dihydroceramide (dhCer) and trihexosylceramide (THC) in descending order. dh Cer (P=0.05, ↑25.4%) and DHC (P=0.03, ↑39.8%) contents were increased in the hypothalamus of HFD vs. LFD mice while Cer, (P=0.08, ↑25.4%) SM (P=0.07, ↑12.9%) and MHC (P=0.08, ↑22.7%) tended to increase (Figure 3.4A). THC was the only sphingolipid lipid increased in *ob/ob* vs. LFD mice (P<0.0001, ↑132%) (Figure 3.4B). Sphingolipids were unchanged in the hypothalamus of HFD ex vs. HFD mice (Figure 3.4C).

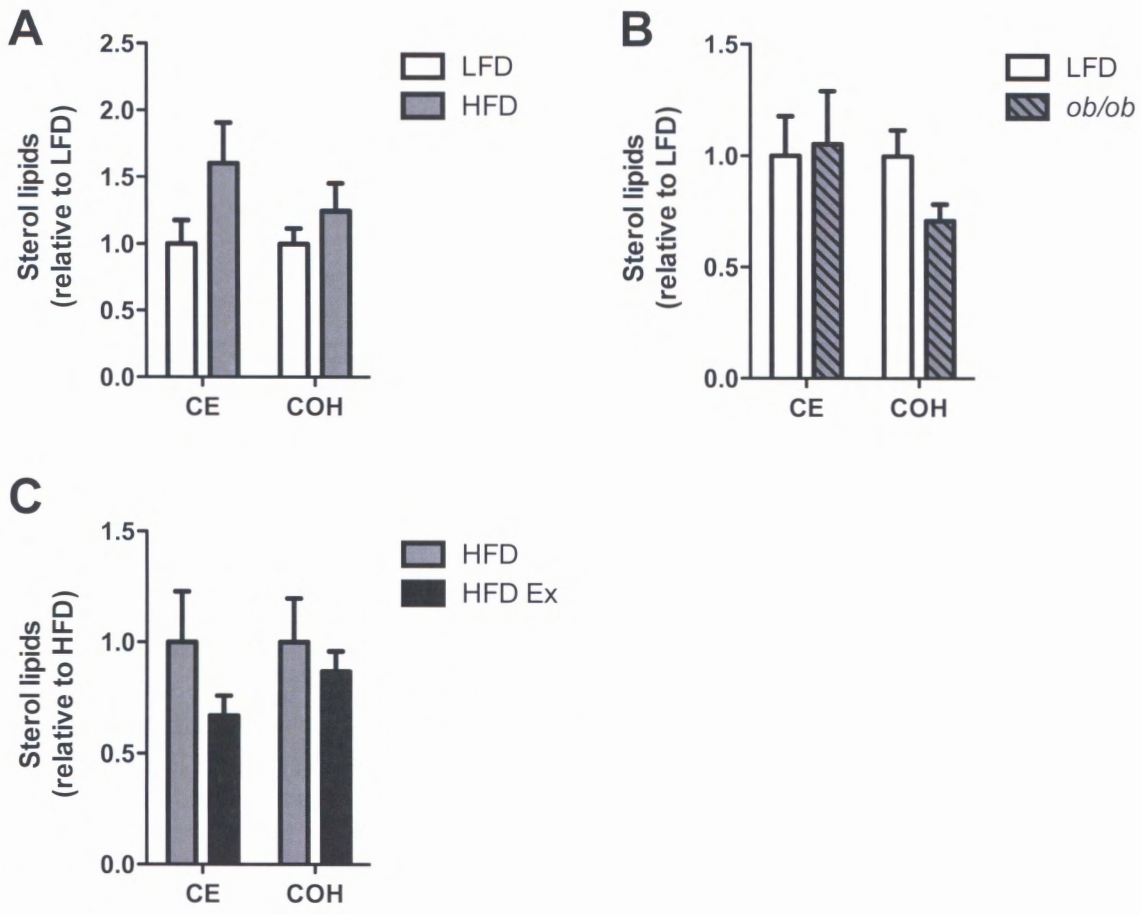


Figure 3.3 Hypothalamic sterol lipid content

The hypothalamus was excised from LFD, HFD, *ob/ob*, and HFD-ex mice and analysed for total sterol lipid content. (A) LFD vs. HFD (B) LFD vs. ob/ob and (C) HFD vs. HFD ex. Cholesterol ester (CE) and free cholesterol (COH). n = 10-14 per group. *P<0.05 vs. relevant control, analysed by an unpaired t-test.

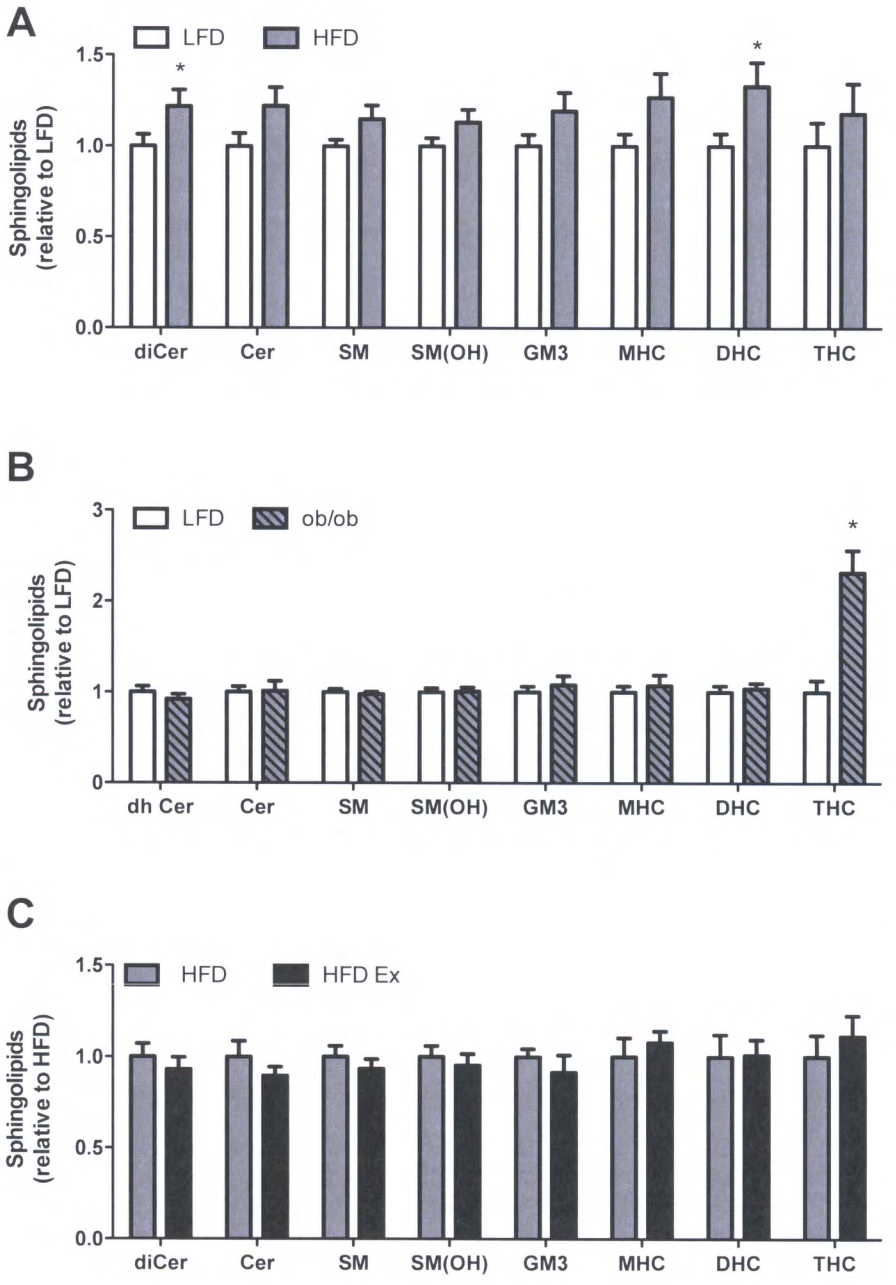


Figure 3.4 Hypothalamic sphingolipid content

The hypothalamus was excised from LFD, HFD, *ob/ob*, and HFD-ex mice and analysed for total sphingolipid content. (A) LFD vs. HFD (B) LFD vs. ob/ob and (C) HFD vs. HFD ex. dihydroceramide (dh Cer), ceramide (Cer), sphingomyelin (SM), hydroxyphingomyelin (SM(OH)), G_{M3} ganglioside (GM3), monohexosylceramide (MHC), dihexosylceramide (DHC) and trihexosylceramide (THC). n = 10-14 per group *P<0.05 vs. relevant control, analysed by an unpaired t-test.

3.3.2.4. Glycerolipids

Diacylglycerol (DAG) (P=0.006, ↑23.1%) and triacylglycerol (TAG) (*P*=0.04, ↑23.9%) content were increased in the hypothalamus of HFD vs. LFD mice (Figure 3.5A). Glycerolipids were unchanged in the hypothalamus of *ob/ob* vs. LFD (Figure 3.5B) and HFD vs. HFD ex mice (Figure 3.5C). Liver TAG is strongly correlated with whole body glucose tolerance ($r^2 = 0.571$ *P*=0.007).

3.3.3. High-fat feeding causes remodelling of hypothalamic lipid species known to cause insulin resistance

The increase in DAG content in the hypothalamus of HFD mice (Figure 3.5A) was attributed to an increase in both saturated and mono-unsaturated fatty acids (MUFA) (Figure 3.6A), which was unexpected because the HFD contained a smaller percentage of MUFA compared with the LFD (32.9% vs. 45.2%, respectively). This suggests post-prandial modification of lipids prior to storage. Similarly, the increased TAG accumulation in the hypothalamus of HFD mice (Figure 3.5A) was attributed to an increase in both saturated and MUFA (Figure 3.6D). There were no further changes observed in the fatty acid content of DAGs or TAGs in obese *ob/ob* or HFD ex hypothalami (Figure 3.6B, C, E, F).

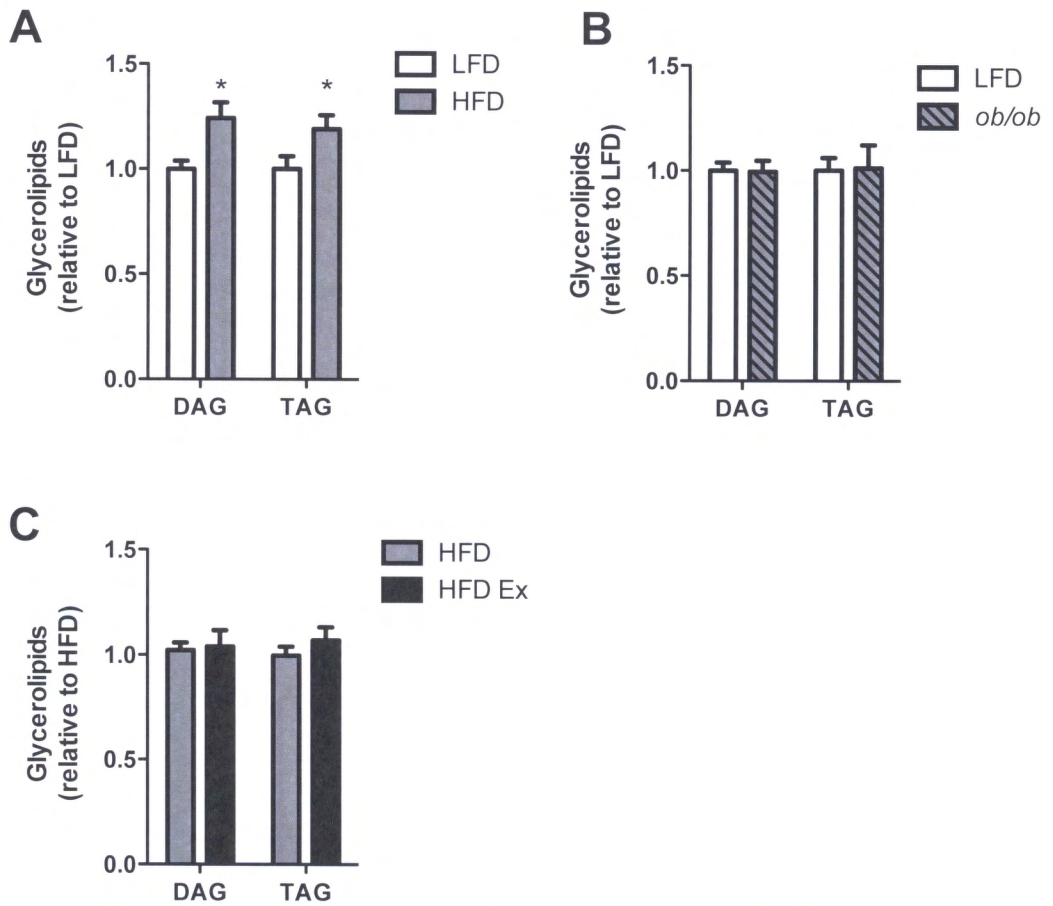


Figure 3.5 Hypothalamic glycerolipid content

The hypothalamus was excised from LFD, HFD, *ob/ob*, and HFD-ex mice and analysed for total glycerolipid content. (A) LFD vs. HFD (B) LFD vs. *ob/ob* and (C) HFD vs. HFD ex. diacylglycerol (DAG) and triaclyglycerol (TAG). n = 10-14 per group *P<0.05 vs. relevant control, analysed by an unpaired t-test

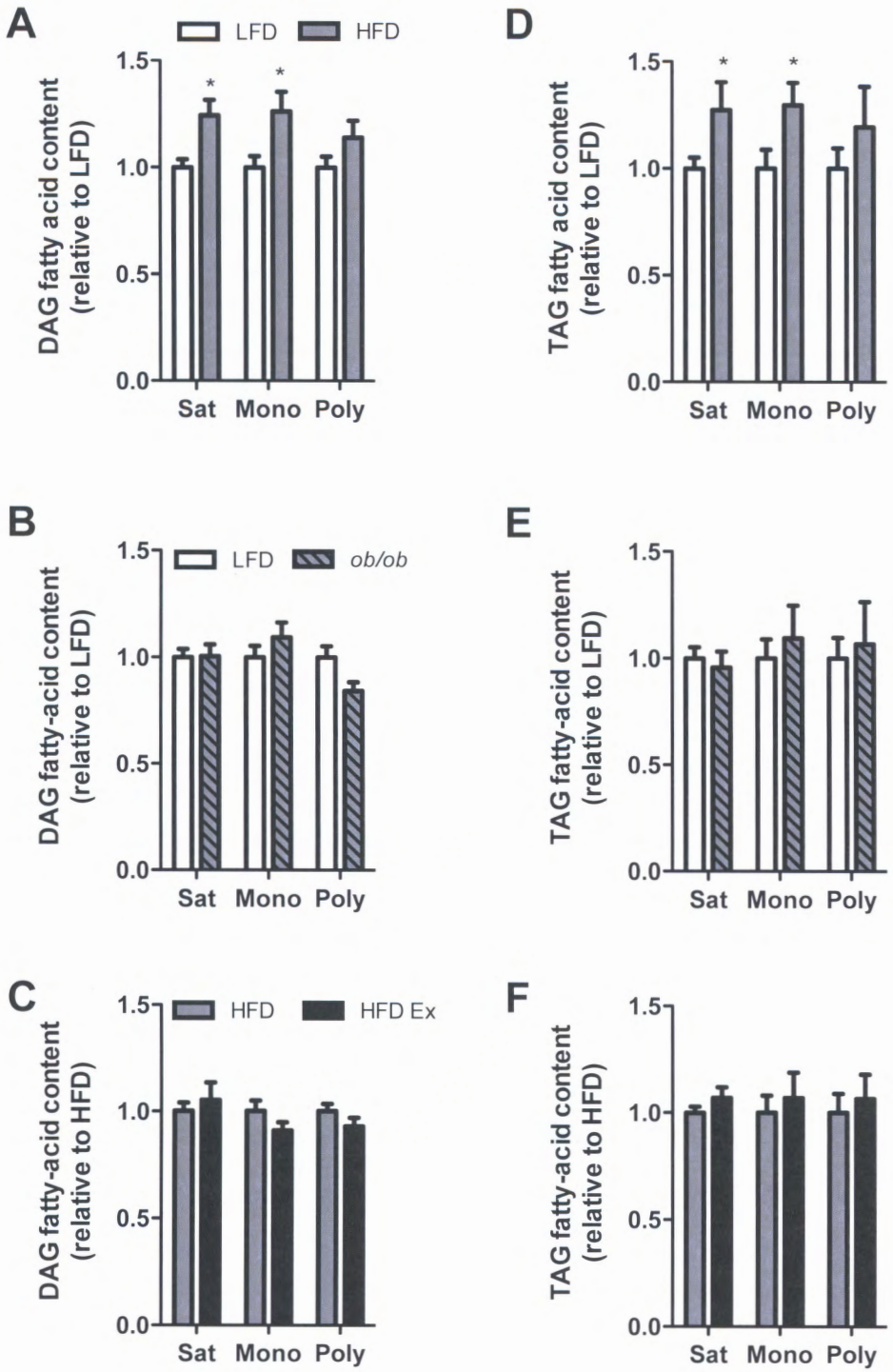


Figure 3.6 Hypothalamic DAG and TAG fatty acid content

Hypothalamic DAG (A, B, C) and TAG (D, E, F) content was analysed for the total amount of saturated (Sat), monounsaturated (Mono) and polyunsaturated (Poly) fatty acids. n=13-14 in each group, *P<0.05 vs. relevant control, analysed by an unpaired t-test.

The accumulation of ceramide and DAG is implicated in the development of insulin resistance in peripheral tissues; therefore we looked more closely at individual species changes of these lipids in the hypothalamus. Ceramide species 18:0 (P=0.05, 24.8%), 22:0 (P=0.01, 29.1%) and 24:0 (P=0.02, 28.9%) were increased in the hypothalamus of HFD vs. LFD mice (Figure 3.7A), while ceramide species 20:0 (P=0.06, ↑12.2%) and 24:1 (P=0.09, ↑38.5%) tended to increase. The 10 most abundant DAG species were analysed. DAG species 14:0 16:0 (P=0.004, 13.9%), 18:1 18:1 (P=0.005, 31.4%), 16:0 20:0 (P=0.003, 22.7%), 18:0 18:1 (P=0.02, 25.0%), 16:0 18:1 (P=0.008, 24.8%), 16:0 16:0 (P=0.0002, 20.2%), 18:0 18:0 (P=0.004, 25.5%), and 16:0 18:0 (P=0.0005, 24.5%) were all increased in the hypothalamus of HFD vs. LFD mice (Figure 3.7B).

3.3.4. Lipid accumulation is associated with ectopic liver accumulation, but not skeletal muscle lipids or increased adiposity

To assess whether the hypothalamus has a similar potential for lipid accumulation as other lipid sensitive peripheral tissue, a Pearson correlation was performed. Hypothalamic TAG content was positively correlated with liver TAG (Figure 3.8A) but not skeletal muscle TAG (Figure 3.8B), or general adiposity, as measured via epididymal fat pad weight (Figure 3.8C). This suggests that the hypothalamus is as sensitive to lipid oversupply as the liver.

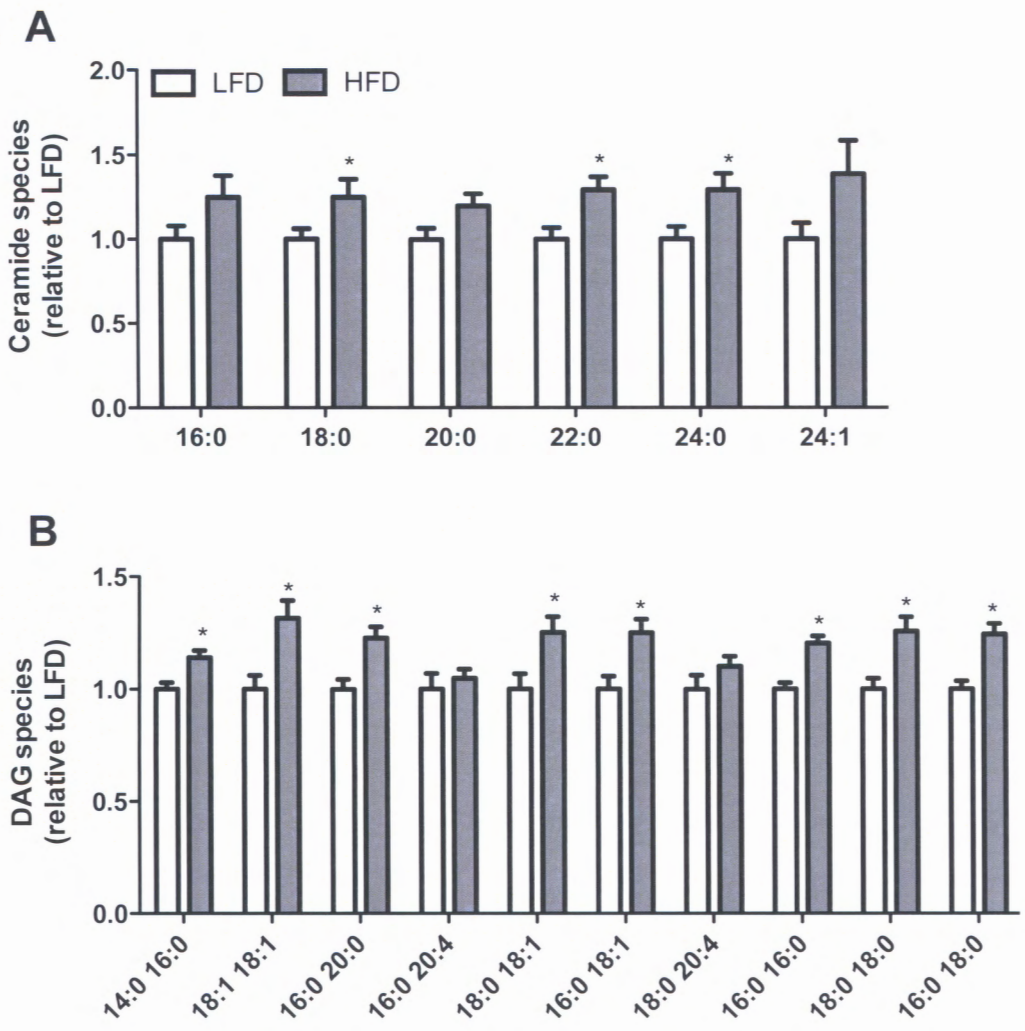


Figure 3.7 Hypothalamic ceramide and DAG species in mice fed a LFD or HFD

(A) Individual ceramide species and, (B) the 10 most abundant diacylglycerol (DAG) species were analysed. n=13-14 in each group, *P<0.05 vs. relevant control, analysed by an unpaired t-test.

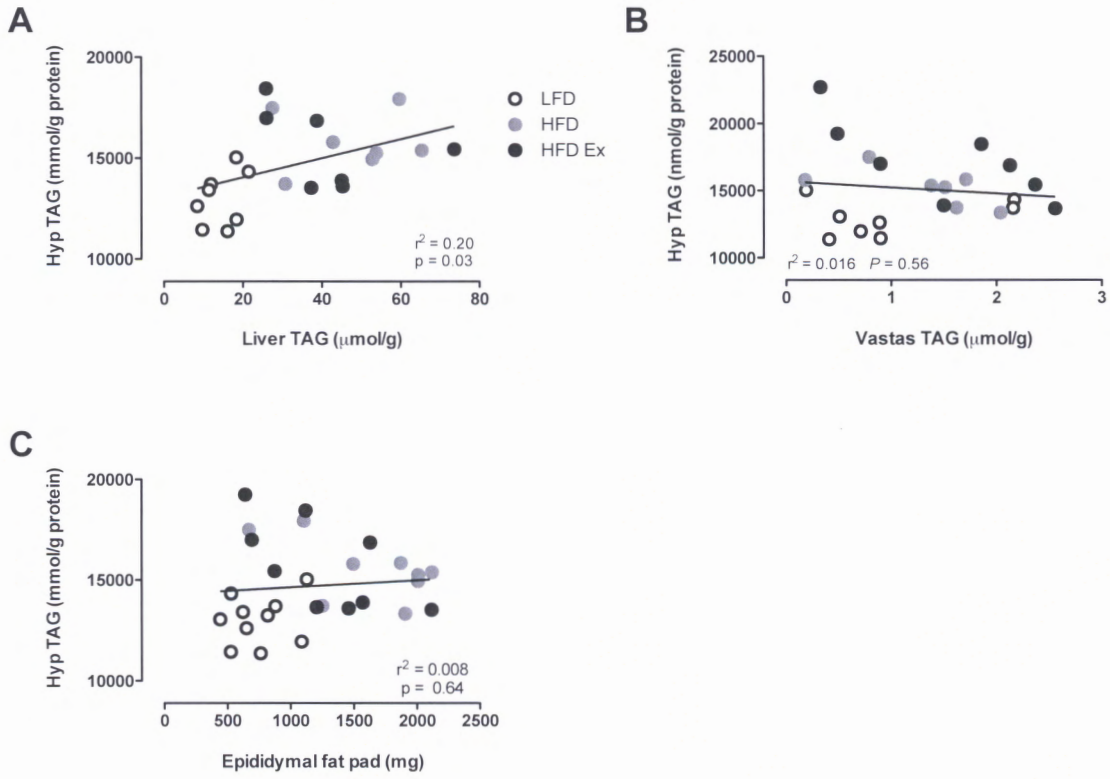


Figure 3.8 Correlations between hypothalamic TAG content and measures of adiposity. Correlations between hypothalamic triglyceride (TAG) content and (A) liver TAG (B) vastas TAG and (C) epididymal fat pad weight. n=8-10 per group. *P<0.05 as determined by Pearson's Correlation R^2.

3.3.5. Hypothalamic serine/threonine kinase signalling in response to high-fat feeding, obesity and exercise training

High-fat feeding and obesity are associated with low grade inflammation and activation of pro-inflammatory serine/threonine kinases (843). The NF-κB pathway was activated in the hypothalamus of HFD vs. LFD (Figure 3.9A) and *ob/ob* mice (Figure 3.9B) as demonstrated by reduced IκBα expression. IκBα expression was increased with exercise training in high-fat fed mice (Figure 3.9C), indicating that regular exercise training was able to partially attenuate hypothalamic pro-inflammatory signalling induced by high-fat feeding. In contrast, hypothalamic JNK signalling was not affected by the consumption of a high-fat diet, exercise training, or in *ob/ob* mice (Figure 3.10A, B, C).

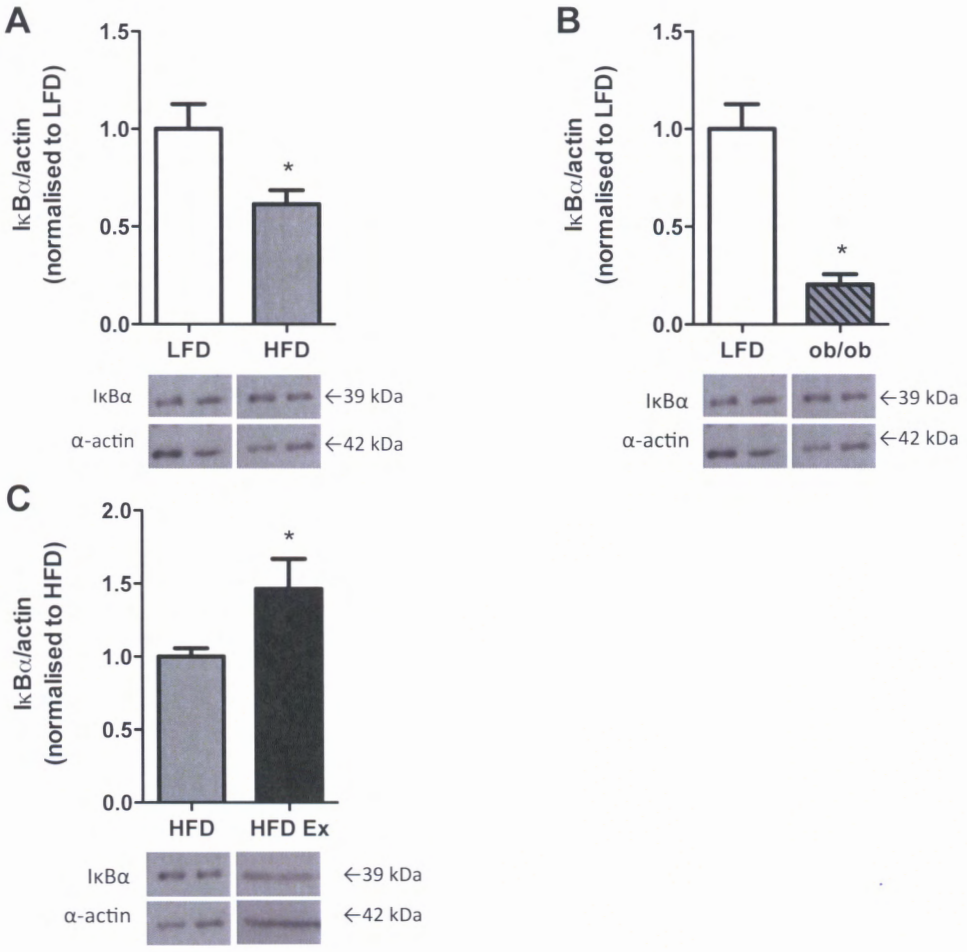


Figure 3.9 Hypothalamic stress signalling (IκBα) in response to high-fat feeding

The hypothalamus was excised and analysed for IκBα protein expression in (A) LFD vs. HFD, (B) LFD vs. *ob/ob*, and in (C) HFD vs. HFD Ex. T n=3-11 per group, *P<0.05 vs. relevant control, analysed by an unpaired t-test

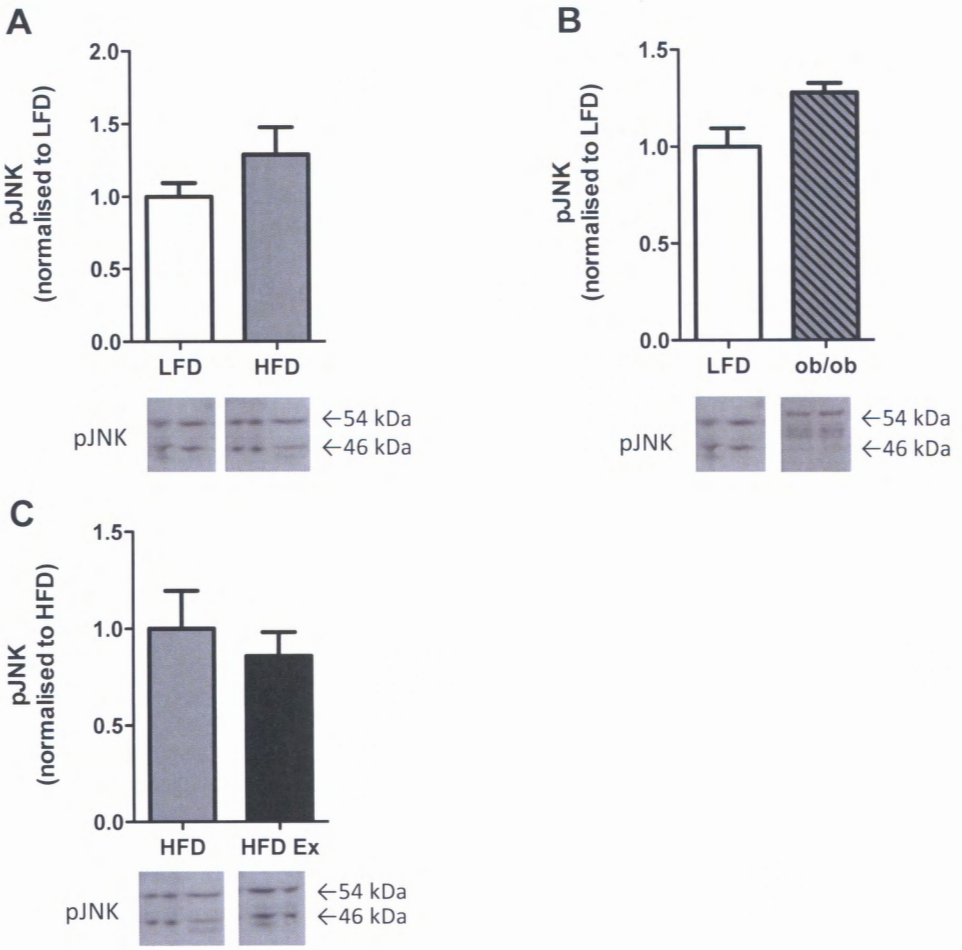


Figure 3.10 Hypothalamic stress signalling (pJNK) in response to high-fat feeding and exercise

The hypothalamus was excised and analysed for pJNK protein expression in (A) LFD vs. HFD, (B) LFD vs. *ob/ob* and (C) HFD vs. HFD Ex. n=3-11 per group, *P<0.05 vs. relevant control, analysed by an unpaired t-test

3.4. Discussion

The excessive storage of lipids in cell types other than adipocytes creates cellular stress leading to cellular dysfunction and sometimes apoptotic cell death (e.g. lipotoxicity), processes that underpin the pathogenesis of disease states such as non-alcoholic steatosis, atherosclerosis and type 2 diabetes. ESI/MS-facilitated lipidomics has provided the opportunity to quantify the lipidome of tissues and thereby enhance the understanding of human disease. Here, this technology has been utilized to demonstrate that increasing dietary fat in mice increases the content of several lipid species in the hypothalamus and that regular exercise training is unable to ameliorate these effects.

Physiological variations of plasma FFA concentrations can be detected and integrated by FA sensing hypothalamic neurons to regulate feeding behaviour and substrate metabolism (413). In this way, it is proposed that FFA fluxes signal the metabolic state of the organism. Short term ICV infusion of palmitate induces inflammatory stress (624), endoplasmic reticulum stress, insulin and leptin resistance (62, 381, 624) and apoptosis (535). However, it is uncertain whether the accumulation of intra-hypothalamic lipid(s) mediates these responses because CNS ablation of the toll-like receptor adaptor protein, MyD88 prevents many of these effects (381). Moreover, the effects of palmitate are unique for this the type of fatty acid (136), and from a physiological perspective, should be interpreted with caution when a mixture of saturated and unsaturated fatty acids naturally perfuse the brain (181). Hence, we asked whether prolonged increases in dietary fat would alter hypothalamic lipid composition. Our experiments demonstrate that high fat feeding increases the total contents of several neutral lipid species such as the phospholipid PC(O) and TAG, whilst also increasing signalling lipids, such as the sphingolipids dh Cer and DHC, and the glycerolipid DAG.

The accumulation of ceramides and DAGs had been implicated in the development of insulin resistance in peripheral tissues such as the liver and skeletal muscle (308). We examined the molecular species of these lipids in the hypothalamus of high fat fed mice. The majority of ceramide species were increased in the hypothalamus with high fat feeding (Figure 3.7A). High-fat feeding causes central insulin resistance as evidenced by decreased insulin signal transduction and an inability of insulin to suppress food intake when delivered directly into the brain (579, 624). The molecular underpinnings of hypothalamic insulin resistance are unclear, although several mechanisms have been proposed including activation of the IKKβ/NF-κB pathway (624), PKCθ (62), JNK (60), p70 S6 kinase 1 (S6K) the major downstream effector of the mammalian target of rapamycin (mTOR) (579), and endoplasmic reticulum stress (647) all of which can directly interfere with components of the insulin signalling cascade. Here, we show that ceramide accumulates in the hypothalamus of high-fat fed mice, and together with previous observations (236, 243) supports a putative role for ceramide in the development of central insulin resistance. DAG is an intermediate of both TAG and phospholipid metabolism, accumulates in the muscle and liver with diet-induced obesity and is postulated to be a key lipid intermediate linking nutrient excess to insulin resistance (336, 534). DAG was elevated in the hypothalamus of high fat fed mice and increases were detected in eight of the 10 species analysed, many of which contain saturated fatty acids (Figure 3.7B). Itani, et al. (336) showed that lipid induced insulin resistance was caused by increase DAG accumulation and membrane-associated PKC-βIII and -δ and a decrease in IκBα. DAG is a potent allosteric activator of both conventional and novel PKC isoforms (92), suggesting a possible role in the aetiology of CNS insulin resistance.

Having confirmed lipid accumulation in the hypothalamus with high-fat feeding, we examined the hypothalamic lipid profile of the *ob/ob* mouse, a monogenic obesity model

characterised by leptin deficiency, severe peripheral insulin resistance and ectopic lipid accumulation. We anticipated that the ectopic lipid accumulation would extend to the hypothalamus of the *ob/ob* mice. Unexpectedly, lipids were not increased in the hypothalamus of *ob/ob* mice, with the exception of a marked increase in THC. The discrepancies between these models of obesity (high-fat feeding and the *ob/ob* mouse) might reflect the differences in dietary composition, with *ob/ob* mice consuming a chow diet. While the absence of leptin may account for the differences in hypothalamic lipid storage between obesity models, it appears that obesity *per se* may not drive hypothalamic lipid accumulation; rather diets enriched in fatty acids may mediate this process.

Exercise training has been used as a model to reduced lipid accumulation in the liver and skeletal muscle of humans (94, 259) and rodents (526, 612). We investigated its use as a means of reducing lipid content in the hypothalamus of high-fat fed mice. Surprisingly, exercise training did not reduce the total lipid content of the hypothalamus in mice fed a high fat diet (Figure 3.2C, 3.3C, 3.4C 3.5C). Unlike studies showing exercise-mediated plasticity of the phospholipid pool in rodent and human muscle (25, 526), and liver (612) and reductions in ceramide, DAG and TAG (94, 259), the hypothalamic lipidome was essentially unaltered with exercise training. Exercise training is associated with increased oxidation of fatty acids in peripheral tissues that are postulated to limit lipid accumulation (94). It is unknown whether the rate of hypothalamic fatty acid oxidation is altered with exercise training. In this regard, one week of endurance exercise training does not alter the expression of several proteins associated with fatty acid metabolism including carnitine palmitoyltransferase 1B, carnitine palmitoyltransferase 1C, medium-chain acyl-CoA dehydrogenase, nuclear respiratory factor 1, Peroxisome proliferative-activated receptor-γ coactivator-1α, uncoupling protein 2 (all fatty acid oxidation), fatty acid translocase, glycerol-

3-phosphate acyltransferase, and diacylglycerol acyltransferase 1 (fatty acid uptake/storage) (data not shown). Indeed, oxidation rates may never be high enough to limit lipid accumulation in the setting of lipid oversupply.

Sustained excessive energy intake adversely influences cognitive function, and a sedentary lifestyle exacerbates these adverse effects of overeating (559). Several neurological disorders are characterised by defective lipid metabolism, and increase in prevalence with obesity. For example, long chain ceramides (C_{18-24}) are increased in the brain in Alzheimer's disease, HIV, arteriosclerosis, stroke and ageing (155, 285, 286, 291, 675) and ceramide accumulation is detrimental to neuronal cell function via the induction of apoptosis (287). From a metabolic viewpoint, hypothalamic ceramide accumulation is linked to insulin resistance (236). The evidence presented in our studies supports a role for altered lipid metabolism in the development of hypothalamic insulin resistance, but does not support a generalised role of "obesity" *per se*.

There were several considerations/assumptions made in the analysis and interpretation of the lipidomics data: (1) the lipids represent the sum of all hypothalamic cell types that include various hypothalamic nuclei, astrocytes, oligodendrocytes, ependymal cells and radial glia, however it has been suggested that the gross lipid composition of neuron and astrocytes are quite similar (566); (2) the lipidomics analysis provides a 'snapshot' of cellular lipid levels and does not assess fluxes, (3) the number of internal standards are limited and assume that the one standard for each class of lipids is representative of all species in that class; and (4) there may be some degradation of lipids during the hypothalamic extraction. However, this is nevertheless unlikely because brains were sectioned on ice and snap-frozen within one minute of decapitation. Despite these limitations, the relative changes between groups are accurate as

all samples were treated the same. It should also be noted that the biological implications of the changes in the lipid compositions are likely to be complex and difficult to predict on the basis of lipidomics data alone. Indeed, the biological effects of lipids depend on their location (membrane vs. cytosolic vs. nuclear) and amount (404, 683) and these detailed questions will be examined in future studies.

In conclusion, high fat feeding results in lipid accumulation in the hypothalamus of mice and hypothalamic lipids remain elevated despite regular endurance exercise training. Furthermore, the hypothalamic lipids remain unchanged in genetically obese mice fed a chow diet. Together, these data suggest that dietary lipids regulate hypothalamic lipid accumulation and this is not readily reversed by an exercise intervention.

Declaration for Thesis Chapter Four

Declaration by Candidate

In the case of Chapter Four, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation	85%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of Contribution	Extent of contribution (%) for student co-authors only
Ahrathy Selathurai	Performed tail vein injections	
Zane B. Andrews	Conceptual design of study, data interpretation, review/editing of manuscript	
Brian Oldefield	Data interpretation, review/editing of manuscript	
Matthew J. Watt	Conceptual design of study. Performed IV injections, data and statistical analysis, data interpretation, manuscript preparation	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contribution to this work

Candidate's Signature	Date
[redacted]	16.12.2013
Main Supervisor's Signature	**Date**
[redacted]	16.12.2013

Chapter Four: Exercise training induces neurogenesis in the hypothalamus of lean and obese mice, which plays a role in insulin-stimulated adipose tissue metabolism

4.1. Introduction

Adult neurogenesis plays a functionally important role for memory and cognitive function. It is most pronounced within the SVZ of the lateral ventricles and the SGZ of the hippocampal formation (240), and adult neurogenesis has been estimated to contribute 10-20% of the total neuronal population in the dentate gyrus (339). Several environmental manipulations influence the number of newborn cells, such as an enriched environment and physical activity, which are associated with improved cognitive function (372, 447, 814, 817, 892). Conversely, aging and stress can impair both neurogenesis and hippocampus-dependent behaviour (855, 892). From a therapeutic viewpoint, neurogenesis appears to be necessary for the positive actions of many antidepressants (339, 671). Therefore, it is now widely recognized that neurogenesis has positive functional changes in brain function and behaviour.

Exercise can stimulate neurogenesis in the hippocampus (814, 815) and mediates learning and memory as demonstrated through loss of/blocking neurogenesis studies (877), thereby indicating a causal effect of neurogenesis on brain functions. In both young and old rodents, exercise stimulates proliferation of the neural progenitor population, increases the number of new neurons, and promotes the survival of these new cells (206, 786, 814). These new neurons become functionally integrated into the hippocampal architecture (817).

Reports indicate that the neuroproliferative potency in the adult extends to other brain structures, including the hypothalamus (387, 522, 601). The hypothalamus has an important series of complex systems that maintain energy homeostasis in order that sufficient energy is available and body weight remains stable. Indeed, BrdU labelling has revealed adult neurogenesis in the hypothalamus under basal (388), CNTF (387) or BDNF (601) stimulated

conditions. Furthermore, hypothalamic neurogenesis appears to play a role in body weight regulation. The postnatal turnover of cells in the ARC is impaired during obesity (502), whilst the number of new born tanycytes in the median eminence is increased in the early stages of high fat feeding (430), suggesting that hypothalamic neurogenesis has an important role in energy balance and body weight maintenance.

Most importantly, growth factor mediated neurogenesis has been linked to metabolic regulation and the maintenance of body mass. Administration of CNTF (387) or BDNF (522, 601) directly into the cerebrospinal fluid of the mouse brain leads to rapid and pronounced weight loss that is maintained in the weeks to months after terminating treatment. In the case of CNTF, these long lasting affects have been attributed to neurogenesis within the hypothalamic feeding circuits, specifically NPY and POMC expressing neurons, which play crucial antagonistic roles in the regulation of energy balance (387). These newborn hypothalamic cells were predominantly neurons, did not die or migrate to other brain regions, and were leptin responsive. Responsiveness to key peripheral signals is the hallmark of hypothalamic cells, such that when resistance to these signals occurs feeding and energy balance are not controlled and obesity states can results. Furthermore, when neurogenesis was chemically blocked in mice, these neurogenic effects of CNTF were prevented, as was the sustained weight loss, indicating that neurogenesis was required for CNTF's anorectic effects. Therefore, the first aim of the current study was to explore the ability of exercise training, a natural adaptive stimulus, to induce hypothalamic neurogenesis,

Signalling events in the CNS play a role in the control of peripheral glucose metabolism. Hypothalamic insulin signalling improves peripheral insulin sensitivity via increased suppression of hepatic glucose production (618, 619). Insulin infusion into the third ventricle

supresses hepatic glucose production, whilst central antagonism of insulin signalling impaired the ability of circulating insulin to inhibit hepatic glucose production (572). Insulin signalling in the hypothalamus, particularly the ARC, is crucial for the maintenance of normal hepatic suppression of glucose output by insulin (569). Central control of peripheral metabolism is not just limited to the liver. Electrical stimulation of the VMH increases glucose uptake into skeletal muscle (712, 760) through intermediation of the sympathetic nerves (524). Furthermore, ICV infusion of insulin increases insulin-stimulated muscle glycogen synthesis (608). Neuron specific insulin receptor and insulin receptor substrate-2 deficient mice are characterised by mild obesity and whole body insulin resistance (95, 403, 462). Therefore, insulin action in the CNS partially mediates peripheral insulin action.

Exercise has been shown to stimulate neurogenesis in the hippocampus where it plays a functional role in learning and memory formation (814, 815). Exercise stimulates the proliferation of the neural progenitor population, increases the number of neurons, and promotes the survival and functional integration of these new cells into the hippocampal architecture (205, 786, 815, 817). Furthermore, recent reports indicate the neurogenesis also occurs in the hypothalamus (387, 388, 429, 502, 601, 606, 615), and that these studies suggest that neurogenesis in the hypothalamus may play a functional role in the control of energy balance, albeit, via unknown mechanism.

Many of the complications associated with type 2 diabetes can be prevented through regular exercise training, healthy diet and weight-loss (383, 411, 791). Exercise training improves peripheral insulin action in humans (38, 293, 680, 854) and rodents (85, 343), in both normal and diabetic states. Therefore, the question arises, could neurogenesis, which is upregulated with exercise training, play a role in improved insulin action with exercise?

It was previously shown that neurogenesis is attenuated in the hypothalamus of obese mice (502); mice that are characterised by impaired insulin action. Therefore, the second aim of the current project was to explore the role of basal, and exercise-induced neurogenesis on energy balance and insulin action by examining these processes following endurance exercise training, which is a situation where both neurogenesis and insulin action increase. It is hypothesized that new cells in the CNS underpin the increased sensitivity of peripheral tissues to circulating insulin after exercise training.

4.2. Methods

4.2.1. Animal Experimental Procedures

Monash University Animal Ethics Committee approved all animal protocols used in this study. Mice (C57Bl/6) were obtained from Monash Animal Services at six weeks of age and maintained on a 12 h light, 12 h dark cycle with lights on at 0700 h at Physiology Department Large Animal Facility, Monash University. Mice were housed five to a cage and allowed to acclimatise for one week with standard rodent chow and water *ad libitum*.

4.2.2. Experimental protocol 1: Assessment of neurogenic potential

To determine the potential for exercise to induce hypothalamic neurogenesis, genes associated with neurogenesis and stem cell activation were assessed after an acute exercise bout. Chow fed mice (Table 4.1) were familiarised to the treadmill three days before the experiment. Mice were run on the treadmill at a 5% slope for 30 minutes at 15 m/min, whereas sedentary mice simply placed on the stationary treadmill for the same amount of time. Mice were culled six hours later and the mediobasal hypothalamus was removed, defined caudally by the mamillary bodies, rostrally by the optic chiasm, laterally by the optic tract, and superiorly by the apex of the hypothalamic third ventricle. A Neurogenesis and Neural Stem Cells RT^2 Profiler^{TM} PCR Array (SA Biosciences, Doncaster, VIC) was then performed.

4.2.2.1. Neurogenesis RT^2 Profiler PCR Array

Total RNA from the hypothalamus was extracted in Qiazol extraction reagent followed by isolation using an RNeasy Tissue Kit (Qiagen, Doncaster, Victoria, Australia) according to the manufacturer's instructions. RNA quality was determined at 260 nm

(NanoDrop p2000 Spectrometer, Biolab), reverse transcribed (Invitrogen, Mt. Waverly, Victoria, Australia) and gene products were determined by real-time quantitative RT-PCR (ep realplex Mastercycler, Eppendorf) using RT^2 profiler PCR Array (SA Biosciences, Doncaster, VIC) looking at the targeted expression of 84 key genes related to neurogenesis and neural stem cell activation. The final reaction mix consisted of 10 µL Master Mix, 10 ng cDNA in 9 µL RNase-free water. PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of PCR reaction at 95°C for 15 sec and 60°C for 60 sec. Hspcb was used as a reference gene and did not vary between groups. The mRNA levels were determined by a comparative C_T method. For each sample, a ΔC_T value was obtained by subtracting Hspcb C_T values from those of the gene of interest. The average ΔC_T value of the control group was then subtracted from the exercise group to derive a Δ-ΔC_T value. The expression of each gene was then evaluated by $2^{(\Delta \Delta CT)}$.

Table 4.1 Details of diets

	Rodent chow	High-fat diet
Manufacturer	Barastoc Irradiated mice cubes	Specialty Feeds SF03-002
Total Crude Fibre	3.2%	4.7%
Total Protein	20%	19.5%
Total fat	6%	36%
Total energy from fats	15%	59%
Digestible energy (kJ/g)	13.2	22.8

4.2.3. Experimental protocol 2: Assessment of neurogenesis in lean mice

To assess whether exercise training can induce neurogenesis in the hypothalamus, mice were placed on a chow diet for 12 weeks. Mice received intracerebroventricular (ICV) osmotic minipump implantation with BrdU infusion (as described below) and underwent treadmill exercise training (Table 4.2). Mice were familiarized to the treadmill for 3 days before surgery, after which the mice exercised for 30 min a day at approximately 1300 h for 7 days (the duration of the osmotic pump) at a 5% slope. Mice were manually encouraged to run by gentle prodding with a metal rod.

To assess the effects of exercise training on the proliferation of cells in the hypothalamus, mice were killed 24 h after the final exercise bout. To assess the survival of the new hypothalamic cells, a separate cohort of mice were killed 28 days after the final exercise bout.

Table 4.2 Seven day exercise training protocol

Day	Protocol
-3	Stand 5min, 7m/min for 5min
-2	7m/min for 5min, 10m/min for 5 min
-1	10m/min for 5 min, 12m/min for 5 min
0	Surgery
1	7m/min for 10min
2	7m/min for 10min
3	7m/min for 20 min, 9 & 11m/min for 5 min each
4	9m/min for 20 min, 11 & 12m/min for 5 min each
5	11m/min for 20 min, 12 & 13m/min for 5 min each
6	12m/min for 20 min, 13 & 14m/min for 5 min each
7	12m/min for 20 min, 13 & 14m/min for 5 min each

4.2.3.1. ICV Cannulation

ICV cannulation and minipump implantation were performed in a single surgical procedure. Cannulation of the left lateral ventricle in the brain allows perfusion of structures lying adjacent to the ventricular system of the brain, i.e. the hypothalamus. Placement of lateral ventricle cannula (Plastics One, Roanoke, VA America) into mice was performed using sterile technique and under general anaesthesia induced and maintained by inhalation of isoflurane. Mice were secured in a stereotaxic apparatus and the surgical area shaved and cleaned with ethanol. A small anterior to posterior incision was made along the midline of the head and cleaned with sterile swabs to expose the skull. The skull was levelled and aligned using bregma as a reference point at the cranial plate junctions. The cannula was targeted to the left lateral ventricle by placing it -0.3 mm anteroposterior and lateral +1.0 mm to bregma and -2.5 mm below the skull. The support plate of the cannula was attached to the skull with superglue and dental cement (methyl methacrylate) was used to further secure the cannula in place. The cannula was connected to an osmotic minipump (model 1007D flow rate 0.5 μL/h, 7 days, Alzet, Cupertino, CA) via 65 mm long vinyl tubing filled with artificial cerebrospinal fluid (aCSF). This length was chosen to allow 2 days of aCSF infusion before the pump contents reached the ventricular system. Each minipump was either filled with vehicle solution alone, which consisted of aCSF containing 1 μg/μL BrdU (Sigma, St Louis, MO) or vehicle solution containing rat CNTF (100 ng/μL, Axokine, provided by Mark Sleeman, Regeneron Pharmaceuticals) for the positive control. Mice were housed singly and monitored daily for body weight and food intake.

4.2.3.2. Tissue processing and antibodies

Either the day after completion of exercise training (7 days) or 28 days after exercise cessation mice were anesthetized under isoflurane inhalation and perfused transcardially with

0.9% NaCl with 10mg/L heperine followed by a 4% neutral paraformaldehyde solution (Sigma, St Louis, MO). Brains were removed and post-fixed in perfusion solution overnight at 4°C, cryoprotected in 20% sucrose solution, and sectioned on a cryostat in the coronal plane. Sections (30 μM thick) were collected in 4 series, and stored at -20°C in cryoprotectant solution (25% glycerol, 30% ethylene glycol, 45% PBS) until further use. Sections were then mounted on Superfrost Ultra Plus slides (Thermo Scientific) and underwent immunohistochemistry.

4.2.3.3. Immunohistochemistry

After mounting and drying overnight, sections were first fixed with 4% PFA for 15 min at room temperature (RT). After rinsing with PBS, sections were incubated with 100% methanol for 20 min, and then rinsed. For antigen retrieval, sections were treated with 2N HCl for 30 min at 37°C. After rinsing with PBS, sections were blocked for 1 h with 5% normal horse serum in PBS/0.02% Triton X-100, then incubated with BrdU antibodies in blocking solution overnight at 4°C (sheep anti-BrdU, Abcam, 1:400). Section were washed in PBS and incubated with secondary antibodies at room temperature for 1 h, washed in PBS and coverslipped with Vectashield with DAPI (Vector Laboratories).

BrdU positive cells within the hypothalamus were counted by eye using a fluorescent microscope and a built-in digital camera (Imager.Z1; Zeiss). Per animal, every 4th coronal section (30 μM thickness) throughout the caudal hypothalamus (-1.22mm to -2.70mm from bregma) was analysed by standard fluorescence microscopy. For each section analysed, all BrdU positive cells within the hypothalamic parenchyma were counted, only excluding cells of the upper most focal plane to avoid over sampling. To obtain the total number of BrdU cells for any given caudal hypothalamus, an average section count was calculated based on all

sections counted and this number was then multiplied by the total number of sections cut per caudal hypothalamus.

4.2.4. Experimental protocol 3: Assessment of neurogenesis in obese mice

To assess whether exercise training was able to induce hypothalamic neurogenesis in the setting of obesity, mice were fed a HFD for 12 weeks (Table 4.1), at which time they received ICV osmotic minipump implantation with BrdU infusion and underwent seven days of exercise training (Table 4.2). Mice were killed as described for lean mice, to assess the proliferation and survival of newborn hypothalamic cells. Neurogenesis was assessed as previously described for lean mice.

4.2.5. Experimental protocol 4: Assessment of neurogenesis in aging mice

To assess whether exercise training was able to induce hypothalamic neurogenesis in the setting of ageing, mice were fed either a chow or a HFD at eight weeks of age. At 12 months of age mice, osmotic minipump were implanted and directed to the lateral ventricle via an ICV cannula. BrdU infusion was commenced and mice underwent exercise training for seven days. This is considered 'middle age' in the lifespan of a mouse (220). Mice were then killed 24 h after the final exercise bout. Neurogenesis was assessed as previously described for lean mice.

4.2.6. Experimental protocol 5: Assessing the role of hypothalamic neurogenesis in peripheral insulin action

To determine the amount of exercise training required to improve whole body insulin action, mice were fed a HFD for 12 weeks (Table 4.1). Mice were then randomized to either a sedentary (n=8) or exercise training group (n=8) and underwent 4 weeks of exercise training

(Table 4.3). An insulin tolerance test (ITT) was performed after 3 and 4 weeks of exercise training.

Table 4.3 Four week exercise training protocol

Week	Day	Exercise protocol	Week	Day	Exercise protocol
1	Mon	12m/min (30min)	3	Mon	15m/min (40min)
	Tue	12m/min (30min)		Tue	15m/min (45min)
	Wed	13m/min (30min)		Wed	15m/min (45min)
	Thu	13m/min (30min)		Thu	15m/min (50min)
	Fri	13m/min (30min)		Fri	15m/min (50min)
	Sat			Sat	
	Sun			Sun	
2	Mon	14m/min (30min)	4	Mon	15m/min (55min)
	Tue	14m/min (30min)		Tue	15m/min (55min)
	Wed	14m/min (40min)		Wed	15m/min (60min)
	Thu	14m/min (40min)		Thu	16m/min (60min)
	Fri	15m/min (40min)		Fri	16m/min (60min)
	Sat			Sat	
	Sun			Sun	

4.2.6.1. Insulin tolerance test

At least 72 hours after the last exercise training bout an insulin tolerance test (ITT) was performed. The ITT measures the blood glucose response to a bolus insulin injection. Following a 4 h fast (0700-1100 h), a tail vein blood sample was taken and mice injected IP with 1 U/Kg of body weight of insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark). Blood glucose was measured by an AccuChek Performa (Roche) from the tail every 15 min up to 90 min.

To determine the role of exercise-induced cell proliferation on whole body insulin action, all mice were placed on a HFD for 4 weeks to induce skeletal muscle insulin resistance (Dr. C. Bruce, personal communication) and then underwent 4 weeks of exercise training (Table 4.3). The drug cytosine-1-β-D-arabinofuranoside (AraC) was utilised to selectively inhibit DNA synthesis. Mice were randomised into either sedentary ± AraC (n=9) or exercise training ± AraC (n=9). After 4 weeks on a HFD mice underwent ICV surgery and osmotic mini-pump implantation as described above. The osmotic pump (model 1004, flow rate 0.11 μL/hr, 28 days, Alzet, Cupertino, CA) contained either vehicle, or AraC reconstituted in aCSF (3.3 μg/μL, Sigma, St Louise, MO). Therefore, mice receive 40 μg of AraC per day. Mice were housed singly and monitored daily for body weight and food intake.

4.2.6.2. Whole body glucose metabolism

4.2.6.2.1. Assessment of tissue specific insulin sensitivity

After 4 weeks of exercise training experiments were performed 72 hours after the final exercise-training bout to exclude any acute exercise effects on insulin sensitivity. 2-deoxyglucose clearance from the blood and into tissues was performed in mice as described (140, 341). After a 3 h fast (0800 h to 1100 h) mice were injected with a 50 μL mixture containing 0.5 U/Kg of insulin, 10 μCi of 2-[1-^{3}H] deoxyglucose (^{3}H-DOG) and 2 μCi of [U-^{14}C] glucose into the tail vein (injections performed by Dr. Ahrathy Selathurai, Physiology Department, Monash University). Blood samples were obtained from a cut in the tail in a capillary tube at 2, 5, 10, 15 and 20 min. Samples were deproteinised immediately by the addition of equal parts blood and H_2O (5-10 μL), 100 μL of 0.3 N barium hydroxide and 100 μL of 0.3 N zinc sulphate. Deproteinised samples were centrifuged (8000 × g, 5 min) and 100 μL of the supernatant was added to 5 mL of Ultima-Gold scintillation fluid, and the ^{3}H-

DOG and ^{14}C-glucose content was counted with a β-scintillation counter. Blood glucose was also determined at these time points by an AccuChek Performa (Roche).

Immediately after collecting the 20 min blood sample, mice were killed via decapitation and trunk blood was collected into EDTA blood collection tubes. The brain was removed and the hypothalamus dissected using the following landmarks; the mamillary bodies, rostrally by the optic chiasm, laterally by the optic tract, and superiorly by the apex of the hypothalamic third ventricle and snap frozen in liquid nitrogen. The gastrocnemius and quadriceps muscles, the epididymal fat pads, liver, heart and brown adipose tissue (BAT) were removed in the listed order and snap frozen within 3 min. Tissue specific glucose clearance was determined from the total ^{3}H-DOG as previously reported (140, 341). Tissues were homogenized in 1.4 mL water and centrifuged at 6000*g* for 10 min. The supernatant (500 μL) was added to 1.5 mL Ultima-Gold scintillation fluid, and the ^{3}H-DOG content was counted with a β-scintillation counter.

4.2.7. Plasma analysis

4.2.7.1. Free fatty acids

See Chapter 3, section 3.2.5.1 for details.

4.2.7.2. Triacylglycerols

See Chapter 3, section 3.2.5.2.for details.

4.2.7.3. Leptin

Plasma leptin concentrations were determined using a commercially available Quantikine® solid phase ELISA: mouse leptin (R&D Systems). Briefly, plasma (50 μL) was

first converted to an immunoreactive form by acid activation (2.5 N acetic acid and 10 M urea) and neutralisation (2.7 N NaOH and 1 M HEPES). Activated plasma was then diluted 20-fold with calibrator diluent. Samples and standards were added to a microplate pre-coated with affinity purified polyclonal antibody specific for mouse leptin. After washing away unbound protein, an enzyme-linked polyclonal antibody specific for mouse leptin was added to the wells, following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution was added. The intensity of the colour measured is in proportion to the amount of leptin bound in the initial step. The absorbance was measured at 450 nm with a wavelength correction at 570 nm to account for optical imperfections in the plate.

4.2.7.4. Insulin

Plasma insulin concentrations were determined using an in house rat/mouse insulin ELISA developed by the Monash Obesity and Diabetes Institute . Briefly, 5 µL of plasma and standards were added to a microplate pre-coated with purified antibody specific for insulin and blocking buffer. Working strength biotinylated antibody was added to the well and incubated for 2 h at room temperature. After washing away unbound protein and antibody, a HRP conjugate was added to the wells. The intensity of colour measured is in proportion to the amount of insulin bound in the initial step. The absorbance was measured at 450 nm with a wavelength correction at 630 nm to account for optical imperfections in the plate.

4.2.8. Immunoblotting

As described in section 3.2.6. with the antibodies described in Table 4.4

Table 4.4 Primary and secondary antibodies used in Chapter 4 analysis

Antibody	Supplier	Catalogue #	Concentration
pAKT (Ser^{473})	Cell signalling	9271	1:1000
AKT	Cell signalling	4685	1:1000
α-actin	Sigma-Aldrich	A5060	1:5000
α/β tubulin	Cell Signalling	2148	1:5000

4.2.9. Statistics

All results are expressed as the means ± SEM. All Statistical analysis was conducted using the statistical package GraphPad Prism version 5.0d (GraphPad Software, San Diego, California, USA, www.graphpad.com). Insulin tolerance tests, body weight and food intake over the 4 week training period were analysed using a repeated measures two-way ANOVA with Bonferroni *post-test*. All other data was analysed using a two-way ANOVA with 'drug' and 'exercise' used as main effect terms. Where a significant interaction was detected, a Bonferroni *post-test* was performed. Significance was established at the $P<0.05$ level.

4.3. Results

4.3.1. An acute exercise bout upregulates hypothalamic genes involved in neurogenesis and stem cell activation.

To firstly investigate the neurogenic potential of exercise on the hypothalamus, mice performed a single exercise bout; 6 h later the hypothalamus was removed and analysed for 84 key genes involved in neurogenesis and stem cell activation. 71% of all genes analysed were significantly unregulated after exercise, compared with sedentary mice. Genes were either upregulated after exercise, or did not change, none were down regulated. Growth factor genes showed some of the largest upregulation after exercise, including Artn (2.7 ± 0.4), Bdnf (3.1 ± 0.5), Bmp15 (3.4 ± 0.6), and Vegfa (3.2 ± 0.5), as well as genes involved in transcription, Apbb1 (4.0 ± 0.7) and Stat3 (3.6 ± 1.1). Overall, the genes upregulated after exercise represent diverse functional categories, including cell proliferation, differentiation motility and migration, transcriptional regulation, growth factors and cytokines. A list of genes that were upregulated more than 2 fold are in Table 4.5, a complete list of all genes analysed and their functional groups can be found in the appendix, Table 8.3. This strongly suggests that exercise is a potent regulator of neurogenic genes in the hypothalamus.

Table 4.5 Neurogenic genes upregulated more than two fold in the hypothalamus after a single exercise bout, as assessed by microarray analysis

Symbol	Gene name	Fold change relative to sed	P value
Adora1	Adenosine A1 receptor	2.7 ± 0.3	0.005
Adora2a	Adenosine A2a receptor	2.3 ± 0.5	0.002
Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1	4.0 ± 0.7	0.002
Artn	Artemin	2.7 ± 0.4	0.005
Bai1	Brain-specific angiogenesis inhibitor 1	3.6 ± 0.5	0.002
Bdnf	Brain derived neurotrophic factor	3.1 ± 0.5	0.004
Bmp15	Bone morphogenetic protein 15	3.4 ± 0.6	0.008
Bmp2	Bone morphogenetic protein 2	2.5 ± 0.3	0.004
Cdk5r1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	2.2 ± 0.2	0.004
Cdk5rap3	CDK5 regulatory subunit associated protein 3	3.4 ± 0.7	0.015
Chrm2	Cholinergic receptor, muscarinic 2, cardiac	2.2 ± 0.2	0.003
Dlg4	Discs, large homolog 4 (Drosophila)	3.1 ± 0.5	0.005
Dll1	Delta-like 1 (Drosophila)	4.2 ± 0.7	0.004
Drd2	Dopamine receptor D2	3.1 ± 0.5	0.002
Drd5	Dopamine receptor D5	2.1 ± 0.3	0.016
Dvl3	Dishevelled 3, dsh homolog (Drosophila)	2.6 ± 0.3	0.013
Efnb1	Ephrin B1	2.1 ± 0.2	0.005
Fgf2	Fibroblast growth factor 2	2.1 ± 0.2	0.003
Gnao1	Guanine nucleotide-binding protein G(o) subunit alpha	2.4 ± 0.4	0.016
Heyl	Hairy/enhancer-of-split related with YRPW motif-like	2.8 ± 0.4	0.001
Mef2c	Myocyte enhancer factor 2C	2.5 ± 0.3	0.001
Ndp	Norrie disease (pseudoglioma) (human)	2.1 ± 0.3	0.007
Nog	Noggin	3.0 ± 0.5	0.005
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
Odz1	Odd Oz/ten-m homolog 1 (Drosophila)	2.0 ± 0.1	0.003
Pax6	Paired box gene 6	2.6 ± 0.4	0.012
Pou3f3	POU domain, class 3, transcription factor 3	2.3 ± 0.2	0.002
Rtn4	Reticulon 4	2.3 ± 0.2	0.001
Slit2	Slit homolog 2 (Drosophila)	2.3 ± 0.6	0.072
Stat3	Signal transducer and activator of transcription 3	3.6 ± 1.1	0.053
Tnr	Tenascin R	4.0 ± 0.9	0.036
Vegfa	Vascular endothelial growth factor A	3.2 ± 0.5	0.004
Ywhah	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	2.5 ± 0.5	0.033

4.3.2. Exercise training induces cellular genesis in the hypothalamus of lean and obese mice

Seven days of exercise training resulted in a 3.5-fold increase in the number of BrdU positive cells in the hypothalamus compared with sedentary controls (P=<0.0001) (Figure 4.1D). Therefore, exercise has a proliferative effect on cells of the hypothalamus. To determine the survival rate of these newborn cells, mice were culled 28 days after BrdU infusion and exercise training ceased. Newborn cells that survive are considered matured and incorporated into the surrounding cellular network, establishing synaptic connections with adjacent cells (1). While the number $BrdU^+$ cells were reduced by 1.7-fold after the initial proliferation period, there was still a 2-fold increase in the number of BrdU cells compared with sedentary mice 28 days after cessation of exercise (P=0.011). The majority of the $BrdU^+$ positive cells were located approximately 200-300 μM away from the ventricle, whilst a few were located approximately 400 μM from the ventricle. These cells were also scattered throughout the different nuclei of the hypothalamus, including the ARC, VMH, DMH and the PVN, whilst very few were located in the LH (Figure 4.1B). Overall, the location of $BrdU^+$ cells was not over represented in any specific region of the hypothalamus. The cytokine CNTF was used as a positive control for hypothalamic neurogenesis (387). While exercise produced a 3.5-fold increase in the number of BrdU positive cells, CNTF treatment resulted in a 17-fold increase in $BrdU^+$ cells, compared with sedentary controls (31.5 ± 4.5 vs. 549 ± 250 $BrdU^+$ cells per section).

Having established that exercise can induce neurogenesis in the hypothalamus of chow fed mice, the neurogenic effects of exercise were investigated in obese mice. Exercise resulted in a 3.2-fold increase in the number of $BrdU^+$ cells in the hypothalamus (P=<0.0001) (Figure 4.1E), which was similar to that seen in chow fed mice. The majority of these new

born cells survived (2.9-fold increase above sedentary mice) (P=0.0001). Therefore, exercise training can stimulate neurogenesis in the hypothalamus of obese mice.

4.3.3. Exercise can induce hypothalamic neurogenesis during middle age

Ageing is associated with reduced neurogenesis in the hippocampus, leading to impaired memory and cognitive ability, which can be improved with exercise (818). Therefore, we investigated whether exercise was able to induce hypothalamic cell genesis in an ageing model. HFD mice were heavier than chow fed mice (44.1 ± 2.2 g vs. 52.9 ± 2.6 g, P=0.028). Exercise training increased the number of $BrdU^+$ cells in the hypothalamus (P=0.0219 main effect) (Figure 4.1F). In the chow fed mice there was a 2.2-fold increase in the number of BrdU positive cells with exercise, while in high-fat fed mice there was a 1.8-fold increase. In the young adult mouse, exercise produced a 3.5-fold increase in BrdU cells in the hypothalamus (Figure 4.1D), however in the ageing mouse a 2.3-fold increase was produced. Basal neurogenesis in the ageing mouse is reduced compared with the young mice (31.5 ± 4.5 vs. 14.1 ± 3.9 $BrdU^+$ cells per section). Therefore, this data shows that exercise can induce cell genesis in the hypothalamus of an ageing mouse, and it is not impaired in a chronically obese model.

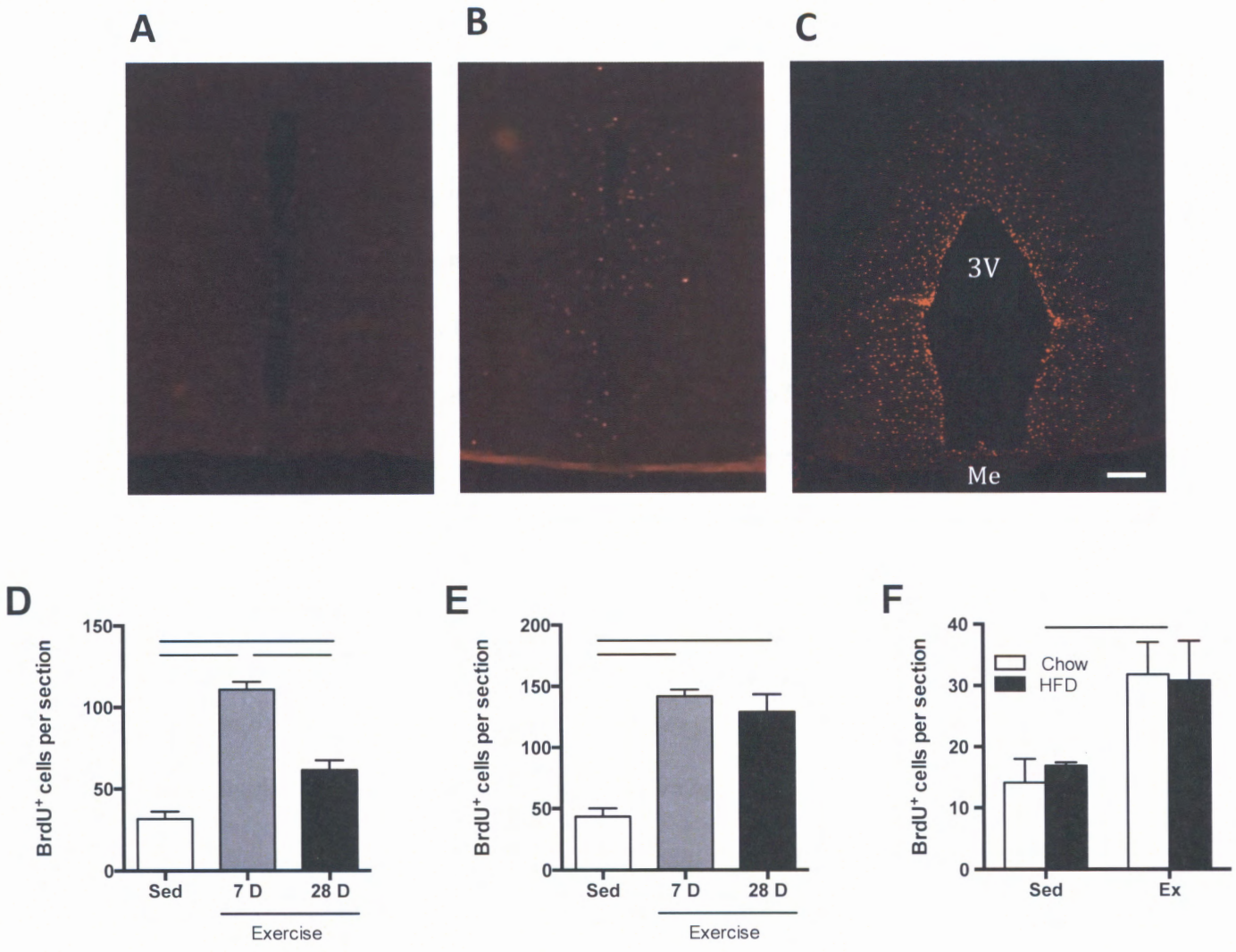


Figure 4.1 Exercise training increases BrdU$^+$ cell number in the hypothalamus

(A, B, C) Representative images of the hypothalamus, immunostained for BrdU. (A) sedentary, (B) exercise and (C) CNTF treated mice. (D) The total number of BrdU positive cells was counted and quantified in chow fed mice, (E) obese mice and (F) aged (~52 week old) mice. n=3-6 per group, connecting lines indicates $P<0.05$ as determined via a one way ANOVA, or two way ANOVA in aging mice. 3V, third ventricle; Me, median eminence; Sed, sedentary; Ex, exercise; D, days. Scale bar, 100 μm

4.3.4. AraC infusion into the brain blocks cell proliferation in the hypothalamus

CNTF increases neurogenesis within the hypothalamus (Figure 4.2). To confirm that AraC treatment can effectively block neurogenesis, mice were infused with either CNTF alone, or CNTF with AraC. AraC infusion at 40 μg/day is successful is blocking neurogenesis in the hypothalamus (Figure 4.2).

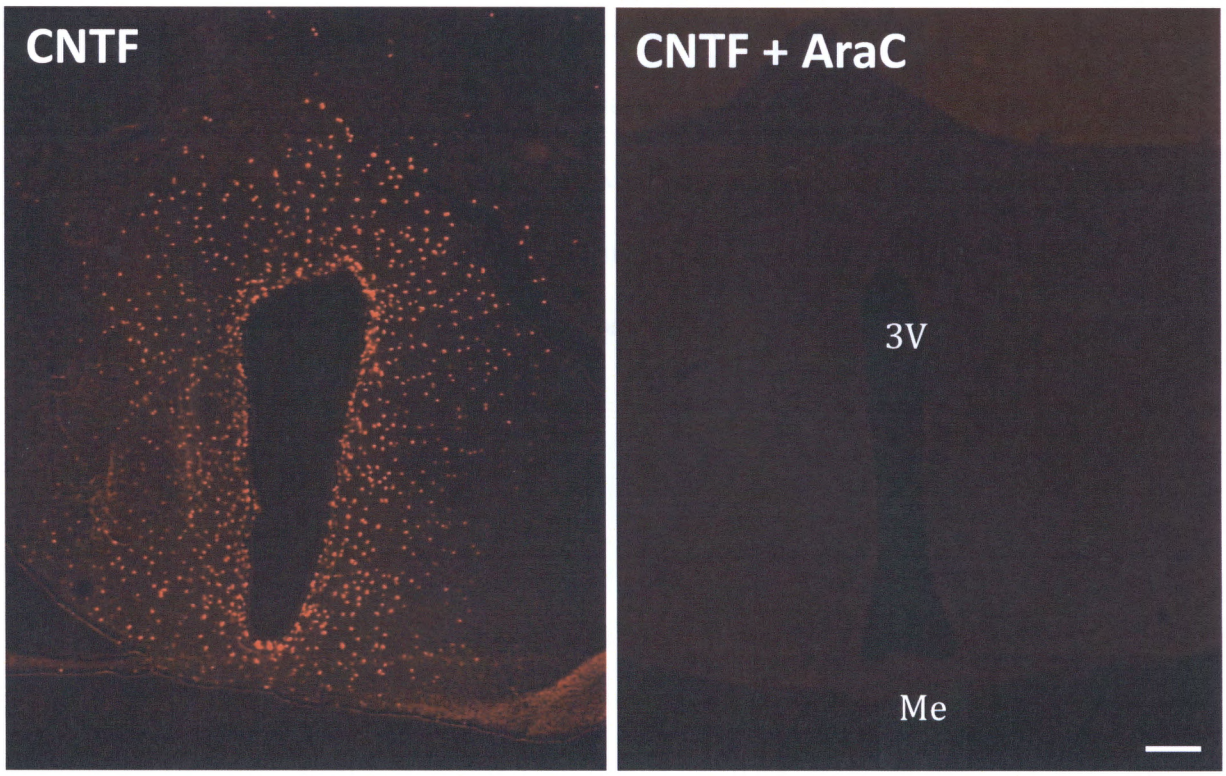


Figure 4.2 AraC infusion blocks neurogenesis

7 days of CNTF infusion into the lateral ventricle induces neurogenesis in the hypothalamus as seen via positive BrdU staining. Co-infusion of CNTF with the mitotic blocker AraC successfully inhibits CNTF's neurogenic effects as seen by a lack of positive BrdU staining. 3V, third ventricle; Me, median eminence. Scale bar, 100 μm

4.3.5. Four weeks of exercise training improves whole body insulin sensitivity, which is blocked with AraC

The shortest amount of exercise training that would improve whole body insulin action in mice fed a high-fat diet was first investigated. Three weeks of exercise training (Table 4.3) did not alter the response to an IP insulin load compared with sedentary animals (Figure 4.3A), which is also reflected by no change in the incremental area under the curve (iAUC) (Figure 4.3B). When an ITT was performed after four weeks of exercise training there were significant improvements in insulin tolerance compared with sedentary mice (Figure 4.3C). This was also reflected in the iAUC (Figure 4.3D). This data demonstrates that 4 weeks of exercise training is the minimum needed to induce detectable improvements in whole body insulin action in HFD mice, and was therefore used for the remainder of the study.

4.3.6. Blocking neurogenesis for 4 weeks does not affect the metabolic characteristics of mice

The role of basal cell proliferation on metabolic characteristics was assessed after 4 weeks of AraC ICV infusion. AraC treatment had no effect on the body weight of mice (Figure 4.4A and B) despite a 14% increase in food intake (Figure 4.4; P=0.047) at the end of the infusion period. The endurance capacity (Figure 4.4E), epididymal fat pad mass (Figure 4.4F) and the plasma profile (FFA, TAG, leptin) (Figure 4.5) were unaffected by 4 weeks of AraC infusion. Plasma insulin levels remained constant between the groups after the IV-ITT (vehicle 234.0 ± 31.14 vs. AraC 255.6 ± 26.94; P=0.614)

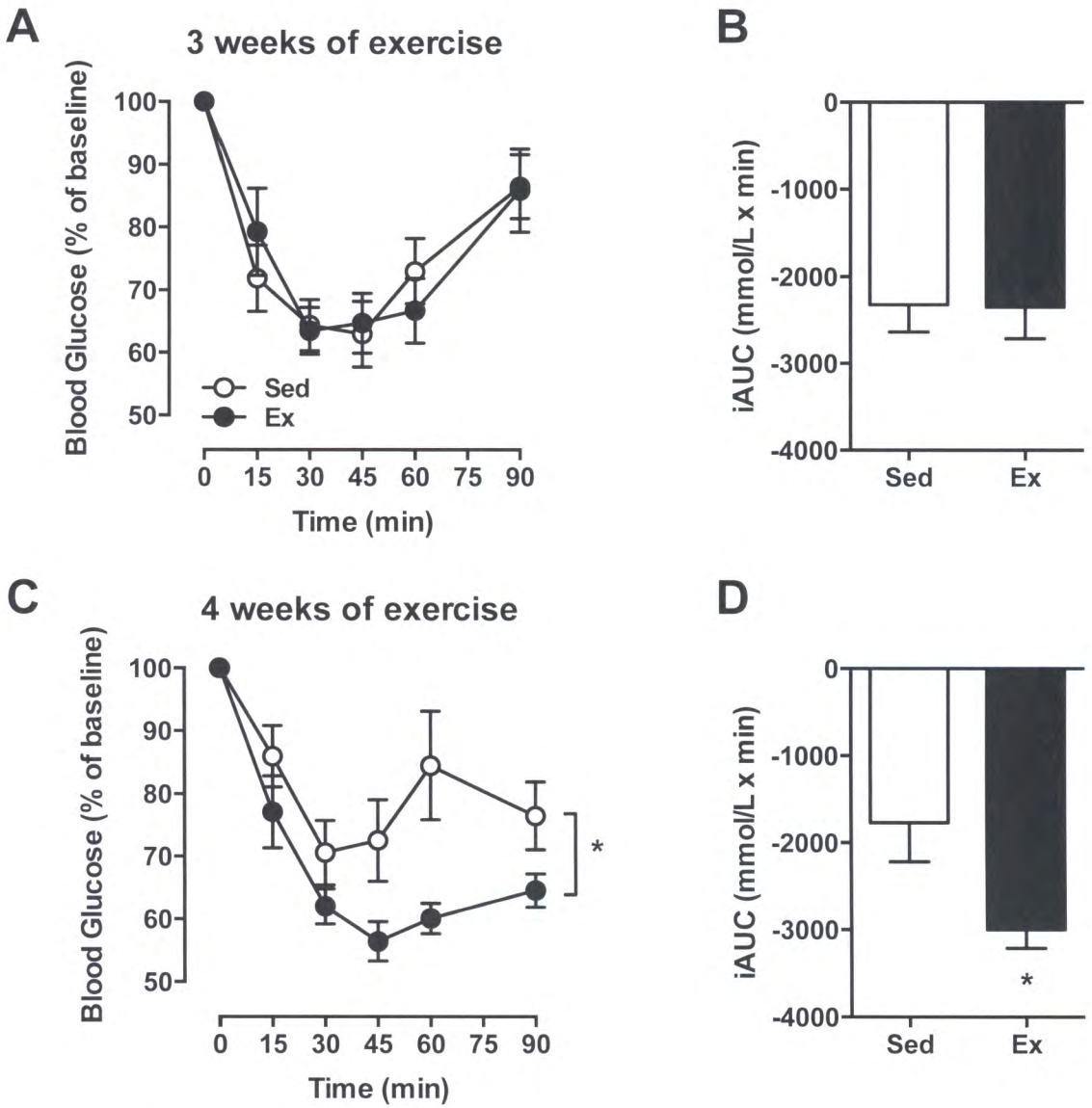


Figure 4.3 Four weeks of exercise training improves whole body insulin tolerance

(A) Insulin tolerance test (ITT) and (B) the blood glucose incremental area under the curve (iAUC) after 3 weeks of exercise training in high fat fed mice. (C) ITT and (D) iAUC after 4 weeks of exercise training in high fat fed mice. n=8 per group, $*P<0.05$ between sedentary (Sed) and exercise (Ex) groups as determined by a two way ANOVA, the iAUC were analysed via an unpaired t-test.

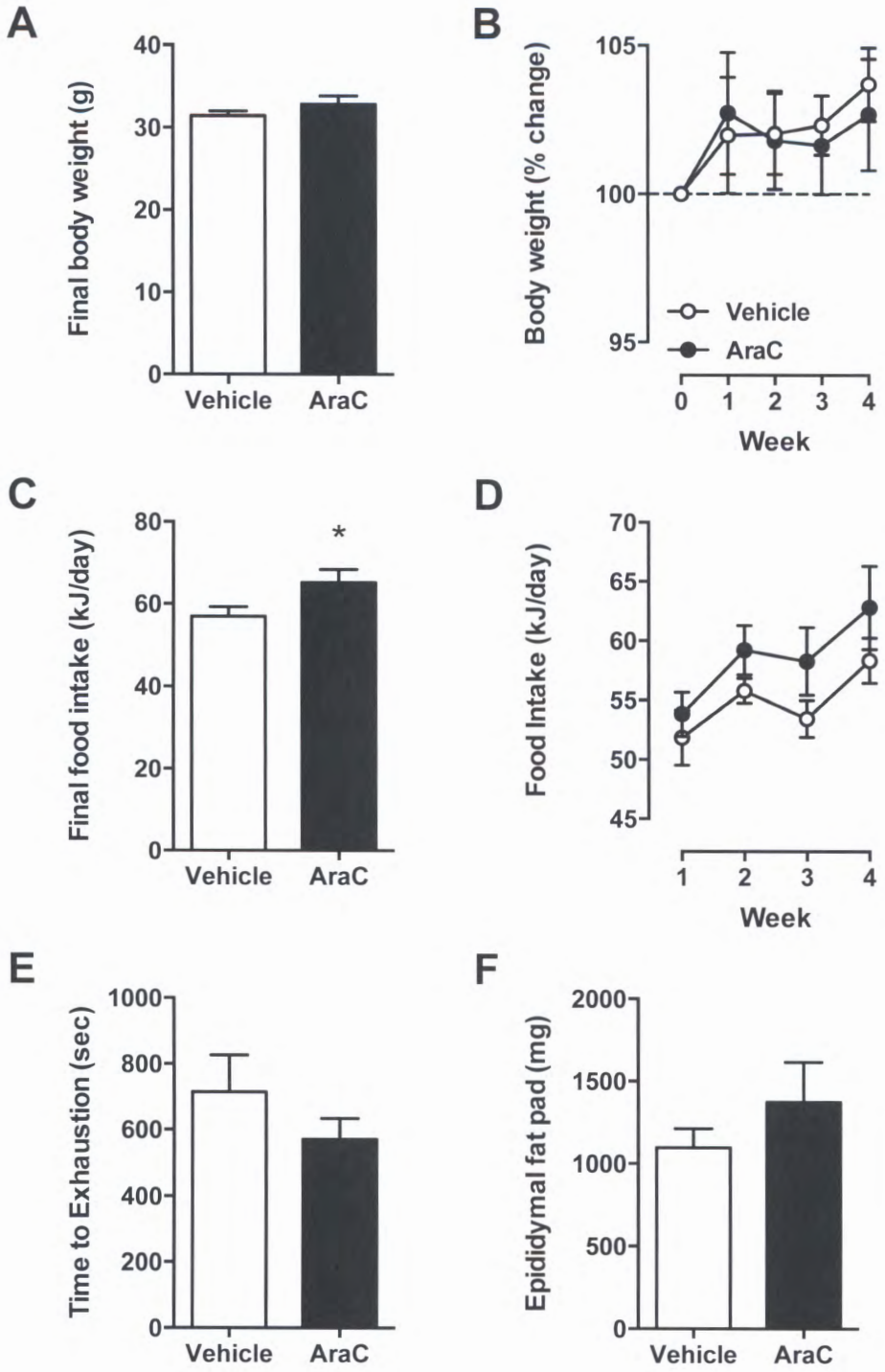


Figure 4.4 Metabolic characteristics of mice after 4 weeks of AraC ICV infusion

(A) Final body weight of mice after 4 weeks of AraC ICV infusion. (B) % change in body weight over the 4 week period. (C) Food intake over the last night of the infusion period (*P<0.05 vs. Vehicle) and (D) weekly food intake during the 4 weeks infusion. (E) The time to exhaustion during an endurance treadmill running test. (F) Epididymal fat mass. n=6-9 per group.

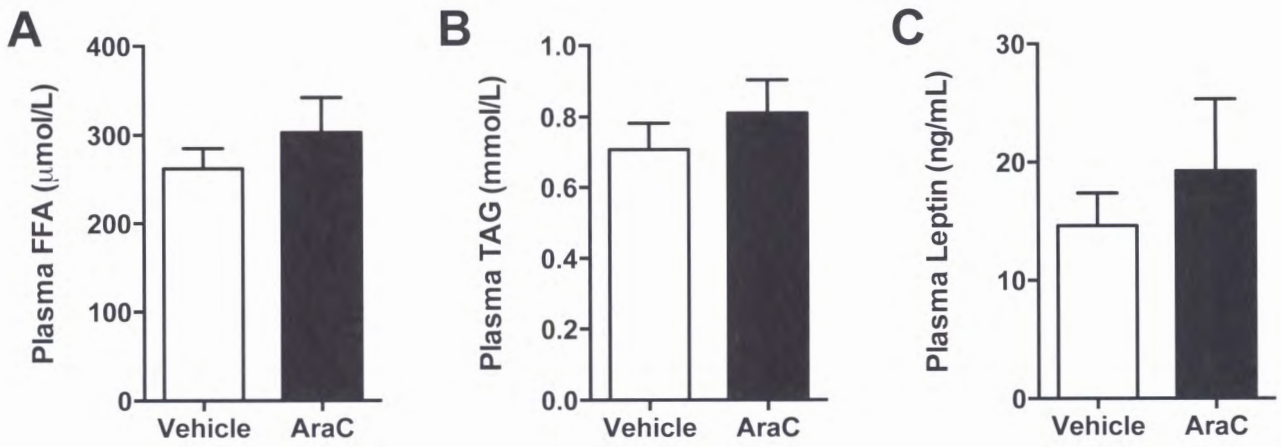


Figure 4.5 Plasma metabolite and hormone profile after 4 weeks of AraC ICV infusion
Whole blood was collected in EDTA tubes at the end of the IV-ITT and the plasma was stored after centrifugation. (A) Plasma free fatty acids (FFA), (B) plasma triacylglycerol (TAG), (C) plasma leptin and (D) plasma insulin. *n*=6-9 per group.

4.3.7. Blocking neurogenesis for 4 weeks does not affect insulin action and glucose uptake.

Insulin action in the brain plays a role in peripheral glucose metabolism, particularly through actions in the liver and skeletal muscle (384, 682). Therefore, the role of neurogenesis on whole body, and organ specific insulin action was investigated by performing an intravenous insulin tolerance test (IV-ITT) with co-administration of ^{3}H-2-deoxyglucose (^{3}H-2DG) and ^{14}C-glucose tracers. Blocking neurogenesis did not alter the blood glucose response to an ITT (Figure 4.6A and B) or the ^{3}H-2DG (P=0.490) (Figure 4.6C) and ^{14}C-glucose (P=0.572) (Figure 4.6D) clearance from the blood. Consistent with this, the glucose uptake into specific organs including quadriceps (P=0.343), gastrocnemius (P=0.325) cardiac muscle (P=0.308) and BAT (P=0.152) was not altered after insulin stimulation (Figure 4.7). Insulin signalling in the hypothalamus was also not changed with AraC infusion (Figure 4.7F). Overall, blocking neurogenesis in the sedentary mice does not affect insulin action or glucose uptake.

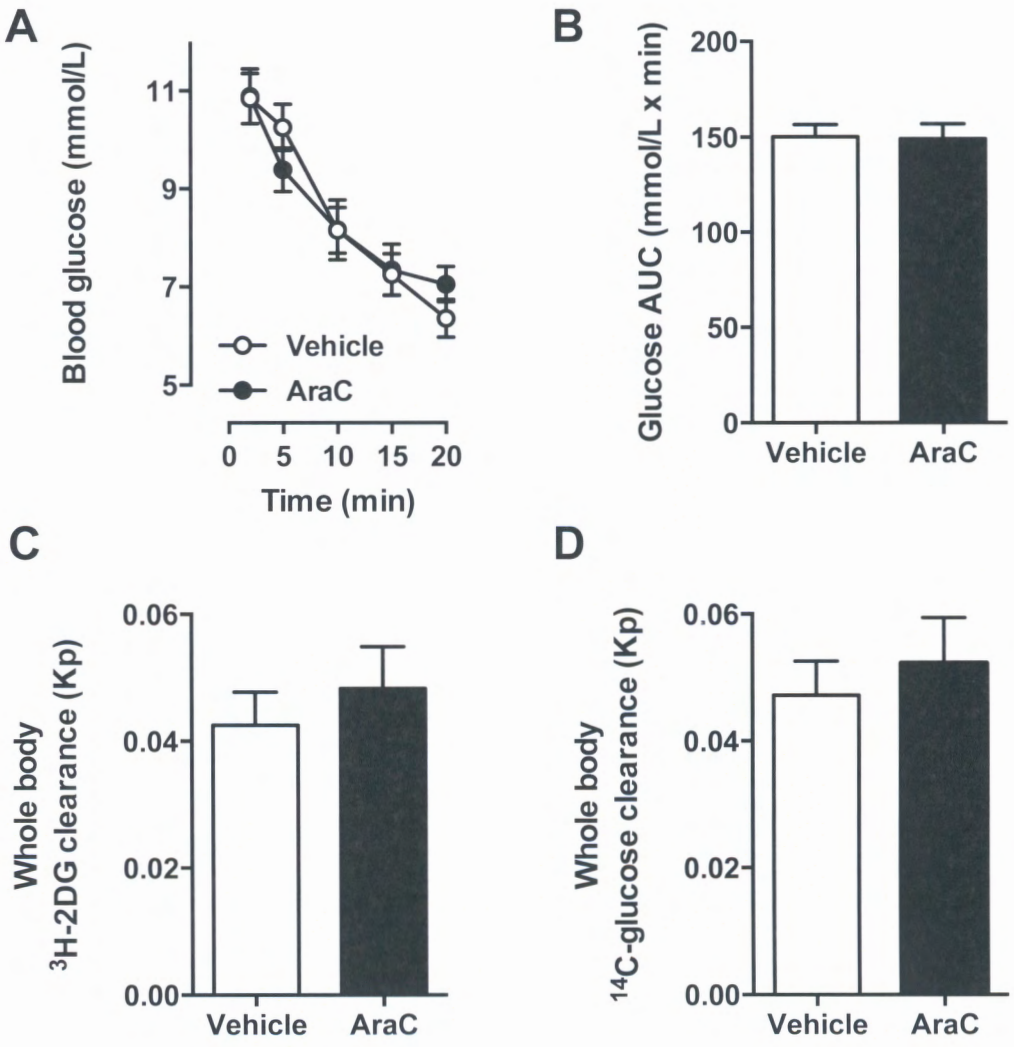


Figure 4.6 Whole body insulin action after 4 weeks of AraC infusion during IV-ITT

(A) Blood glucose levels during intravenous insulin tolerance test (IV-ITT) and the resulting (B) area under the curve (AUC). (C) The whole body clearance of ^{3}H -2deoxy glucose (DG) and (D) ^{14}C -glucose from the blood. *n*=6-9 per group.

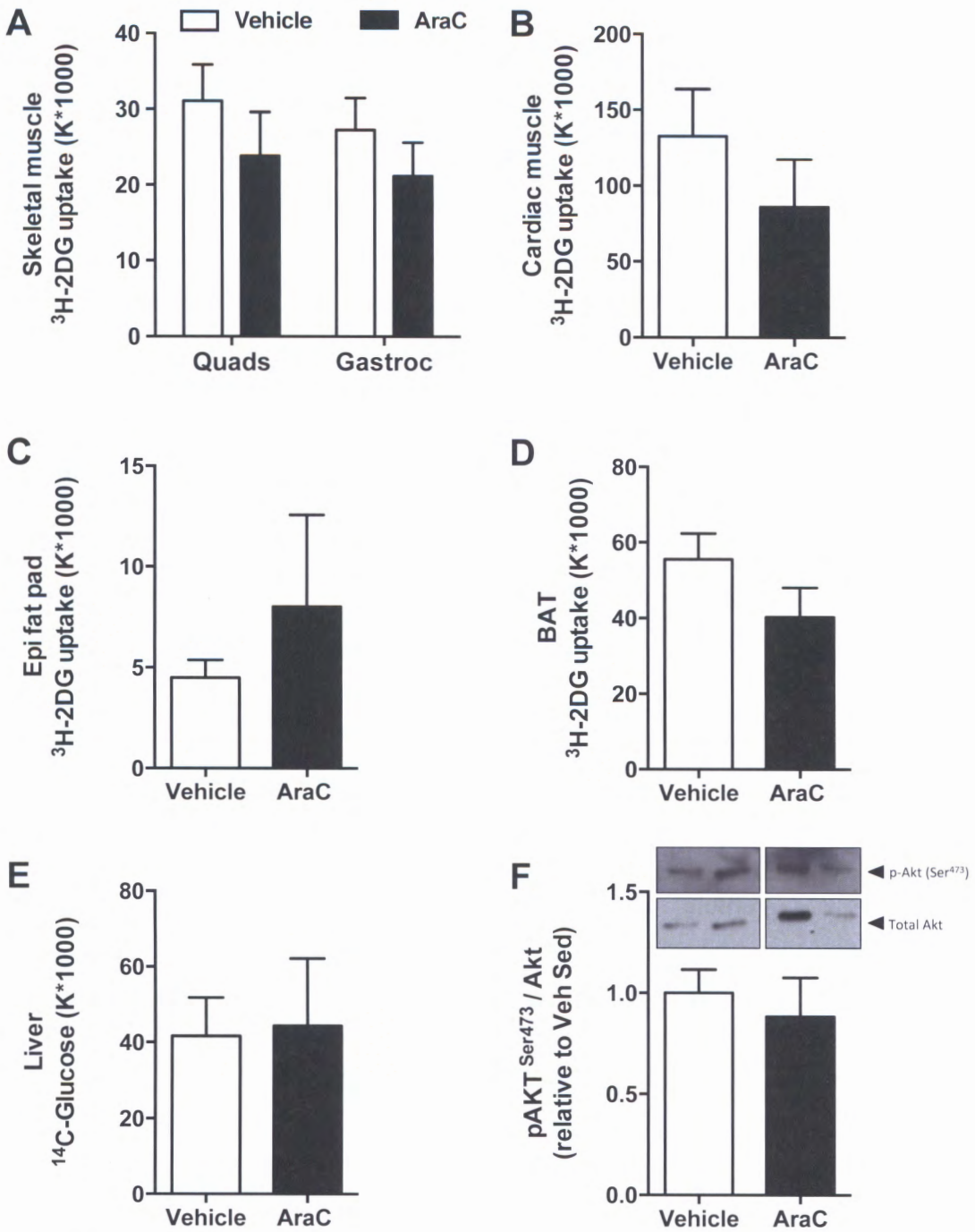


Figure 4.7 Insulin stimulated glucose uptake into peripheral tissues during IV-ITT after 4 weeks of AraC ICV infusion

Insulin stimulated glucose uptake into (A) Skeletal muscle (quadriceps and gastrocnemius), (B) heart, (C) and epididymal fat (D) brown adipose tissue and (E) liver. (F) Insulin-stimulated Akt phosphorylation (Ser473) [in] the hypothalamus, all groups analysed on the same exposure from the same immunoblot. Membranes were stripped and re-probed for total Akt *P<0.05 vs. vehicle. n=6-9 per group.

4.3.8. Exercise-induced neurogenesis does not affect the metabolic characteristics of mice

Having established that basal neurogenesis does not play a significant role in body weight regulation or insulin action, we next examined a situation where neurogenesis is enhanced in the hypothalamus, exercise training (Figure 4.1). Blocking neurogenesis did not affect the body weight during and after 4 weeks of exercise training (Figure 4.8A and B), and unlike the basal condition, food intake was unaffected (Figure 4.8C and D). After the 4 weeks of exercise training, AraC mice had a 33% reduction in their endurance capacity compared with the vehicle mice (P=0.0002) (Figure 4.8E), whilst the epididymal fat pad mass remained the same (Figure 4.8F). Blocking neurogenesis during exercise training resulted in a 32% increase in plasma FFA (P=0.043) (Figure 4.9A), whereas plasma TAG (Figure 4.9B) and leptin (Figure 4.9C) remained constant. Plasma insulin levels also remained constant after the IV-ITT (Vehicle 185.3 ± 19.1 vs. AraC 215.1 ± 33.5; P=0.413). Therefore, exercise-induced neurogenesis plays a role in insulin-mediated suppression of lipolysis.

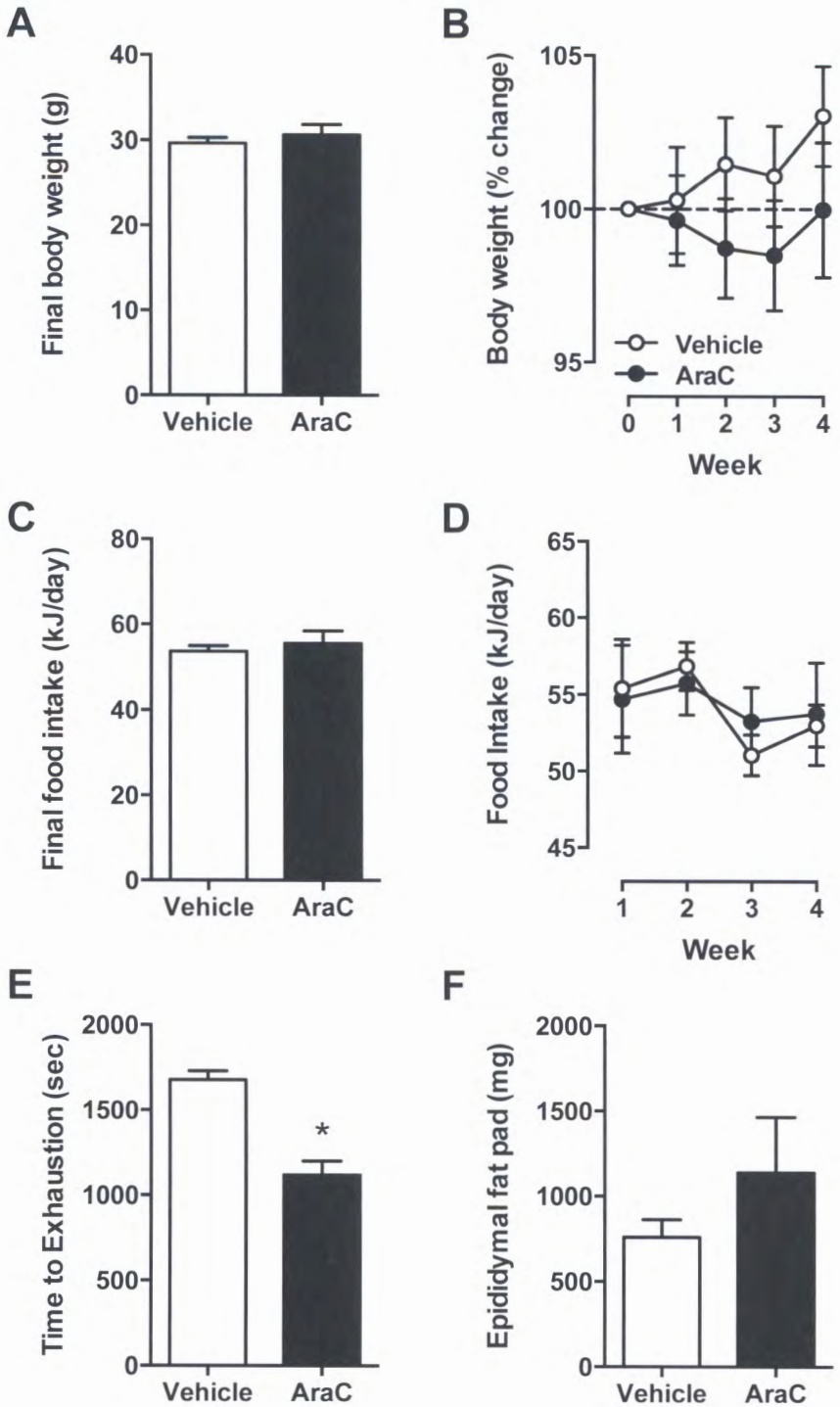


Figure 4.8 Metabolic characteristic of exercise-trained mice with or without ICV administration of AraC

(A) Final body weight of mice after 4 weeks of AraC ICV infusion. (B) % change in body weight over the 4 week period. (C) Food intake over the last night of the infusion period (D) food intake over the 4 weeks infusion. (E) The time to exhaustion during an endurance treadmill running test (*P<0.05 vs. Vehicle). (F) Epididymal fat mass. n=6-9 per group.

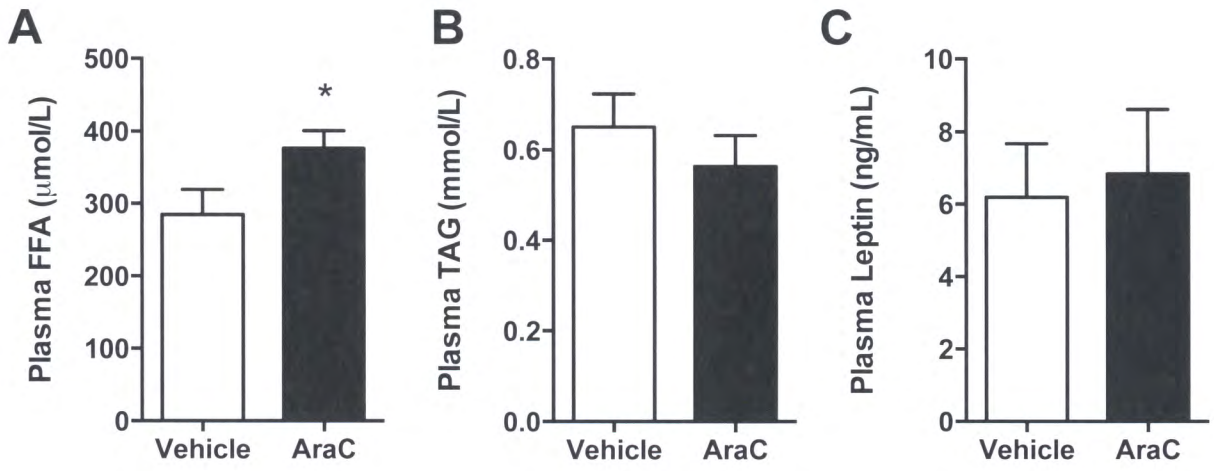


Figure 4.9 Plasma profile of exercise-trained mice with or without ICV administration of AraC

Whole blood was collected in EDTA tubes at the end of the IV-ITT and the plasma was stored at -80°C after centrifugation. (A) Plasma free fatty acids (FFA), (B) plasma triacylglycerol (TAG), (C) plasma leptin and (D) plasma insulin. *P<0.05 vs. Vehicle. n=6-9 per group.

4.3.9. The role of exercise-induced neurogenesis on insulin action and glucose metabolism

Exercise training improves insulin tolerance (Figure 4.3), therefore we investigated the role of central neurogenesis on peripheral insulin action. This was achieved once again by performing an IV-ITT with co-administration of ^{3}H-2DG and ^{14}C-glucose tracers. After 4 weeks of exercise training, AraC treated mice tended to have a blunted blood glucose response during the IV-ITT (P=0.10 main effect) (Figure 4.10A) and this is reflected in the AUC (P=0.08) (Figure 4.10B), however there was no difference in the tracer clearance from the blood (Figure 4.10C and D). AraC infusion during exercise training tended to reduce the insulin stimulated glucose uptake into the epididymal fat pad (P=0.059) (Figure 4.11C) whilst the uptake into other organs remained unchanged (Figure 4.11). Insulin signalling in the hypothalamus was also not changed with AraC infusion (Figure 4.11F). Overall exercise-induced neurogenesis may play a role in whole body insulin tolerance, specifically affecting insulin stimulated glucose uptake into epididymal adipose tissue.

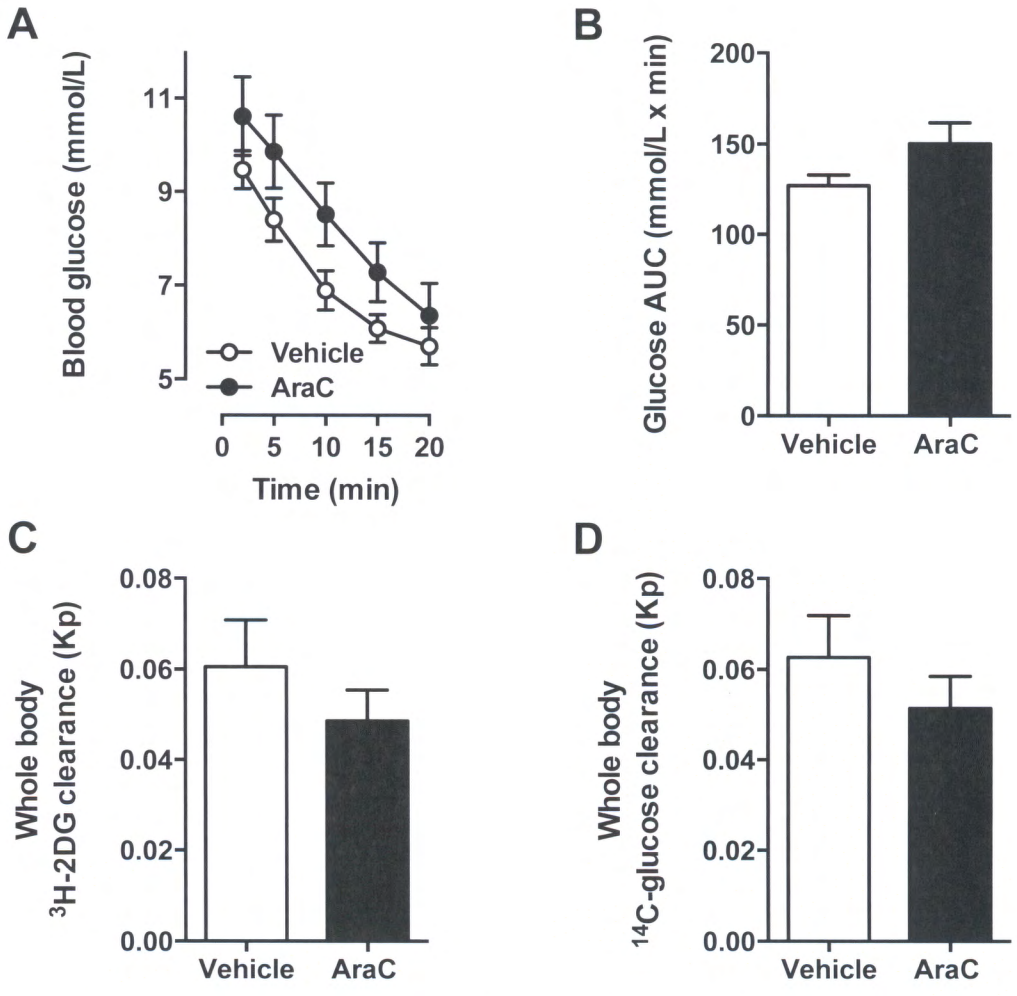


Figure 4.10 Whole body insulin sensitivity after 4 weeks of exercise training with AraC infusion during IV-ITT

(A) Blood glucose levels during intravenous insulin tolerance test (IV-ITT) and the resulting (B) area under the curve (AUC). (C) The whole body clearance of ^{3}H -2deoxy glucose (DG) and (D) ^{14}C -glucose from the blood. *n*=6-9 per group.

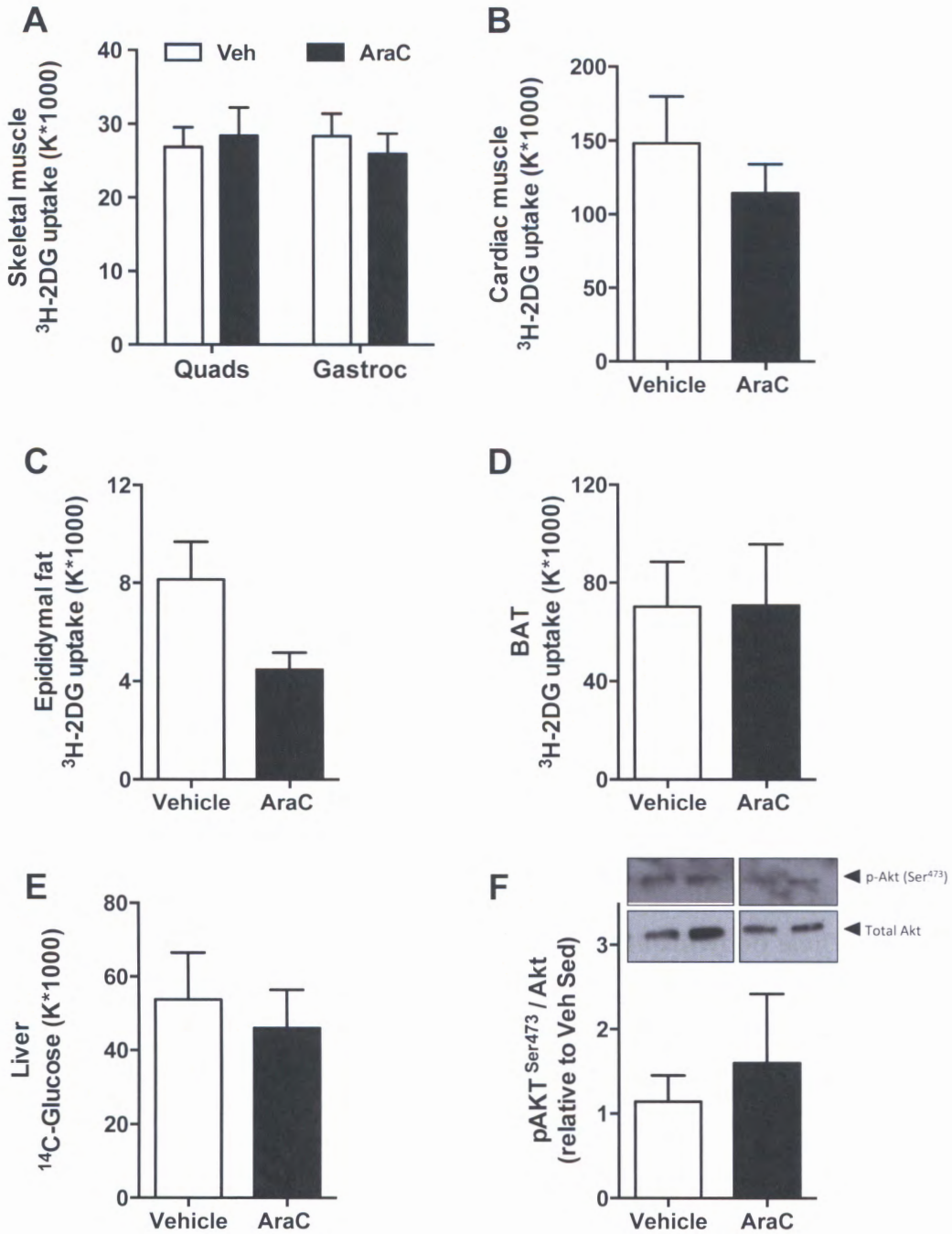


Figure 4.11 Insulin-stimulated glucose uptake into peripheral tissue during IV-ITT after 4 weeks of exercise training with AraC ICV infusion

Insulin-stimulated glucose uptake into (A) Skeletal muscle (quadriceps and gastrocnemius), (B) heart, (C) and epidiymal fat (D) brown adipose tissue and (E) liver. (F) Insulin-stimulated Akt phosphorylation^{Ser473} in the hypothalamus, all groups analysed on the same exposure from the same immunoblot. Membranes were stripped and re-probed for total Akt. *n*=6-9 per group.

4.4. Discussion

Recent reports show that neurogenesis in the hypothalamus extends into adulthood, and highlights its potential importance in body weight regulation (387, 430, 455, 502, 503, 615). In addition, exercise training induces neurogenesis in the hippocampus, and leads to improved learning and memory. Therefore it was hypothesised that exercise training would also induce neurogenesis in the hypothalamus. Just seven days of exercise training increased the number of newborn cells in the hypothalamus, the majority of which survived. This is the first study to demonstrate the induction of hypothalamic neurogenesis by exercise training. Recent studies examining the role of neurogenesis in energy metabolism are equivocal with some reporting reduced hypothalamic neurogenesis in obesity and type 2 diabetes (502), whilst others have shown increased neurogenesis during obesity, suggesting that this is a compensatory mechanism (430). Seven days of exercise training in obese mice increased the number of newborn cells in the hypothalamus, to a similar extent as lean mice, suggesting that the stimulation of hypothalamic neurogenesis by exercise is not reduced in obese mice.

Exercise training was shown to induce hypothalamic neurogenesis in both lean and obese mice. However, the metabolic consequences of this exercise-induced neurogenesis were unknown. In the current study, the physiological impact of neurogenesis on the metabolic profile and insulin-stimulated glucose metabolism was investigated. This was achieved by pharmacological blockade of CNS cell division via ICV infusion of the mitotic blocker AraC. After high-fat feeding, blocking cell division had minimal impact on the metabolic profile, or on insulin-stimulated glucose uptake in sedentary mice. However, blocking cell division during exercise training tended to reduce whole-body insulin action and running endurance capacity. Furthermore, AraC treatment in exercise-trained mice resulted in an increase in

plasma FFA and reduced glucose uptake into the epididymal fat after insulin stimulation. This suggests that exercise-induced neurogenesis may be important in mediating insulin's suppression of lipolysis, combined with reduced glucose uptake, which may contribute to ectopic fat deposition and insulin resistance in other tissues.

Recent lines of evidence suggest that neurogenesis is supressed in obese mice. Specifically, McNay, et al. (502) showed that the number of newborn neurons in the ARC was severely reduced in obese mice compared with lean controls. However, in the current study, there were no differences seen between BrdU cell number between chow and high fat diet sedentary mice. A point of difference between the two studies is the timing of $BrdU^+$ cell counting after the ICV infusion of BrdU. In the current study, cell counting was performed immediately after the 7 days infusion period, and thus only assessed the proliferation of these cells, while McNay, et al. (502) counted the number of newborn cells 4 weeks after the BrdU infusion to allow time for proliferating cells to adopt mature fates. This may account for the difference between the two studies.. In a separate study McNay, et al. (503) once again showed that adult neurogenesis was supressed during obesity. BrdU was administered intraperitoneally, and positive cells were only counted in the ARC. These studies highlight the controversial nature of assessing and quantifying hypothalamic neurogenesis in adulthood, and in response to obesity.

The study of hypothalamic neurogenesis is still a relatively new field, and as such, no consensus on obesity's effects on neurogenesis has been reached. There are increased numbers of new born tanycytes in the median eminence in response to high fat feeding (430) which can differentiate into NPY cells of the ARC (274). Whereas others suggest that *de novo* neurogenesis provides a new level of neural plasticity in reshaping the hypothalamic feeding

circuits (615), and as mentioned previously, BrdU cell number appears to be reduced during obesity (502, 503). Adding the results of the current study, with normal BrdU cell number during obesity, it is clear to see that with all the different time points for assessing neurogenesis, the different hypothalamic nuclei studied, and the different methods employed, that the true physiological role of adult hypothalamic neurogenesis requires further investigation.

Whilst the ability for residential neural stem cells of the adult hypothalamus to proliferate appears to be low compared with the SVZ and SGZ (388, 502), a number of growth, and neurotrophic factors such as FGF2, BDNF, CNTF, VEGF and TGFα have been shown to regulate neural stem cells and neural progenitor proliferation in the adult rodent brain (276, 387, 464, 601). These studies suggest that neurogenesis in the adult hypothalamus can be stimulated under certain conditions. In the current study, just 7 days of exercise training was shown to stimulate neurogenesis in the hypothalamus of lean, obese, and ageing mice. Furthermore, a single exercise bout upregulated genes in the hypothalamus that are involved with neurogenesis and stem cell proliferation. And whilst other studies suggest that adult neurogenesis is reduced in obesity and during ageing (502, 503, 818), exercise training is still able to stimulate hypothalamic neurogenesis under these conditions. These results suggest that exercise training is a potent activator of hypothalamic neurogenesis.

The functional role of hypothalamic neurogenesis has only been investigated in a small number of studies. To establish a functional role for newly formed neurons one must inhibit neurogenesis and analyse physiological outputs. Such experiments have been successfully performed with the use of antimitotic drugs or agents. In the current study blocking neurogenesis in sedentary mice had minimal effects on peripheral insulin action or the

metabolic profile. To date, only a small number of studies have examined the role of spontaneous (basal) hypothalamic neurogenesis on measures of energy balance. In a similar experimental design, 4 weeks of AraC ICV infusion had no effects on body weight or food intake (615). Ablation of hypothalamic neural stem cells via IKKβ/NFκB activation leads to a reduced number of BrdU cells and impaired neuronal differentiation within the ARC (455). This manifested as glucose intolerance, hyperinsulinemia, hyperphagia and obesity. Lee, et al. (430) used focal computed tomography-guided irradiation to selectively inhibit adult neurogenesis in the median eminence, a proposed neurogenic niche of the hypothalamus. Mice with radiation treatment showed reduced weight gain on a HFD compared with control mice, which corresponded with increased energy expenditure and total activity. This suggests that basal hypothalamic neurogenesis is involved in body weight regulation, albeit with conflicting results. Both studies used sophisticated, targeted ablation of neurogenesis in specific areas of the hypothalamus, predominantly the ARC and median eminence. In the current study, AraC infusion into the ventricle was used to inhibit neurogenesis. Thus, inhibition was not localised to a specific nuclei of the hypothalamus, or to the hypothalamus itself, but potentially to the entire brain. As such, the lack of a metabolic phenotype with AraC infusion in the basal state (sedentary mice) in the current study compared with the aforementioned studies may be due to differences in the methodology used to inhibit neurogenesis.

Having established the role of hypothalamic neurogenesis in sedentary mice, we next sort to explore the role of increasing neurogenesis on metabolic function. Exercise is an established model to not only increase hippocampal neurogenesis (806, 814, 815, 817), but to increase hypothalamic neurogenesis (Figure 4.1). Exercise training also enhances insulin action and glucose metabolism in the periphery, with evidence of CNS input (139, 608, 712,

760). Therefore, it was hypothesised that enhanced neurogenesis during exercise would, in part, play a role in energy balance and increased insulin sensitivity in peripheral tissues. In the current study, similar to the sedentary mice, AraC infusion had no effect on the metabolic characteristics of the exercise-trained mice. The effects of enhancing hypothalamic neurogenesis on energy metabolism were examined in one other study. When CNTF was co-administered with AraC into the lateral ventricles, cell proliferation in the hypothalamus was abolished and the long term decrease in body weight induced by CNTF alone was inhibited (387). While the absolute amount of neurogenesis was not quantified in the current study, exercise training induces 5 fold less $BrdU^+$ cells compared with CNTF treatment (Figure 4.1). Therefore, exercise induced neurogenesis may not be high enough to induce metabolic changes over sedentary mice.

Adipose tissue is highly innervated by both the sympathetic and parasympathetic nervous system. Fat mobilisation in adipose tissue is extremely sensitive to suppression by insulin. Both sympathetic (adrenergic) (31) and parasympathetic (cholinergic) (26) activation affects adipose tissue lipolysis. In the current study, AraC infusion during exercise reduced insulin stimulated glucose uptake in adipose tissue whilst increasing plasma FFA, suggesting that neurogenesis is involved in mediating insulin's ability to increase glucose uptake and supress lipolysis in adipose tissue. These findings also suggest that the efferent signals from the CNS that modulates glucose metabolism in adipose tissue are affected by neurogenesis/cellular genesis. Numerous studies have suggested that the CNS, in particular the hypothalamus, can regulate glucose metabolism (423, 446, 554). These studies also suggest that alterations in sympathetic tone may mediate this effect (554). Furthermore, insulin action in the brain appears to play an important role in adipose tissue metabolism. Brain insulin action restrains lipolysis by reducing sympathetic outflow to adipose tissue and controls *de novo* lipogenesis

in adipose tissue (682). Chronic ICV treatment of insulin increases fat mass, fat cell size, and adipose tissue LPL expression, indicating that CNS insulin action promotes lipogenesis (384). We did not observe enhanced insulin activation in the hypothalamus as measured through Akt phosphorylation. This may be due to the route of insulin administration, IP vs. ICV, and that IP delivered insulin has impacts on glucose levels, which also signal to the hypothalamus. Together, these studies show that insulin action in the brain can have direct effects on adipose tissue metabolism, and the results of the current study suggest that enhanced neurogenesis during exercise training can play a role in the regulation, albeit via unknown mechanisms which warrant further investigation.

Neurogenesis in the hypothalamus of sedentary mice was relatively low in the hypothalamus; as such it was not surprising that blocking CNS cell division had no effect on energy balance and insulin action. Previous studies have shown the existence of relatively slow dividing progenitors in the hypothalamus (455), compared with other neurogenic regions of the brain, such as the SVZ. As such, it was expected that increasing hypothalamic neurogenesis through exercise training would lead to the development of an impaired metabolic phenotype, although this was not the case. A recent study suggests that new born cells in the hypothalamus do not acquire a pronounced neuropeptide secretory profile until 30 days after BrdU infusion (455). In the current study 4 weeks of training was performed with IV-ITT preformed 2 days later. Therefore, it is possible that the lack of neurogenesis in the AraC treated mice was not long enough to produce an effect. As such, a longer training period with AraC infusion may reveal a metabolic role for exercise-induced neurogenesis. However, this seems unlikely as 7 days of AraC infusion blocked the sustained weight loss effects of CNTF treatment in mice (387).

Due to the invasive nature of ICV surgery with mini-osmotic pump implantation, the exercise training protocol was designed to be of a moderate intensity. And as such, the neurogenic stimulus may have been insufficient enough to promote metabolic improvements, despite the 3.5-fold upregulation of neurogenesis after just 7 days of exercise. Therefore the question arises, how much neurogenesis is required for a significant effect on physiology? Very small numbers of neurons in the classically neurogenic region of the hippocampus are critical to the regulation of memory formation (284). However, neither the critical number of hypothalamic neurogenesis on physiological output, nor the importance of neurogenesis in specific hypothalamic nuclei is yet to be assessed and requires further investigation. Furthermore, CNTF appears to induce hypothalamic neurogenesis equally in hypothalamic nuclei, whilst having profound effects on body weight and food intake, indicating that neurogenesis may not need to be confined to specific nuclei, ie. the ARC, to have an effect on metabolism.

The hypothalamus can have profound effects on the control of glucose homeostasis through the regulation of hepatic glucose output (569, 572, 618, 619). The experimental design of the current study (IV-ITT vs. hyperinsulinemic-euglycemic clamp) means that while insulin tolerance and organ specific insulin stimulated glucose uptake was assessed, hepatic glucose output was not. Therefore, the effect of hypothalamic neurogenesis in both the basal and exercised state on hepatic glucose production is not known and could provide a point of regulation of cell proliferation in the CNS. Furthermore, the phenotype of $BrdU^+$ cells was not assessed in the current study. Work performed in the hippocampus shows that exercise training preferentially induces the proliferation of neurons, and not other cells of the CNS such as astrocytes and microglial cells (814, 815, 818). Stimulation of cell proliferation by CNTF is seen mostly in neurons (387, 388) and results of the gene array in the current

study suggest that exercise would be inducing neurogenesis in the hypothalamus. Future studies will need to determine explicitly the cell type proliferating during exercise training, and whether these cells express neuropeptides involved in body weight regulation.

The study of hypothalamic neurogenesis, although in its infancy, has revealed a potential role in body weight regulation. Exercise training stimulates adult neurogenesis in the hypothalamus in lean, obese and ageing mice. Furthermore, insulin mediated suppression of lipolysis and glucose uptake was impaired, suggesting that exercise induced neurogenesis is required to maintain insulin sensitivity in adipose tissue. Insulin resistance in adipose tissue is a major defect of obesity and diabetes, leading to elevated plasma lipids and ectopic lipid accumulation, and thus provides a rationale for the use of neurogenesis promoters as targets for obesity and diabetes treatment.

Declaration for Thesis Chapter Five

Declaration by Candidate

In the case of Chapter Four and Five, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation	85%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of Contribution	Extent of contribution (%) for student co-authors only
Zane B. Andrews	Contributed to study conception	
Matthew J. Watt	Study design, carried out IV injections, interpretation of data, manuscript preparation	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contribution to this work

Candidate's Signature	Date
[redacted]	16.10.2013
Main Supervisor's Signature	**Date**
[redacted]	16.10.2013

Chapter Five: Cessation of cilliary neurotrophic factor treatment causes rebound weight gain and impaired insulin action

5.1. Introduction

Obesity is a strong risk factor for the development of insulin resistance and type 2 diabetes. The anti-obesity agent CNTF leads to weight loss and improved insulin action by modulating key metabolic and inflammatory pathways in major tissues such as skeletal muscle, adipose tissue and the liver (76, 151, 730, 835, 836). In addition to these actions in metabolic tissues of the periphery, CNTF induces pronounced neurogenesis in the hypothalamus (387, 388). Many of these new adult-born neurons express POMC, a major regulator of energy balance that suppresses food intake and increases energy expenditure via the actions of the melanocortin system (112, 305, 328, 590)

Intriguingly, CNTF induces pronounced and sustained weight loss several weeks after ceasing administration and chemical blockade of neurogenesis with AraC abrogates these effects, suggesting that neurogenesis is a critical regulator of energy balance (387). The interpretation that neurogenesis contributes to the plastic control of energy balance has since been supported (431, 615). Notably, POMC neurons also regulate glucose metabolism through the activation of central MC4R signalling (546) in the sympathetic nervous system to control hepatic insulin action (44, 66, 305, 328, 590). Therefore, it was hypothesised that CNTF-mediated neurogenesis would improve insulin action in obesity.

5.2. Methods

5.2.1. Animal experimental procedures

Monash University Animal Ethics Committee approved all animal protocols used in this study. Mice were obtained from Monash Animal Services at six weeks of age and maintained on a 12 h light, 12 h dark cycle with lights on at 0700 h at Physiology Department Large Animal Facility, Monash University. Mice were housed five to a cage and allowed to acclimatise for one week with standard rodent chow and water *ad libitum*.

To determine the role of enhanced neurogenesis on whole body insulin action and glucose metabolism, 6-week-old mice were placed on a HFD for a period of 4 weeks (Table 5.1). Mice then underwent ICV surgery and osmotic mini-pump implantation (model 1007D flow rate 0.5 µL/h, 7 days, Alzet, Cupertino, CA) (as described in Chapter 4), containing either vehicle (aCSF and 1 µg/µL BrdU (n=7)) or vehicle and CNTF ((Axokine, 100 ng/µL) (n=5)). Axokine (developed by Regeneron Pharmaceuticals) is a truncated form of CNTF with the last 15 c-terminal amino acids removed, glutamine is replaced by arginine at position 63 and the free cysteine at position 17 is replaced by alanine (729). Mice were housed singly and monitored daily for body weight and food intake.

Table 5.1 Details of diet

	High-fat diet
Manufacturer	Specialty Feeds SF03-002
Total crude fibre	4.7%
Total protein	19.5%
Total fat	36%
Total energy from fats	59%
Digestible energy (kJ/g)	22.8

5.2.2. Whole body glucose metabolism

To assess the effects of centrally administered CNTF on peripheral insulin action, an IV-ITT was performed with radioactive glucose tracers as described in Chapter 4.

5.2.3. Plasma analysis

5.2.3.1. Free fatty acids

See chapter 3, section 3.2.5.1 for details

5.2.3.2. Triacylglycerol

See Chapter 3, section 3.2.5.2 for details

5.2.3.3. Insulin

See Chapter 4, section 4.2.7.4. for details

5.2.4. Statistics

All results are expressed as the means ± SEM. All Statistical analysis was conducted using the statistical package GraphPad Prism version 5.0d (GraphPad Software, San Diego, California, USA, www.graphpad.com). Body weight and food intake over the 28 days were analysed using a repeated measures two-way ANOVA with Bonferroni *post test*. All other data was analysed using a students t test. Significance was established at the $P<0.05$ level.

5.3. Results

5.3.1. CNTF reduces body weight and food intake, only during the treatment period

CNTF induces a powerful anorexigenic response (748, 866). We recorded a 45% decrease in food intake during the CNTF administration period (Figure 5.1A). This was associated with a progressive reduction in body mass (Figure 5.1B). CNTF was previously reported to result in a sustained reduction in body mass lasting for at least 36 days (387). We observed an immediate increase in food intake and body mass after the cessation of CNTF administration. Food intake was normalised within 2 days and body mass restored after 5 days (Figure 1A and B). In contrast to the improvement in glycaemia and lipid profiles during short-term CNTF treatment (730, 835), neither blood glucose, plasma FFA nor TAG were different 3 weeks after treatment (Figure 5.2). Overall, central CNTF reduced food intake and body weight, without any sustained effects after treatment.

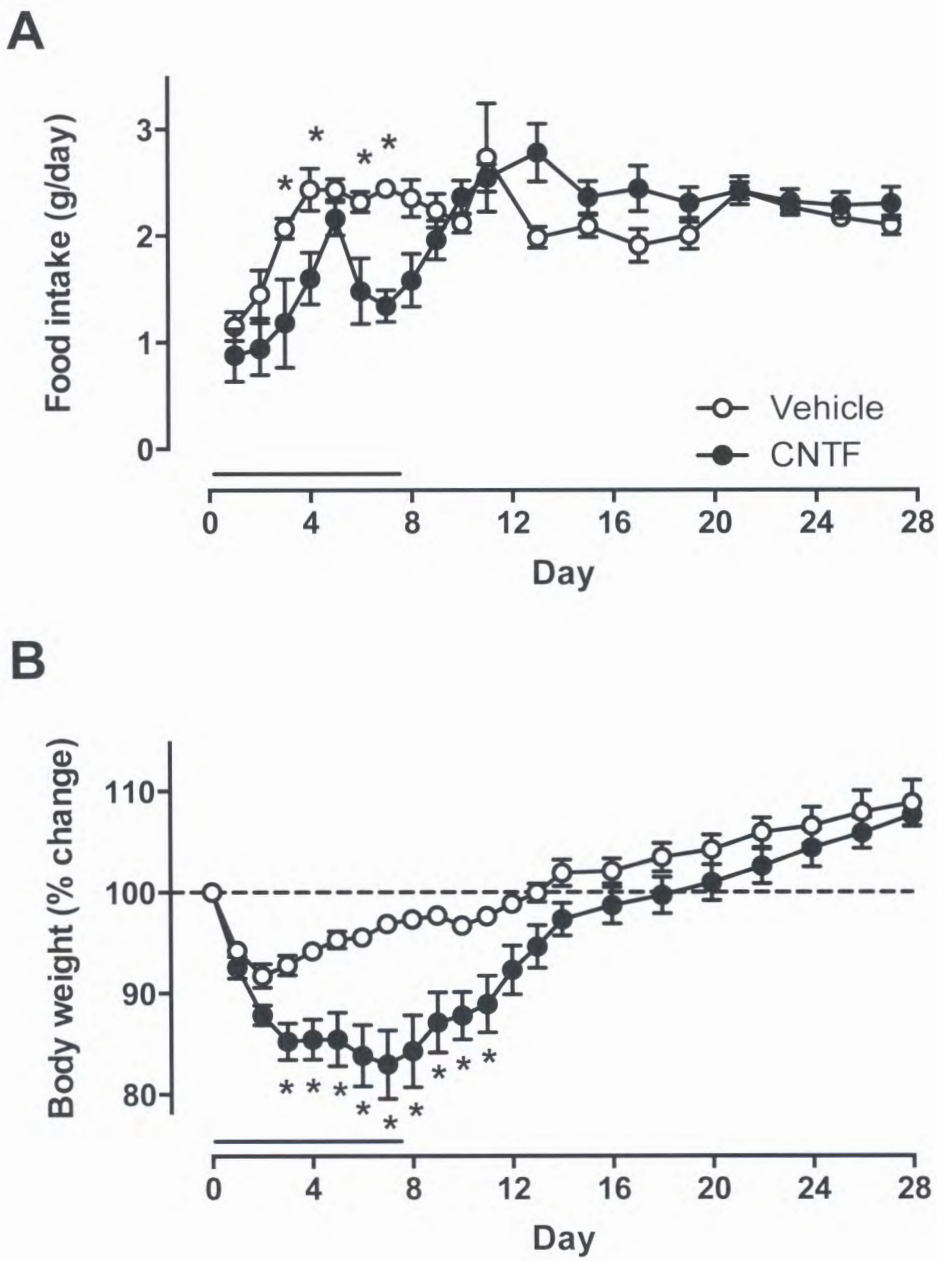


Figure 5.1 Central CNTF effects on body weight and food intake

(A) Food intake and (B) body weight expressed as a percentage of starting weight over a 28-day period. Black line denotes infusion period. n=5-7 per group. *P<0.05 vehicle vs. CNTF.

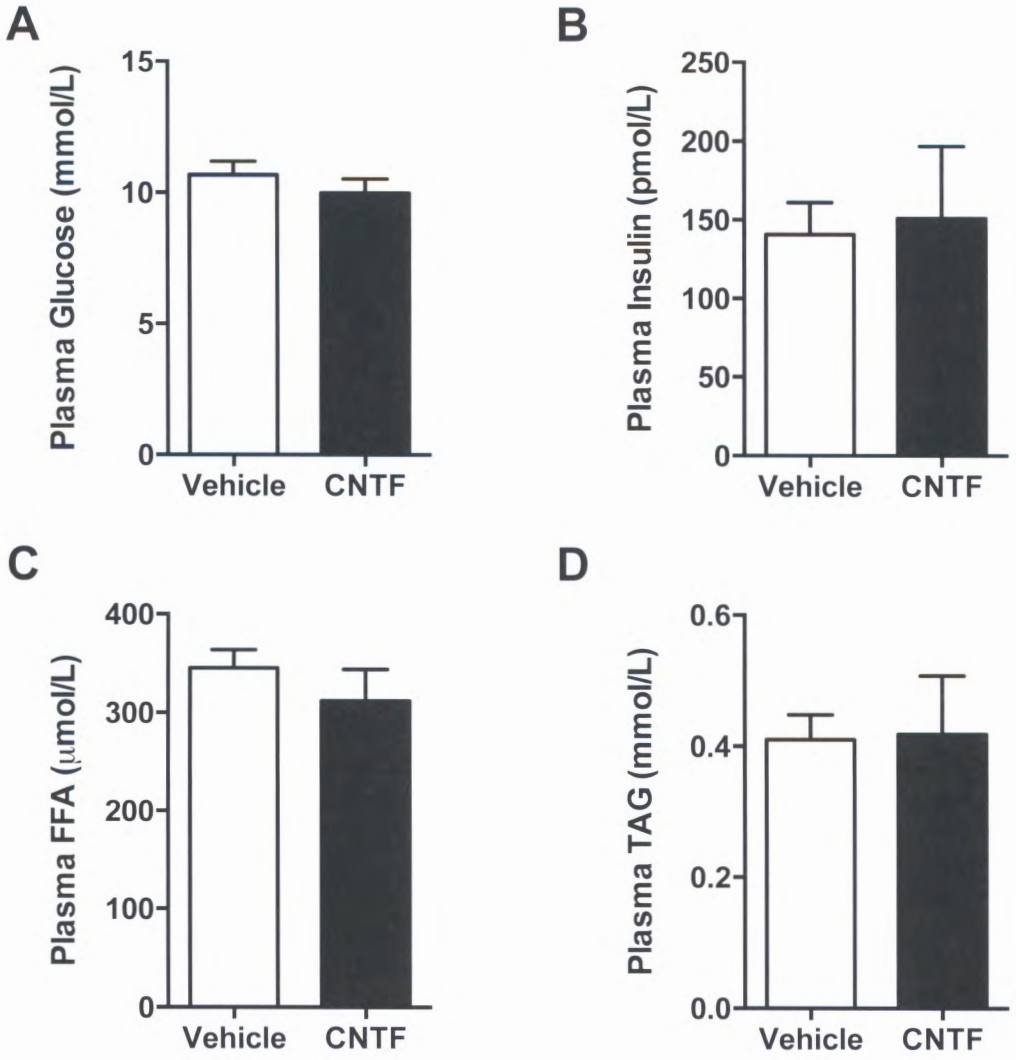


Figure 5.2 Plasma profile after central CNTF treatment

Whole blood was collected in EDTA tubes at the end of the IV-ITT and the plasma was stored after centrifugation. (A) Plasma glucose, (B) plasma insulin, (C) plasma free fatty acids (FFA), and (D) plasma triacylglycerol (TAG). *n*=5-7 per group.

5.3.2. CNTF mice have reduced whole body insulin sensitivity

Peripheral CNTF improves skeletal muscle insulin sensitivity, and reduces inflammatory signalling cascades associated with lipid accumulation in the liver and skeletal muscle (730, 835). However, the effect of centrally administered CNTF on peripheral metabolism and insulin action is less clear. Insulin action was assessed by intravenous administration of insulin (0.5 U/kg) and the use of radioactive glucose tracers. Whole body glucose clearance during insulin stimulation was reduced by 32% (P=0.06) in CNTF treated mice (Figure 5.3A). This was associated with a tendency for reduced glucose uptake into the quadriceps muscle (42%, P=0.08), cardiac muscle (29%, P=0.15) and brown (27%, P=0.11) and white adipose tissue (24%, P=0.18) (Figure 5.4FA-E). Overall, this suggests that central CNTF promotes insulin resistance, which may be more related to the rebound in body weight after treatment.

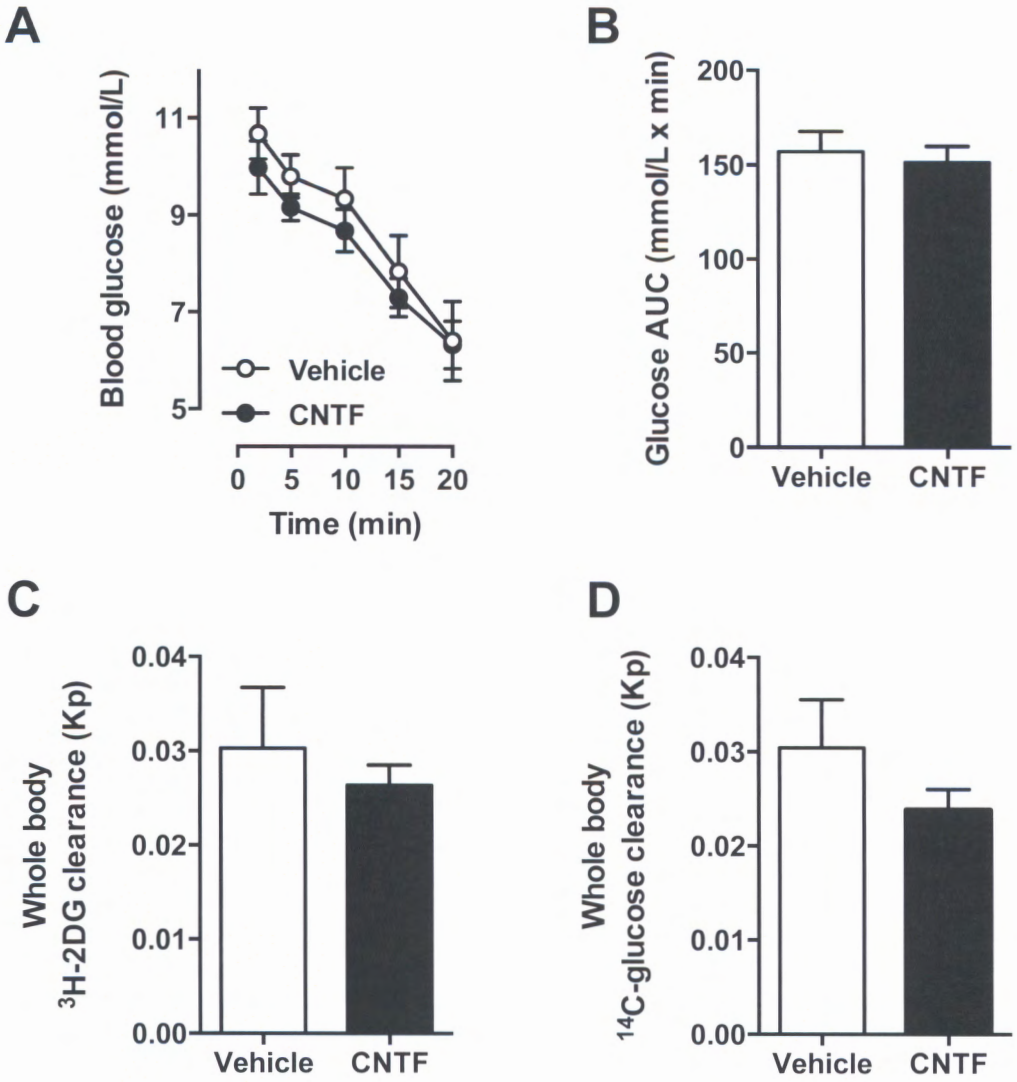


Figure 5.3 Whole body insulin sensitivity after central CNTF during IV-ITT

(A) Blood glucose levels during intravenous insulin tolerance test (IV-ITT) and the resulting (B) area under the curve (AUC). (C) The whole body clearance of ^{3}H -2deoxy glucose (DG) and (D) ^{14}C -glucose from the blood. *n*=5-7 per group.

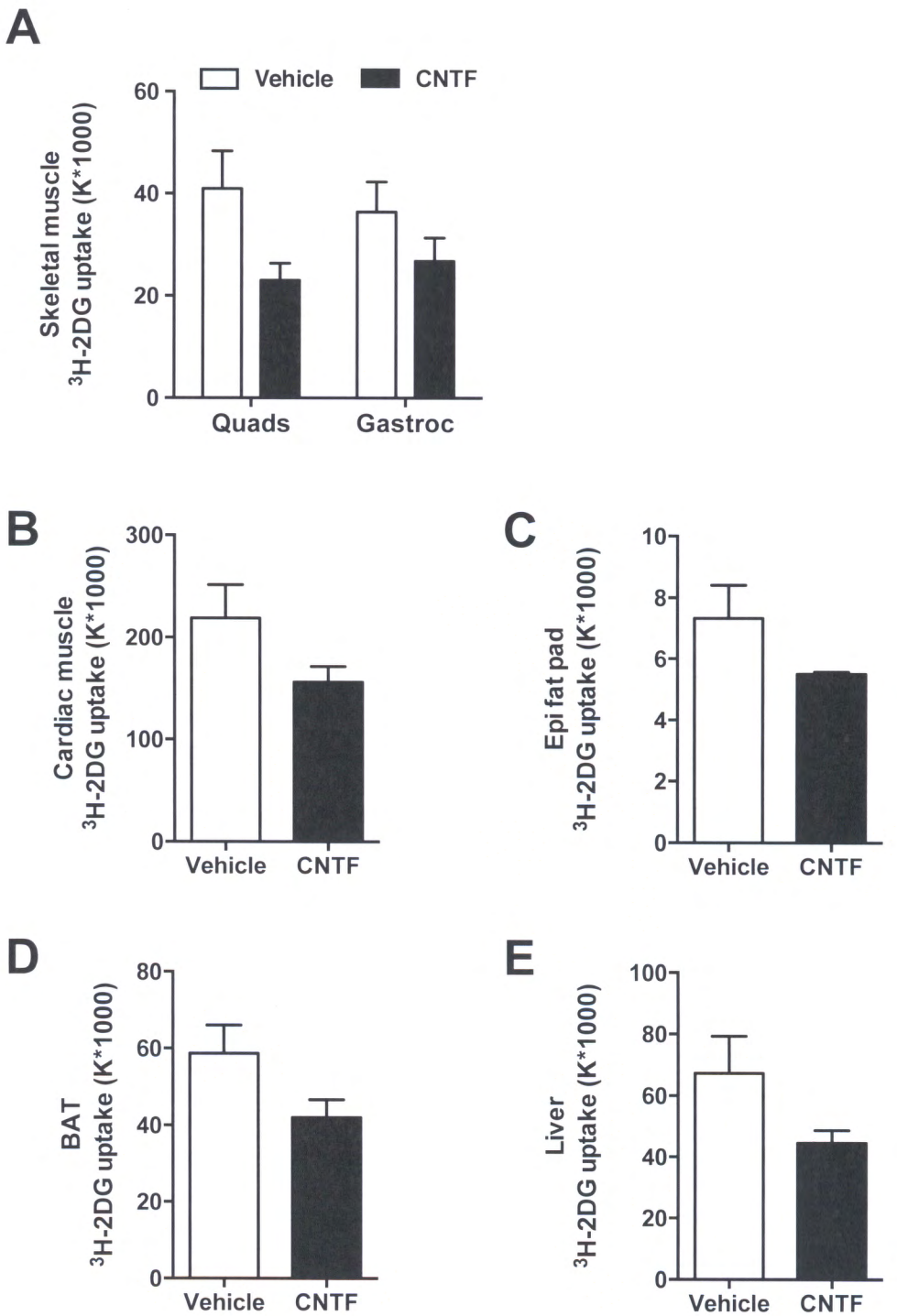


Figure 5.4 Insulin stimulated glucose uptake into peripheral tissues during IV-ITT after central CNTF

(A) Skeletal muscle (quadriceps and gastrocnemius), (B) heart, (C) and epidiymal fat (D) brown adipose tissue and (E) liver insulin-stimulated glucose uptake. *P<0.05 vs. vehicle. n=5-7 per group.

5.4. Discussion

The sustained weight-reducing effect of CNTF was previously linked to hypothalamic neurogenesis (387), thereby implying that the activation of a select subset of hypothalamic neural stem cells could provide a therapeutic intervention to treat obesity. Opposing this view is the observation that high-fat feeding concomitantly induces obesity and neurogenesis in the median eminence of the hypothalamus, suggesting that neurogenesis in this hypothalamic region promotes a pro-obesity phenotype (431). We find that 7 days of central CNTF administration leads to rapid weight loss and reduced food intake in obese mice, but cessation of CNTF administration results in rebound weight gain and impaired insulin action. This suggests that hypothalamic neurogenesis induced by CNTF does not regulate energy balance as previously reported (387) and questions the role of hypothalamic neurogenesis in regulating substrate metabolism and energy homeostasis.

CNTF induces rapid and sustained weight loss, which is ascribed to hypothalamic neurogenesis (387). Therefore it was hypothesised that alongside weight loss, CNTF-induced neurogenesis would also have peripheral, insulin sensitising effects. The methods described by Kokoeva, et al. (387) for the induction of hypothalamic neurogenesis and weight loss were replicated in the current study. We find that central CNTF administration causes rebound gain and impaired insulin action after cessation of treatment. In support of this finding, just 2 weeks of overeating in normal weight humans leads to whole body inulin resistance (144) while 2 weeks of HFD in mice models results in a considerable increase in fat mass weights and a profound reduction in hepatic insulin sensitivity (379). Furthermore, low birth weight in infants leads to catch-up weight gain and the development of insulin resistance (207, 330, 395). These studies demonstrate how rapid weight gain and over nutrition can lead to

metabolic dysfunction and impaired insulin action. A point of different between the current study and that of Kokoeva, et al. (388) is the use of the CNTF analogue Axokine instead of recombinant CNTF, which appears to be more specific for the CNTF receptor (687, 729), and therefore should not negatively impact the results. Additionally, a higher concentration of CNTF was infused into the ventricles (100ng Axokine), as lower doses failed to induce hypothalamic neurogenesis (data not shown).

While CNTF-induced neurogenesis was not shown the in the current study, the previous chapter has clearly demonstrated that neurogenesis is present in the hypothalamus directly after seven days of ICV infusion (Figure 4.1 and 4.2), recapitulating the work of others (387, 388). Furthermore, these studies show that these newborn neurons are present in the hypothalamus for 3 weeks and longer after the end of the treatment period (387), so it is reasonable to assume that these newborn neurons would still be present at the time of the IV-ITT. The current study also recapitulated the rapid weight loss effects of central CNTF, although the sustained weight loss after the treatment has ended was not apparent. Instead we observed rapid rebound weight gain; suggesting that CNTF induced neurogenesis does not always result in sustained weight loss. To that end, numerous growth factors such as BDNF (601), IGF-1 (605), FGF (869) induce hypothalamic neurogenesis after central administration. Additionally, ICV infusion of BDNF induces rapid weight loss in rats (600, 823, 824), and in accordance with the current study, results in rebound weight gain at cessation of treatment (600). Therefore, there is evidence to suggest that stimulating hypothalamic neurogenesis does not always result in sustained weight loss.

In conclusion, central administration of CNTF causes reduced food intake and robust weight loss, while cessation of treatment results in immediate rebound weight gain, which

manifests as reduced insulin action in the periphery. These results question the role of stimulating hypothalamic neurogenesis through growth factors as a pharmacological treatment for obesity.

Declaration for Thesis Chapter Six

Declaration by Candidate

In the case of Chapter Seven, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Study design, animal husbandry, performed experiments, analysed samples, data and statistical analysis, data interpretation, manuscript preparation	85%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of Contribution	Extent of contribution (%) for student co-authors only
Zane B. Andrews	Study conception. Critical analysis and interpretation of data, review/edited manuscript	
Matthew J. Watt	Study conception. Critical analysis and interpretation of data, manuscript preparation	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contribution to this work

Candidate's Signature	Date
[redacted]	16.12.2013
Main Supervisor's Signature	**Date**
[redacted]	16.12.2013

Chapter Six: Exercise training does not produce long-term changes in hypothalamic sensitivity to leptin or ghrelin in obese mice

6.1. Introduction

The hypothalamus of the central nervous system detects neural, endocrine and metabolic signals from the periphery to regulate whole-body energy homeostasis. Pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons of the arcuate nucleus (ARC) are critically involved in the regulation of food intake and energy expenditure (28, 270). Leptin and ghrelin are two such peripheral signals/hormonal cues that influence POMC or AgRP neurons in the ARC (27, 44). Leptin is a critical hormone linking adiposity with food intake and energy expenditure (891). Leptin is secreted by white adipose tissue and provides feedback of the body's fat stores to the hypothalamus, invoking anorexigenic actions by activating POMC neurons and inhibiting NPY/AgRP neurons (44, 691). On the other hand, ghrelin is secreted from the stomach and is one of the most potent orexigenic factors. It increases appetite before the onset of a meal, predominantly though the activation of NPY neurons, and the indirect inhibition of POMC neurons via GABA input from NPY neurons onto POMC neurons (27, 37). Thus, under normal circumstances leptin suppresses and ghrelin stimulates food intake. Obesity is characterised by a state of both leptin (201, 549) and ghrelin resistance (87, 245) in the brain, whereby POMC and AgRP neurons of the ARC fail to elicit the appropriate food intake response to circulating levels of these hormones. Restoring leptin responsiveness has been suggested as a therapeutic approach to treat obesity (778).

A sedentary lifestyle contributes to the development of obesity and obesity-associated disorders such as cardiovascular disease, type 2 diabetes, atherosclerosis and stroke (762). Physical activity is considered a cornerstone for the treatment for obesity because exercise increases energy expenditure, reduces adiposity and improves glycaemic control (108, 269,

296, 832). Some of these beneficial actions of exercise might be mediated by enhanced responsiveness of hypothalamic neurons to endocrine signals that modulate feeding and energy expenditure. For example, a single swimming exercise bout improves the anorexigenic effects of leptin, as well as hypothalamic leptin signalling in lean (218, 646) and obese rats (648). In agreement, six weeks of voluntary wheel running improves leptin signalling in the hypothalamus of obese mice (393). Notably, the aforementioned studies assessed leptin sensitivity directly after an exercise bout, and as such, the long-term consequence of exercise training on leptin responsiveness, independent of acute exercise-induced signalling, is unknown.

Exercise increases the formation of new neurons in various brain regions, most notably the hippocampus (578, 602, 818), which integrate into existing neural networks and impact CNS functions (127). Because hypothalamic neurogenesis modulates feeding behavior and energy balance (274, 387), we reasoned that exercise might induce changes to the hypothalamic parenchyma to influence leptin or ghrelin sensitivity. The effect of exercise training on ghrelin sensitivity in the hypothalamus is yet to be determined, but given ghrelin's central role in energy metabolism this warrants further investigation.

Therefore, the purpose of the current study was to assess whether endurance exercise training enhances hypothalamic sensitivity to leptin and ghrelin, independent of acute exercise-mediated effects. It was hypothesised that exercise would increase leptin and ghrelin sensitivity in chow fed mice, and restore sensitivity in obese mice.

6.2. Methods

6.2.1. Animal Experimental Procedures

The experiments were approved by the Monash University School of Biomedical Sciences Animal Ethics Committee. Male C57Bl6/J mice were obtained from Monash Animal Services at 6 weeks of age and maintained on a 12 h light, 12 h dark cycle with lights on at 0700 h at the Physiology Department Large Animal Facility, Monash University. Mice were housed 5 to a cage and allowed to acclimatise for 1 week with standard rodent chow (Table 6.1) and water *ad libitum*.

6.2.2. Study Design

The experimental procedures described herein were performed on two occasions in separate cohorts of mice. Numbers refer to the combined data set. Mice were randomised at 8 weeks of age to four groups with the following diet and exercise interventions: chow sedentary, chow exercise, HFD sedentary, and HFD exercise (n=20 per group). After 6 weeks on their respective diets, mice remained sedentary or completed a progressive exercise-training program. Mice were maintained on their respective diets during this 6-week period (Figure 6.1A). For the exercise training groups, mice ran on a motorised treadmill once daily, 5 times a week for 6 weeks, at a 5° incline, and at a final speed of 18m/min for 70 minutes (Table 6.2). A run to exhaustion test was performed on all mice before and after the exercising training period to assess improvements in running capacity. Body weight was monitored throughout the training period.

Table 6.1 Details of diets

	Chow	High-fat diet
Manufacturer	Barastoc Irradiated mice cubes	Specialty Feeds SF03-002
Total Crude Fibre	3.2%	4.7%
Total Protein	20%	19.5%
Total fat	6%	36%
Total energy from fats	15%	59%
Digestible energy (kJ/g)	13.2	22.8

Table 6.2 Six week exercise training protocol

Week	Day	Exercise protocol	Week	Day	Exercise protocol
1	Mon	Endurance test	4	Mon	15m/min (55min)
	Tue	12m/min (30min)		Tue	15m/min (55min)
	Wed	13m/min (30min)		Wed	15m/min (60min)
	Thu	13m/min (30min)		Thu	16m/min (60min)
	Fri	13m/min (30min)		Fri	16m/min (60min)
	Sat			Sat	
	Sun			Sun	
2	Mon	14m/min (30min)	5	Mon	16m/min (60min)
	Tue	14m/min (30min)		Tue	16m/min (65min)
	Wed	14m/min (40min)		Wed	16m/min (65min)
	Thu	14m/min (40min)		Thu	16m/min (70min)
	Fri	15m/min (40min)		Fri	17m/min (70min)
	Sat			Sat	
	Sun			Sun	
3	Mon	15m/min (40min)	6	Mon	17m/min (70min)
	Tue	15m/min (45min)		Tue	17m/min (70min)
	Wed	15m/min (45min)		Wed	18m/min (70min)
	Thu	15m/min (50min)		Thu	18m/min (70min)
	Fri	15m/min (50min)		Fri	Endurance test
	Sat			Sat	
	Sun			Sun	

6.2.3. Run to Exhaustion

Running endurance was assessed on a treadmill (Columbus Instruments, Columbus, OH) before and after the exercise training/dietary intervention. Mice commenced running at 10 m/min for 2 min (5% incline) and the speed was increased by 2 m/min every 2 min until exhaustion. Exhaustion was defined as mice spending 10 sec at the base of the treadmill without attempting the reengage running with manual prompting. The change in the time taken to reach exhaustion between baseline and the end of the study was taken as a measure of the change in work capacity.

6.2.4. Glucose Tolerance Test

Glucose tolerance testing was conducted on the Monday of the 6th week of training, allowing 2 exercise free days before the test, and was performed according the methods outlined in Chapter 3, section 3.2.4

6.2.5. Plasma Analysis

6.2.5.1. Free fatty acids

See Chapter 3, section 3.2.5.1 for details.

6.2.5.2. Triacylglycerol

See Chapter 3, section 3.2.5.2.for details.

6.2.5.3. Leptin

See Chapter 4, section 4.2.7.3 for details.

6.2.5.4. Insulin

See Chapter 4, section 4.2.7.4 for details.

6.2.6. Food intake in response to peripheral hormones

Mice were housed individually two days before the last exercise bout, and hormone sensitivity was assessed at least 24 h later to avoid the acute effects of the exercise, while minimising the initial stress of individual housing. One hour before the start of the dark phase, mice were given an IP injection of leptin (2 mg/kg body weight, Preprotech) or saline, and food intake was monitored 4-5 and 24 h later. The reciprocal condition was completed 24 h later (i.e. 48 h after the first injection). In a separate cohort, mice received an ip injection of ghrelin (1 mg/kg body weight, NeoMPS) or saline in a randomised order at 0900 during the light cycle and food intake was monitored 4-5 and 24 h later (Figure 6.1B). Experiments were randomized and counter-balanced.

6.2.7. Signalling pathways

Hypothalamic leptin and ghrelin sensitivity was assessed by measuring activation of downstream signalling molecules in response to acute hormone administration.

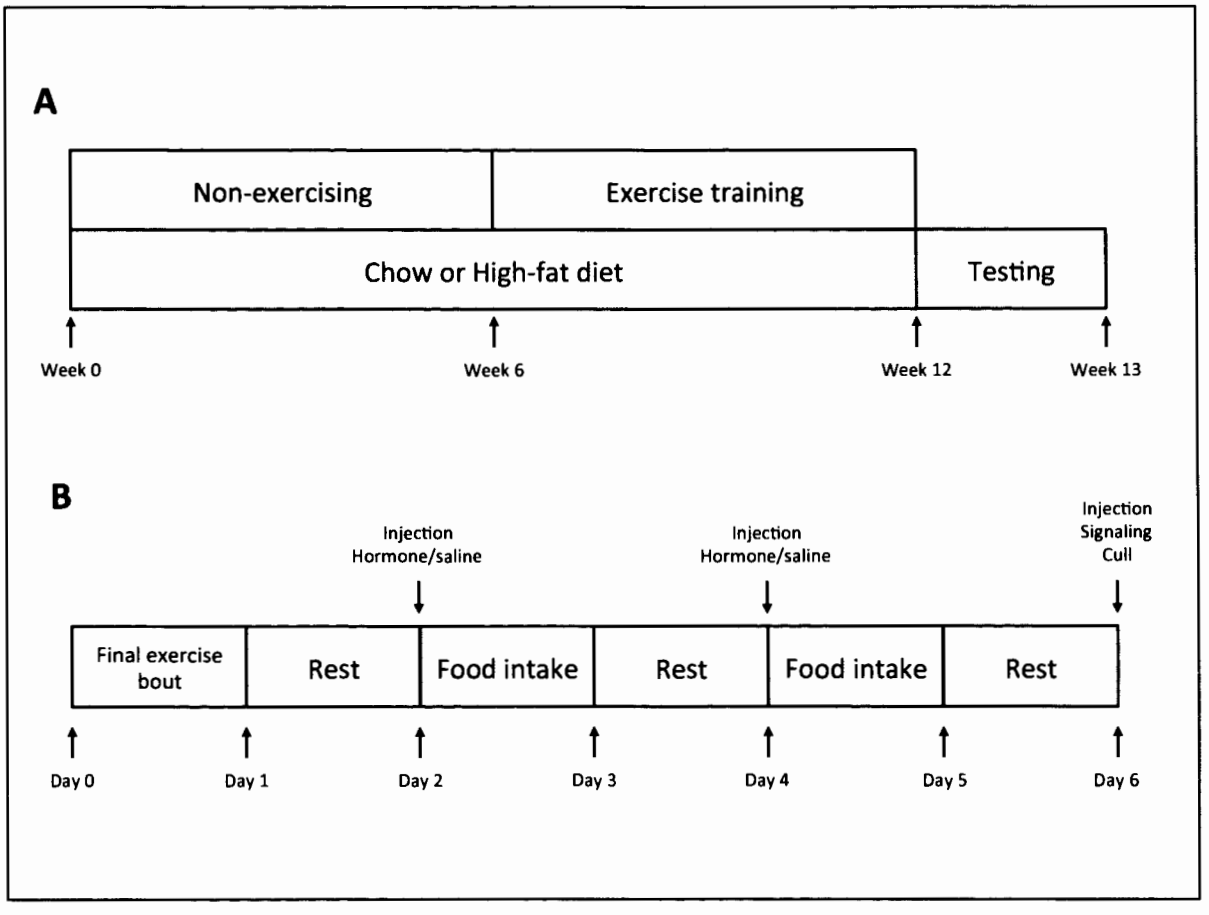


Figure 6.1 Experimental design

(A) Mice were placed on their respective diets (chow or high fat) for 6 weeks before being separated into sedentary or exercise training for another 6 weeks whilst remaining on the diet. This was followed by a week of assessing hormone sensitivity. (B) The testing period consisted of began at least 24 hours after the final exercise bout. Mice were injected IP with either leptin or saline at 1800 or in a separate cohort of mice, ghrelin or saline at 0900, food intake was monitored for the subsequent 24h. Following a 24h wash out period, mice were injected again with the other hormone or saline and food intake monitored again. Therefore all mice received a hormone and a saline injection and serve as their own control. After a 24h wash out period, mice were injected with either leptin or ghrelin, and hypothalamic signalling was assessed.

6.2.7.1. Assessment of central leptin sensitivity

Once food intake measurements were completed, the same mice received an IP injection of leptin (2mg/kg body weight) and were killed 30 min later. For ghrelin experiments, mice received and IP injection of ghrelin (1mg/kg body weight) and were killed 60 min later. Mice were anesthetised with isoflurane and perfused with 0.9% saline, followed by 4% paraformaldehyde. The brain was postfixed in 4% paraformaldehyde overnight at 4°C, and then placed in 30% sucrose. Brains were cut at 30 μm on a cryostat and every fourth section through the hypothalamus was collected and stored in cryoprotectant at -20°C.

Immunohistochemisty was performed to assess the phosphorylation of STAT3 at Tyr^{705}, a well-documented leptin target. Sections were washed in 0.1M PBS and incubated with 0.5% sodium borohydride for 15 min. This was followed by 1% NaOH and 1% H_2O_2 washes for 20 min, 0.3% glycine for 10 min and 0.03% SDS (all w:v) for 10 min, all with PBS washing in between. Sections were blocked with 4% normal horse serum and 0.02% Triton X-100 incubated with biotin-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:200 for 1 h at room temperature. Sections were washed and incubated with Avidin-Biotin Complex (1:200) for 1 h. To visualise immunoreactivity, sections were incubated with a solution containing 1% nickel ammonium sulphate, 1% diaminobenzidine, and 30 μL of 0.1% H_2O_2. Sections were then washed in PBS, mounted and cover slipped.

6.2.7.2. Assessment of central ghrelin sensitivity

Once food intake measurements were completed, the same mice received an IP injection of ghrelin (1mg/kg body weight) and were killed 60 min later. Mice were anesthetised with isoflurane and perfused with 0.9% saline, followed by 4% paraformaldehyde. The brain was postfixed in 4% paraformaldehyde overnight at 4°C, and then placed in 30% sucrose. Brains were cut at 30 μm on a cryostat and every fourth section through the hypothalamus was collected and stored in cryoprotectant at -20°C.

Immunohistochemisty was performed to assess for c-Fos content. Sections were washed in PBS and blocked for 1hr with 5% normal horse serum in 0.03% triton X100 and PBS followed by incubation with c-Fos rabbit polyclonal IgG at 1:1000 in blocking solution, overnight at 4°C. Sections were washed and incubated with Alexa-flura conjugated 488 goat anti-rabbit IgG at 1:1000 for 1 hr at room temperature, then washed and cover slipped.

pSTAT3 and Fos-positive neurons in the arcuate nucleus (bregma -1.22 mm to -2.06 mm) and ventromedial nucleus (VMN) (bregma -1.06 mm - -2.06 mm) were identified using a mouse brain atlas (594). Cells were visualized by a Zeiss microscope (Zeiss, Oberkochen, Germany) and counted using a grid eyepiece. We assessed six sections per mouse and five mice for each treatment group.

6.2.7.3. Immunoblotting analysis

In a cohort of mice exercise trained for 6 weeks, the mice were killed by decapitation and the brains were quickly removed. Immunoblotting was performed as described in Chapter 3, section 3.2.6 using the antibodies outlined in Table 6.3.

Table 6.3 Primary and secondary antibodies used in Chapter 6 analysis

Antibody	Supplier	Catalogue #	Concentration
c-Fos	Santa Cruz Biotechnology	sc-52	1:1000
pSTAT3 (Tyr^{705})	Cell signalling	9131	1:1000
Goat anti-rabbit (alexa fluor 488)	Abcam	ab150077	1:1000
Goat anti rabbit (biotin conjugated)	Jacksons ImmunoResearch Laboratories	111-065-045	1:200
GHSR	Santa Cruz Biotechnology	sc-20748	1:1000
α -actin	Sigma-Aldrich	A2066	1:5000

6.2.8. Statistics

Results are expressed as the means ± SEM. Statistical analysis was performed by employing a two-way ANOVA with Bonferroni *post hoc* test. Body weight (% change), time to exhaustion, blood glucose, food intake and ghrelin/leptin sensitivity data were analysed using a repeated measures two-way ANOVA with Bonferroni *post hoc* test using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Significance was established at $P<0.05$.

6.3. Results

6.3.1. Metabolic consequences of exercise training in lean and obese mice

High fat feeding increased the body weight of mice compared with chow fed mice (P<0.0001 main effect), whilst six weeks of exercise training reduced body weight (P=0.0008 main effect) (Figure 6.2A). When expressed as a percentage of starting body mass, both chow and HFD sedentary mice continued to gain weight over the final 6 week period, whereas the chow and HFD exercise mice maintained their body weight (*P*<0.0001 main effect) (Figure 6.2B). Epididymal fat pad mass was increased with high fat feeding (*P*=0.0004 main effect) and reduced with exercise training (*P*=0.014 main effect) (Figure 6.2C). Despite this reduced body weight, exercise trained mice consumed more kilojoules than the sedentary mice (*P*=0.0005 main effect), and HFD mice consumed more kilojoules than chow fed mice (*P*=<0.0001 main effect) (Figure 6.2D). Exercise training increased running capacity in both chow (38% above baseline) and HFD mice (48% above baseline) compared with sedentary mice (*P*<0.0001 main effect) (Figure 6.2E).

Fasting blood glucose was increased with high fat feeding (*P*<0.0001 main effect) and reduced with exercise training (*P*=0.0018 main effect) (Figure 6.3A). Plasma insulin was also increased with high-fat feeding (*P*=0.025 main effect) but there was no effect of exercise training (Figure 6.3B). Glucose tolerance was vastly improved after exercise training in both chow and HFD mice (Figure 6.3C and D). Plasma FFAs were increased with high-fat feeding (*P*=0.0084 main effect) but there was no effect of exercise training (Figure 6.4A). Plasma TAG was unaffected by diet and exercise (Figure 6.4B). Plasma leptin was increased with high-fat feeding (*P*=0.037 main effect) and there was no effect of exercise training (Figure 6.4C).

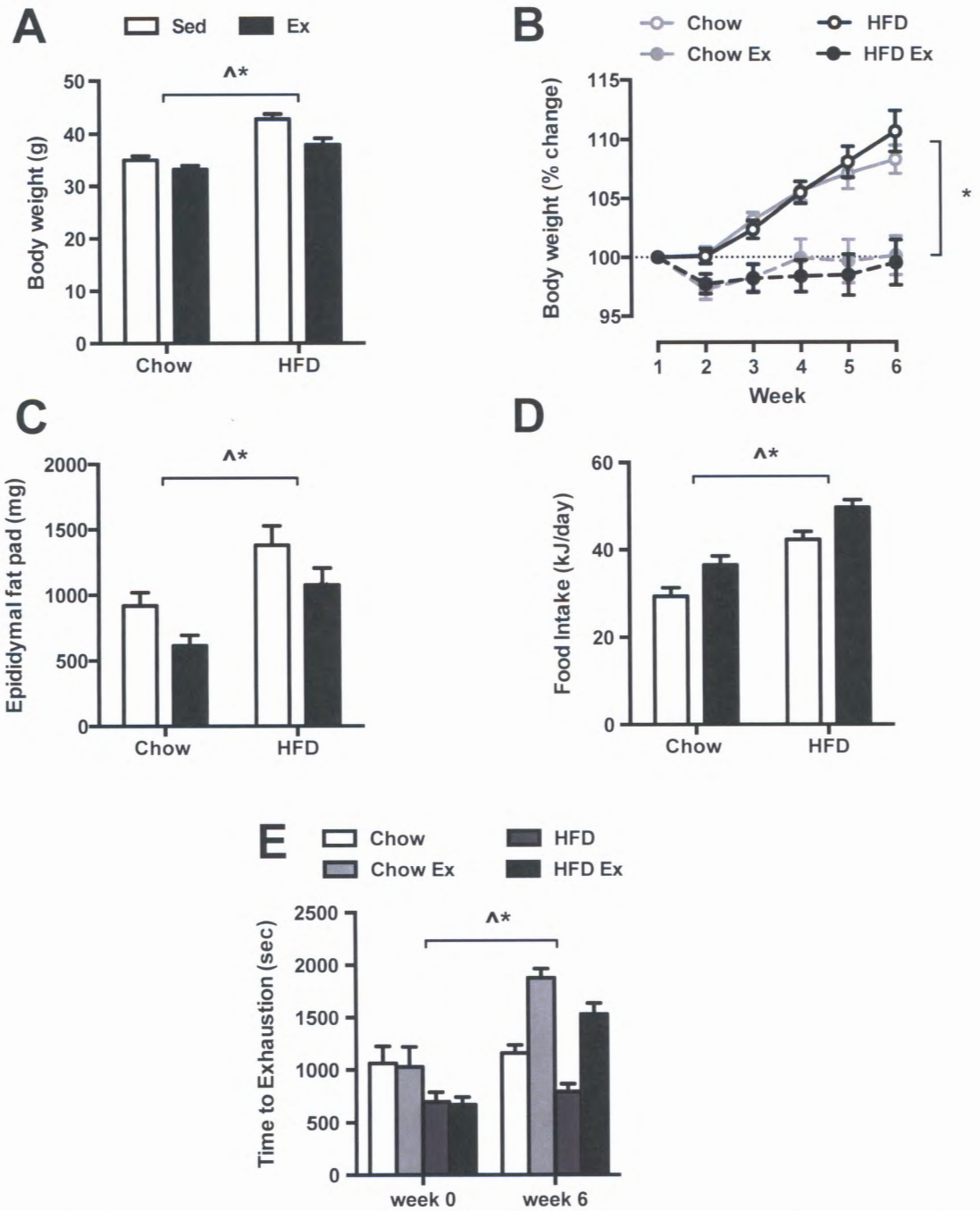


Figure 6.2 Exercise training decreases body weight and adiposity while increasing food intake.

(A) Final body weight of mice after exercise training. *n*=20 per group. (B) Changes in body weight presented as a percentage of starting body weight over the final 6 week period. *n*=20 per group. (C) Epididymal fat pads was excised and weighed at the time of killing. *n*=10 per group (D) Food intake was measure every night and averaged over a 4-day period. *n*=10 per group. (E) An endurance test was performed before and after exercise training. Week 0 (*n*=5) Week 6 (*n*=10). **P*<0.05 main effect for exercise. ^*P*<0.05 main effect for diet.

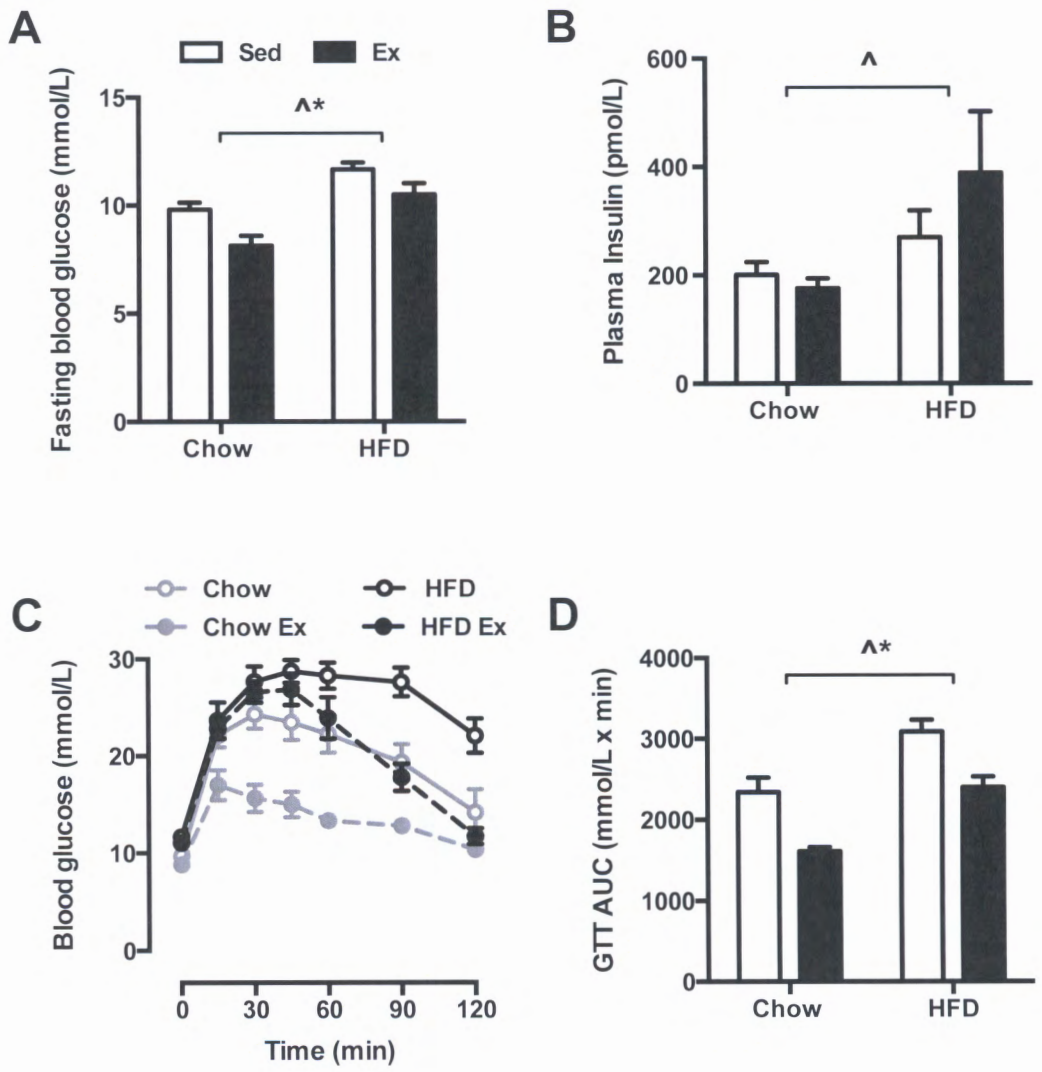


Figure 6.3 Exercise training improved glucose tolerance and fasting blood glucose, despite unchanged insulin levels

(A) Blood glucose was determined after a 4 hour fast. (B) Plasma insulin was determined at the time of killing. (C) A glucose tolerance test was performed at the end of the training period. n=10-13 per group (D) The area under the curve was calculated from the glucose tolerance test data. n=10-13 per group. *P<0.05 main effect for exercise. ^P<0.05 main effect for diet.

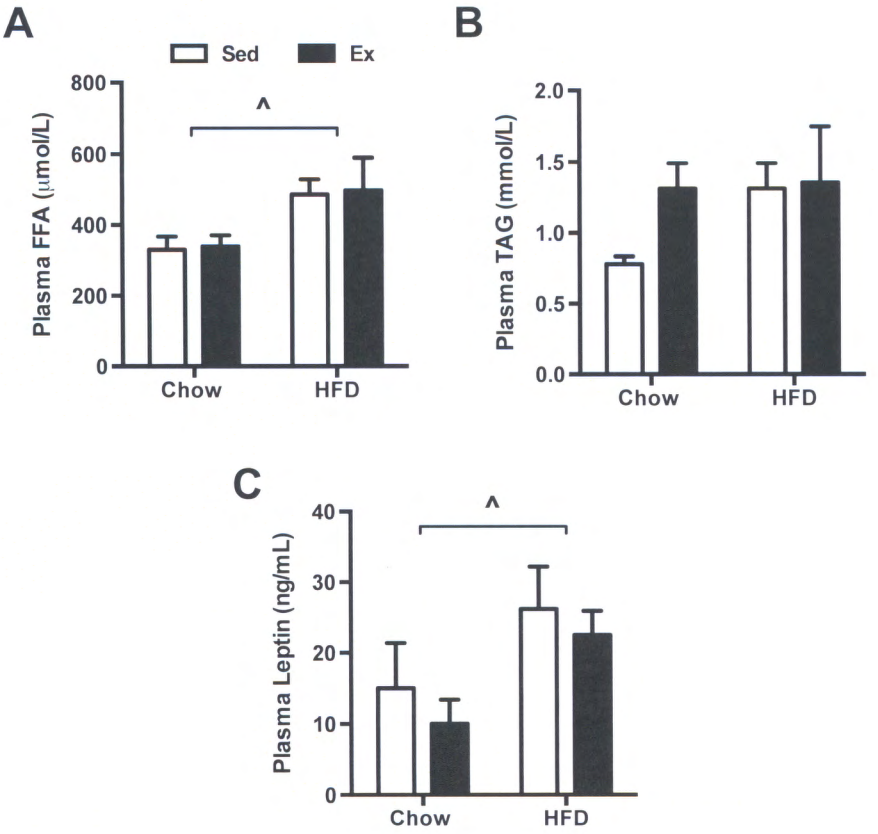


Figure 6.4 Plasma lipids and leptin after six weeks of exercise training in chow and HFD mice.

Blood was collected at the time of perfusion in EDTA tubes and the plasma separated before analysis. (A) Plasma free fatty acids (FFA), (B) plasma triacylglycerols (TAG), and (C) plasma leptin, n=5 per group, *P<0.05 main effect for exercise. ^P<0.05 main effect for diet.

6.3.2. Exercise training and the anorexigenic effects of leptin

After six weeks of exercise training, mice were injected with leptin at the onset of the dark period (1800 h). The assessment of the anorexigenic effects of leptin is primarily performed in overnight fasted animals, and is a major physiological challenge. Because mice eat the majority of their food during the dark phase, leptin was injected immediately before the dark phase in order to examine the effect on normal physiological feed patterns. Leptin administration reduced food intake (P=0.043 main effect) but there was no effect of diet or exercise either 5 or 24 hours after administration (Figure 6.5A). (P=0.022 main effect) (Figure 6.5B). When energy intake was expressed as the difference between leptin and saline for each mouse (i.e. each mouse acting as its own control), there was no effect for diet or exercise (Figure 6.5C). Overall, the anorexigenic effects of leptin were not influenced by high-fat feeding or sustained exercise training.

6.3.3. Exercise training and central leptin signalling

After we established the anorexigenic effect of leptin, we next investigated whether diet or exercise training impacted leptin-signalling pathways within the ARC and VMH. Phosphorylation of STAT3 at Tyr^{705}, was assessed to demonstrate the activation of leptin signalling. In accordance with the food intake data (Figure 6.5), there were no differences observed between any of the treatment groups for $pSTAT3^{Tyr705}$ activation within the ARC (Figure 6.6B) and the VMH (Figure 6.6C). Notably, leptin-induced STAT3 Tyr^{705} phosphorylation tended to be reduced in high-fat fed vs. low-fat fed mice when assessed by unpaired t-tests (P=0.10). When coupled with the increased plasma leptin levels observed in the high-fat fed mice (Figure 6.4C), this indicates that the high-fat diet induced a mild leptin resistance that was insufficient to impact food intake under physiological conditions.

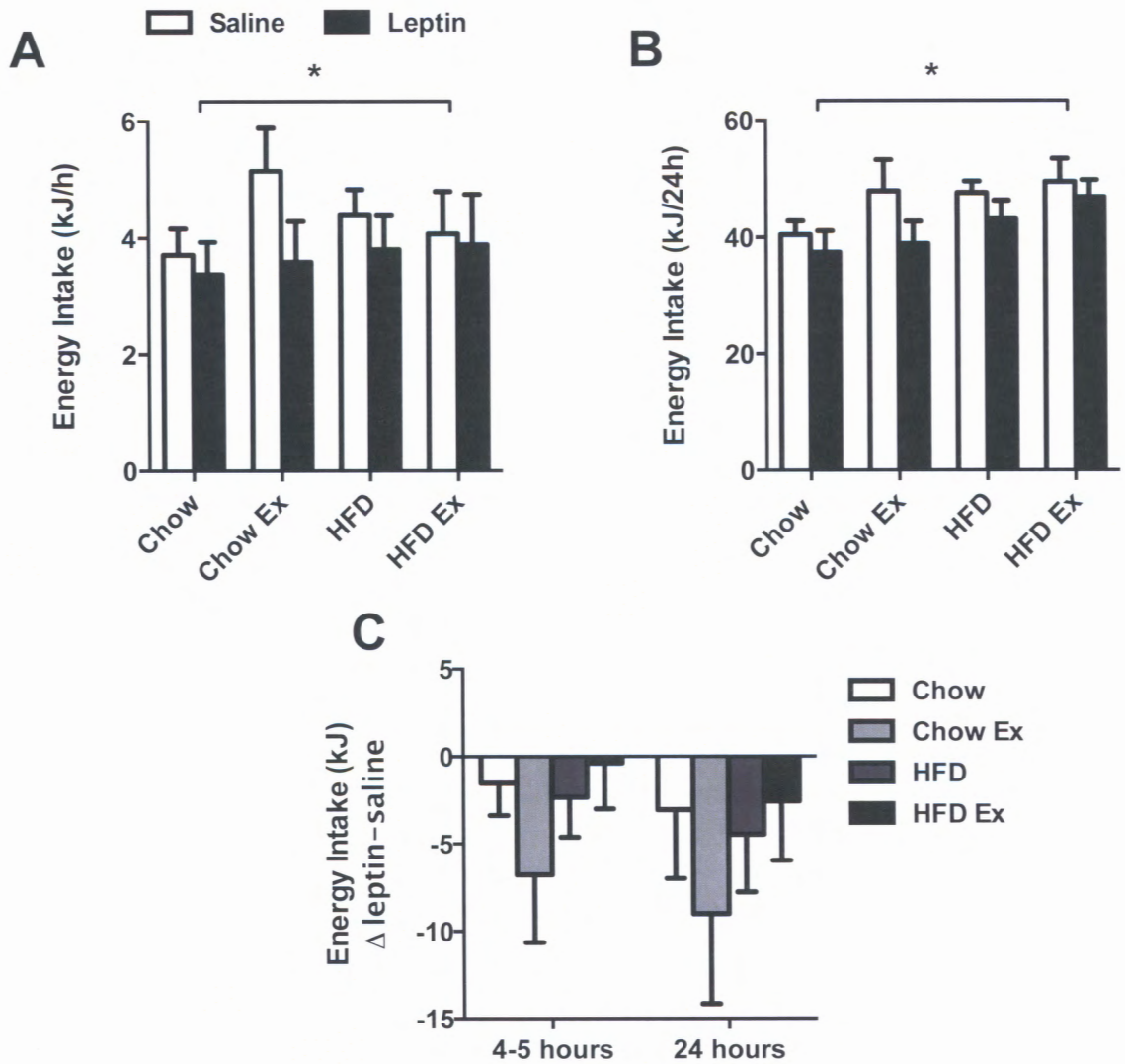


Figure 6.5 Food intake responses to saline or leptin in sedentary and exercise-trained mice.

(A) Food intake measured 4-5 hours after saline and leptin injection during the dark phase, expressed as kilojoules consumed per hour. (B) Food intake measured 24 hours after saline and leptin injection. (C) Food intake expressed as the difference consumed during leptin and saline injections. n=10 per group, *P<0.05 main effect for injection.

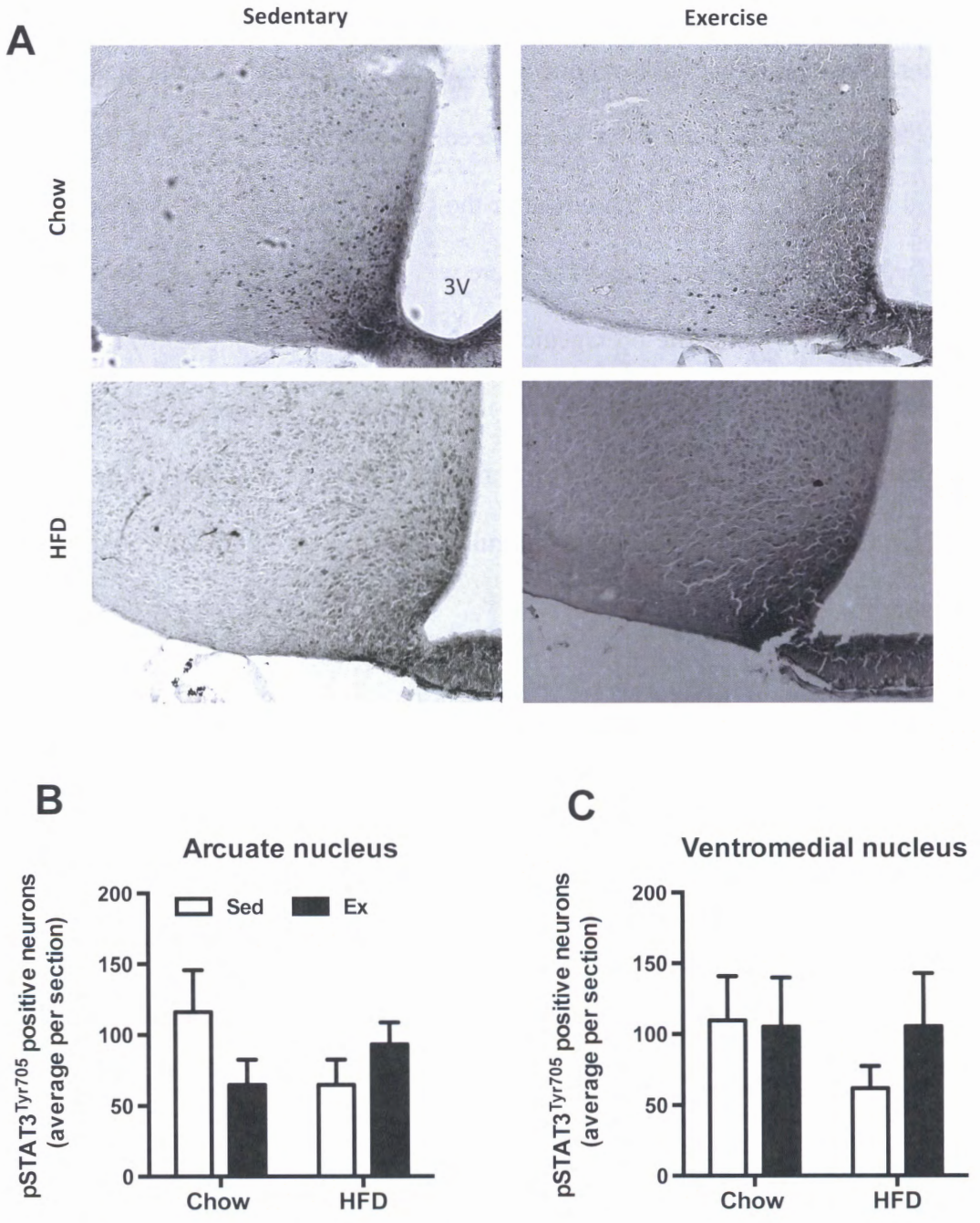


Figure 6.6 Leptin activates neurons in the hypothalamus, irrespective of diet and training status.

(A) Representative images of pSTAT3^{Tyr705} positive neurons in the arcuate nucleus and ventromedial hypothalamus. 3V = third ventricle (B) pSTAT3^{Tyr705} positive neurons were counted and quantified in the acuate nucleus and (C) the ventromedial hypothalamus. *n*=5 per group

6.3.4. Exercise training and the orexigenic effects of ghrelin

After six weeks of exercise training, mice were injected with ghrelin at the start of the light cycle, when mice are sated from normal feeding during the dark period, to induce food intake. Food intake was increased 5 hours after the ghrelin injection compared with the saline injection (P=0.0001 main effect); however, there was no specific effect of diet or exercise on this response (Figure 6.7A). The orexigenic effect of ghrelin was not evident 24 h after the injection (P=0.159 main effect) (Figure 6.7B). When energy intake was expressed as the difference between ghrelin and saline for each mouse, there was no effect for diet or exercise (Figure 6.7C). Overall, high fat feeding nor regular exercise training influenced the orexigenic effects of ghrelin.

6.3.5. Exercise training and central ghrelin signalling

After we established the orexigenic effect of ghrelin, we next assessed whether the function was accompanied by activation of neurons within the ARC. The presence of c-Fos, the protein product of the immediate-early gene c-Fos, is a commonly used marker to identify and localize activated neurons. HFD mice had a 49% reduction in the number of c-Fos positive neurons compared with chow fed mice (P=0.0002 main effect) (Figure 6.8A and B). There was no effect of exercise training in either the chow or HFD mice. The ghrelin receptor mediates the orexigenic effect of acyl ghrelin on food intake and activation of ARC neurons (898), therefore we assessed whether diet or exercise affected GHSR protein expression. There was a trend for GHSR to be reduced with high-fat feeding (P=0.078, main effect) and to be increased with exercise training (P=0.077, main effect) (Figure 6.9A and B).

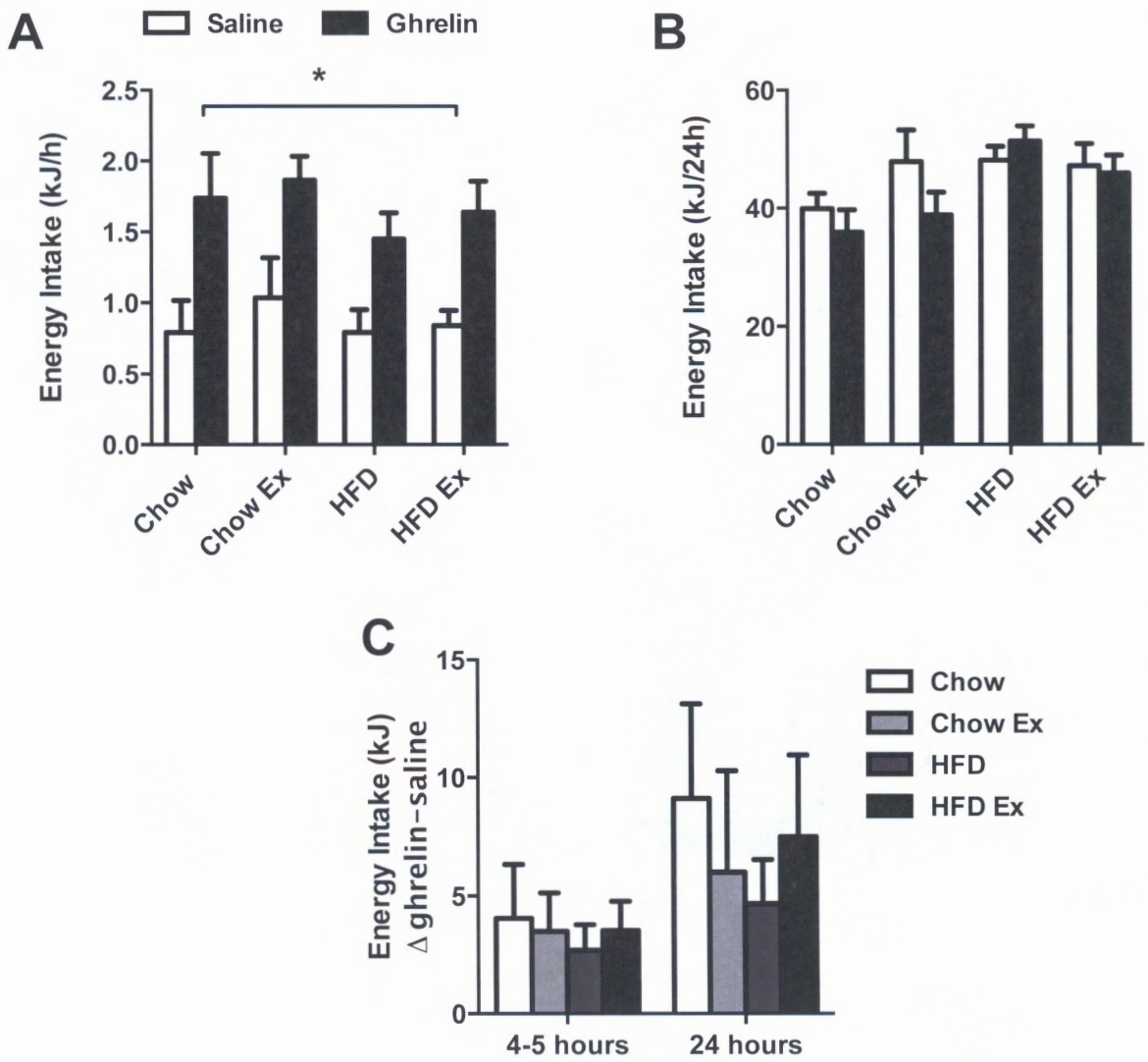


Figure 6.7 Ghrelin-induced food intake after six weeks of exercise training in chow and HFD mice

(A) Food intake measured 4-5 hours after saline and ghrelin injection during the light phase, expressed as kilojoules consumed per hour. (B) Food intake measured 24 hours after saline and ghrelin injection. (C) Food intake expressed as the difference consumed during ghrelin and saline injections. n=10 per group, *P<0.05 main effect for injection.

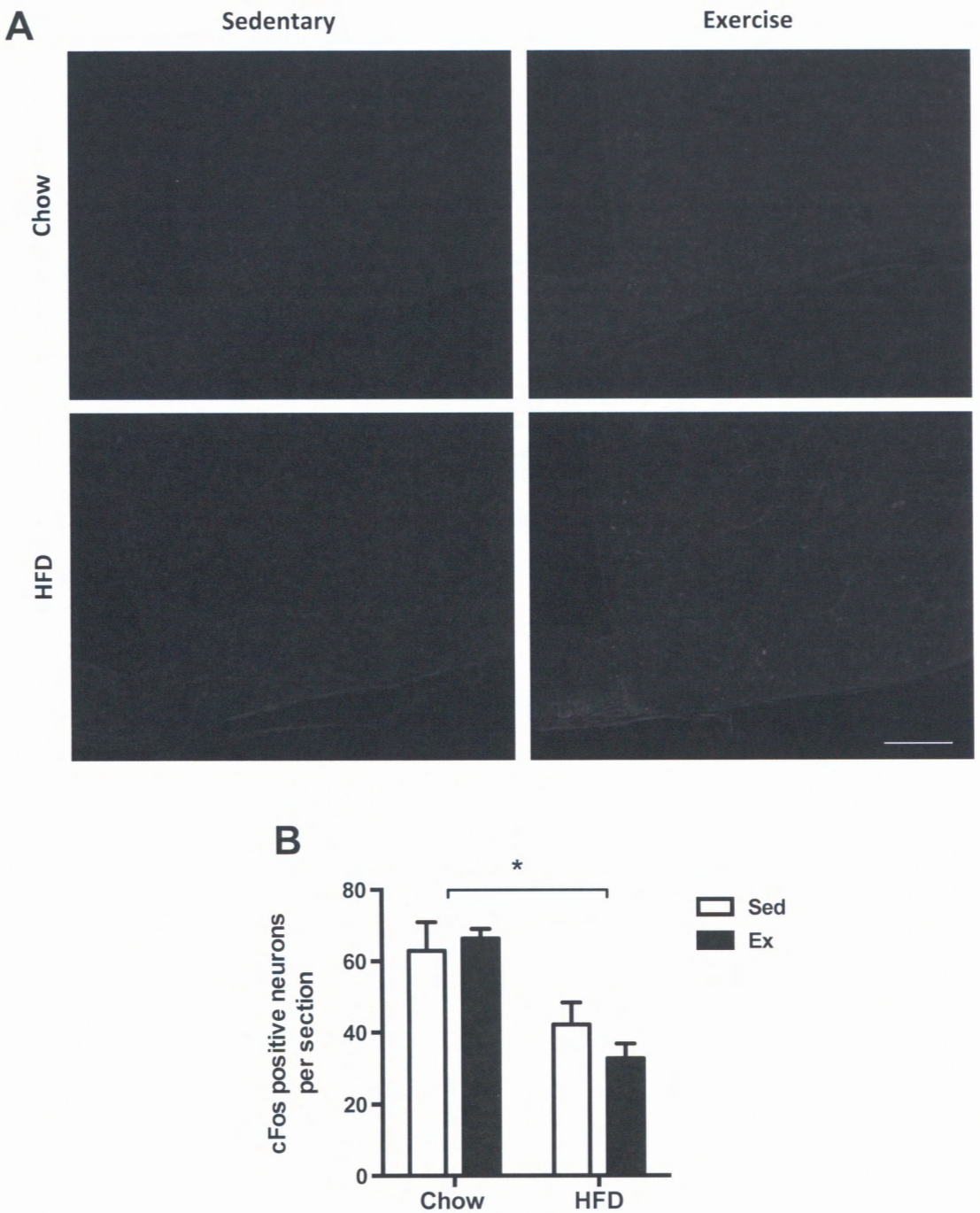


Figure 6.8 Ghrelin activates neurons within the arcuate nucleus of chow but not HFD mice, irrespective of exercise training status

(A) Representative images of c-Fos positive neurons in the arcuate nucleus. (B) c-Fos positive neurons were counted and quantified. n=5 per group, *P<0.05 main effect for diet.

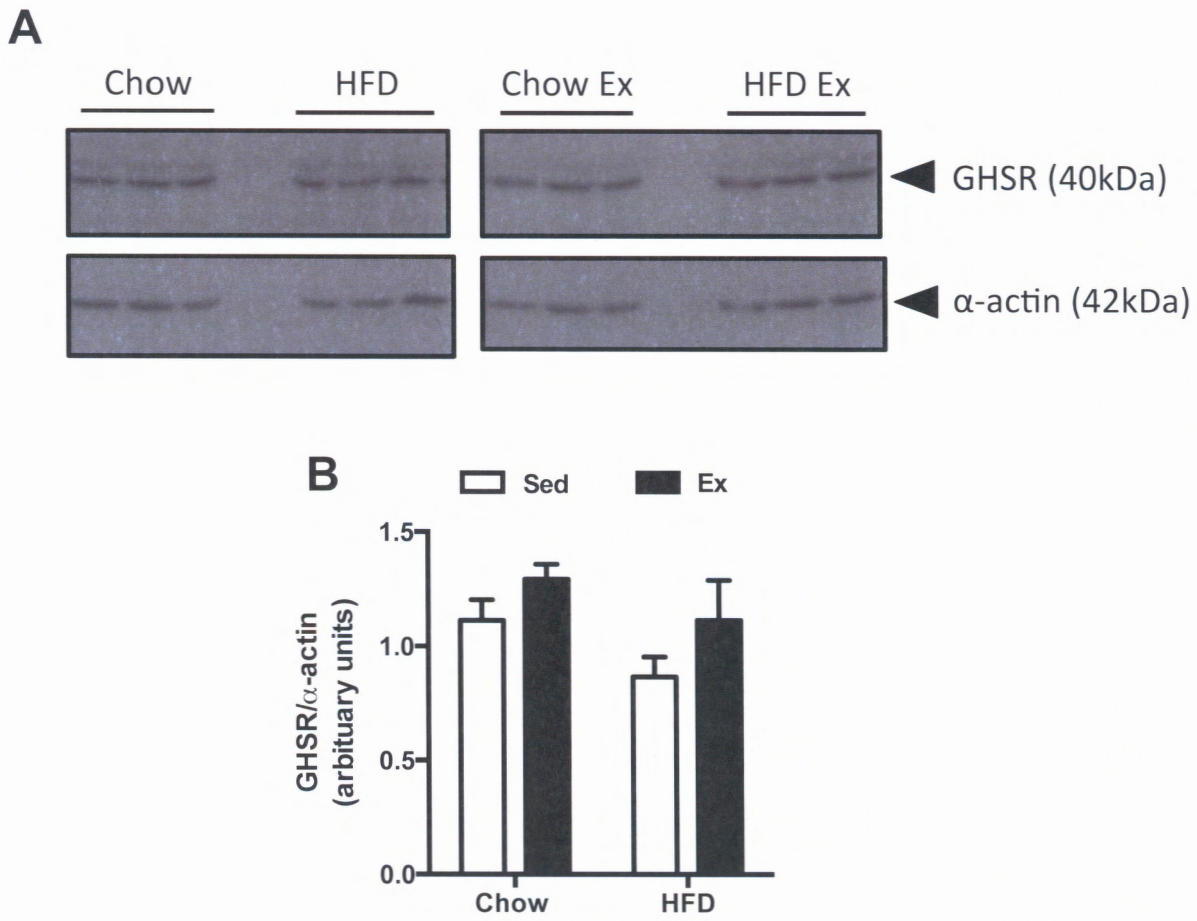


Figure 6.9 Ghrelin receptor (GHSR) in the hypothalamus after six weeks of exercise training in chow and HFD mice

(A) Representative immunoblots for GHSR and the loading control α-actin. (B) Quantification of GHSR expression. *n*=5 per group.

6.4. Discussion

The ARC of the hypothalamus senses and integrates peripheral and central signals of energy stores to regulate feeding and energy balance. Impaired hypothalamic detection and/or responsiveness to peripheral signals, such leptin and ghrelin, contributes to hyperphagia and altered energy homeostasis in obesity. Hence, improving the hypothalamic sensing of peripherally derived hormones in obesity may help treat obesity and related disorders. While exercise induces weight loss and acute exercise enhances leptin sensitivity (219, 393, 646, 648), the long-term effects of exercise training on hypothalamic responsiveness to peripherally-derived hormonal signals are unknown. In the present study, we assessed the effects of regular endurance exercise training on leptin and ghrelin sensitivity. We designed the study to assess stable changes to hypothalamic function, not immediate post-exercise effects. Neither diet nor exercise training affected hypothalamic signalling or food intake in response to peripheral leptin and ghrelin administration. Hence, any sustained changes to hypothalamic parenchyma or cell signalling induced by regular physical exercise is insufficient to improve sensitivity to leptin and ghrelin, and exercise-induced improvements in hypothalamic signalling are most likely the direct result of acute signalling events.

Leptin sensitivity in the hypothalamus is improved immediately after an acute exercise bout in lean and obese rodents. A single swimming exercise bout representing an ultra-endurance event (6 h exercise) improved leptin-induced food intake and STAT3 phosphorylation in the hypothalamus of lean (219) and high fat fed rats (648). Also, voluntary wheel running for six weeks in both lean and obese mice chronically activated neurons in the VMH, but not the ARC, and improved leptin's anorexigenic effect on food intake (393). In the current study, we asked whether moderate-intensity exercise training, similar to that

performed for weight loss, would enhance leptin action independent of the acute exercise effects. We reasoned that this was physiologically relevant given that most meals are consumed in the hours to days after an exercise bout. Exercise training did not increase the anorectic effects of leptin or leptin signalling in the ARC and VMN of the hypothalamus. This contrasts a previous study that reported increased leptin-receptor binding, STAT3 phosphorylation and anorexigenic effects of leptin in rats 10 weeks after the cessation of an exercise program (591). However, in this previous study, exercise training was initiated in the immediate post-weaning period, at a time where hypothalamic energy-balance circuitry is plastic (84, 502), which may contribute to enhanced modification of leptin-responsive pathways that persists into adulthood. In the present study, exercise training commenced at 14 weeks of age, when energy-balance circuitry is well established and neuronal turnover of ARC neurons declines (502). Ageing results in a precipitous decline in neurogenesis (822) and others have shown that voluntary wheel running did not enhance leptin sensitivity in the hypothalamus of aged, obese rats (704). Taken together, these results suggest that exercise training in adulthood does not improve hypothalamic leptin sensitivity and that the leptin sensitizing effects of exercise are only present in the immediate post-exercise period.

The development of leptin resistance in the hypothalamus during obesity appears to be confined to the ARC, with other regions such as the VMH and the DMH remaining leptin responsive (549). The VMH has long been implicated in the control of energy balance and glucose homeostasis (519) and leptin action in the VMH decreases body weight and improves glucose metabolism (172, 523). Neither high fat feeding nor exercise training effected leptin-induced STAT3 phosphorylation in the VMH, suggesting that leptin signalling in the VMH may not be essential for the weight loss or improved glucose tolerance associated with exercise training.

Ghrelin is a stomach-derived hormone that initiates food intake by activating NPY/AgRP neurons in the ARC (27). It plays a critical role in regulating appetite, body mass, fuel substrate preference, and glucose homeostasis (109). Ghrelin resistance develops in NPY/AgRP neurons of the ARC during obesity (87) and we hypothesized that exercise training would reverse central ghrelin resistance concomitant with an improved metabolic profile in obese mice. Ghrelin-induced c-Fos activation in the ARC was reduced in obese vs. lean mice but there was no impairment in ghrelin-induced food intake. The lack of ghrelin resistance during obesity in this study may be related to ghrelin's route of administration. Ghrelin resistance is observed when administered ICV during the light and dark phase or ip during the dark phase. However, when administered ip during the light phase, ghrelin's orexigenic actions are maintained (87), consistent with our results herein (Figure 6). Because central ghrelin does not elicit food intake during the light phase, the ip effect of ghrelin may be related to peripheral actions of ghrelin on the vagal nerve at this time (164, 426).

Why the signaling changes did not manifest changes in food intake is puzzling, and may be related to inherent redundancy, in which above a certain signaling threshold behavioral changes remain unaffected. Further work is required to test this hypothesis, however similar phenomena have been observed in other neuroendocrine hypothalamic systems, such as the reproductive system (300). This study shows that between 12% and 34% of total GnRH neurons are required for normal puberty, ovulation and fertility.

Exercise training did not improve ghrelin induced food intake or hypothalamic neuronal activation, in either the lean or obese state. These finds were surprising because obesity results in the down regulation of several components of the ghrelin system, including stomach GOAT expression, ghrelin secretion and hypothalamic GHSR mRNA expression (87). While

we observed reduced hypothalamic GHSR content in obesity that was increased with exercise training, this was insufficient to produce changes in feeding.

There are a number of caveats in the interpretation of the current data. Leptin resistance is a common feature of high-fat feeding in rodents and is attributed to decreased leptin transport across the BBB (50, 781) and decreased activation of leptin receptor signalling (774). In the present study, the direct comparison of low-fat and high-fat fed sedentary mice indicated the development of very mild leptin resistance in obesity. Plasma leptin was increased with high-fat feeding (Figure 6.4C), indicating that the obese mice were insensitive to endogenous leptin (225), and there was a strong trend (P=0.10) for leptin resistance at the level of hypothalamic signalling with obesity (Figure 6.6B). However, when assessed by two-way ANOVA, there was no significant effect of high-fat feeding on leptin's actions when assessed via physiological (food intake) or molecular (ARC STAT3 signalling) outputs. While clear leptin resistance has been reported in murine models using a similar high-fat feeding protocol (187, 461), we and others have observed mild leptin resistance in male mice after 12 weeks high-fat feeding (257, 748). It is apparent that resistance to peripheral leptin administration develops in mice after a given length of treatment and/or a critical body mass or leptin level is reached (811). In addition, specific types of dietary sugars and fats are capable of inducing leptin resistance in experimental rodent models, even in the absence of obesity. Thus, several factors could account for the very mild leptin resistance observed in our study. Leptin resistance is complex and (860) have recently highlighted this issue, stating that food intake is not under stringent homeostatic control, and therefore, variance in food intake responses is context dependent. Nevertheless, the absence of marked leptin resistance does not impact the interpretation that exercise training does not enhance hypothalamic leptin sensitivity.

The design of the study was such that the effects of leptin/ghrelin on feeding were assessed between 1.5 and four days and hypothalamic signalling six days after the last exercise bout. Detraining is the loss of training-induced adaptations in response to a training stimulus and can occur rapidly in some body systems. For example, detraining can reduce blood volume within two days (152) and skeletal muscle mitochondrial protein content by 12 days (150). The effects of detraining on hypothalamic signalling are unknown and we cannot exclude the possibility that training-induced adaptations regressed before our functional assessments of hormone sensitivity and hence masked potential training effects.

Overall, our data establish that prolonged, regular exercise training does not enhance leptin or ghrelin signaling in the hypothalamus or impact the feeding responses to these hormones in lean or obese mice. This suggests that the sensitizing effects of exercise on hypothalamic signaling and food intake responses are limited to the period immediately post-exercise.

Chapter Seven: General Discussion

The primary aims of this thesis were two-fold. The first aim was to investigate the dysfunction of the hypothalamus during obesity; namely lipid accumulation, dysfunctional neurogenesis and the resistance to two major appetite-regulating hormones. The second aim was to determine whether exercise training could alter the structure and/or function of the hypothalamus in association with improved whole-body metabolic function.

The drive to consume food is one of the most primitive instincts promoting survival. It has been shaped by millions of years of evolution, providing powerful mechanisms for adapting and responding to times of nutrient scarcity. In more recent times, the increased availability of high kilojoule foods, coupled with decreasing rates of physical activity, have contributed to obesity becoming one of the greatest public health challenges of the 21st century. Obesity has profound consequences on one's life, spanning from mental health issues such as depression, to serious comorbidities that markedly diminish both quality and length of life. Lifestyle interventions such as diet and exercise are two useful, non-medical methods for controlling or limiting obesity development. However, the incidence of obesity and obesity related complications continue to rise. Indeed, research has shown that obesity development is not as simple as kilojoule intake and energy expenditure, but instead is a complex neurological process involving the integration of the gastrointestinal tract, hormones derived from tissues of the periphery and the CNS (162). New strategies to combat the obesity epidemic are urgently needed, but gaps in our understanding of obesity pathogenesis continue to limit progress toward this goal.

In peripheral tissues, the deleterious metabolic consequences of obesity arise in part via lipotoxicity triggered by nutrient excess. Therefore, we tested the hypothesis that lipotoxicity in CNS, namely the hypothalamus, is a pathophysiological feature of obesity in

mice. It was demonstrated that high-fat feeding in mice results in lipid accumulation in the hypothalamus. Specifically, 12 weeks of high-fat feeding increased the amounts of lipids known to interfere with insulin signalling, such as DAG and ceramide, and the more neutral lipid, TAG. We then sort to 'correct' this lipotoxic environment in the hypothalamus through the use of exercise training. Whilst 6 weeks of treadmill exercise training improved endurance capacity and whole body glucose homeostasis, it was unable to reduce the amount of lipids in the hypothalamus. Together, these studies demonstrated that high fat feeding results in lipotoxicity in the hypothalamus, and that regular exercise training is unable to correct this deficit.

Much of the early work involving lipids and the hypothalamus has focused on lipid 'sensing' and not on lipid storage/accumulation. The information on lipid accumulation in the hypothalamus is scarce, and has only been addressed in a number of studies. Specifically, a high fat diet leads to long-chain fatty acyl-CoA accumulation in the hypothalamus, while palmitoyl-CoA accumulates after short-term ICV palmitate infusion (624). Similarly, intravenous infusion of lard oil, which increases plasma fatty acids to high yet physiological levels, increases ceramide and DAG content in the hypothalamus (307). Therefore, the results of this thesis provide the first in-depth lipidomics investigation of lipid accumulation in the hypothalamus after high-fat feeing.

The field of hypothalamic dysfunction during obesity focuses on inflammation (474, 515, 624), gliosis (321, 779) and peripheral hormone resistance (168, 201, 549, 740) as mechanisms in the aetiology of obesity. The link between lipid accumulation in the periphery, such as skeletal muscle and the liver, and the induction of insulin resistance is clear (837). However, the role of lipid accumulation in the hypothalamus and the induction of insulin

resistance is a link that is yet to be fully elucidated. Therefore, the results presented in this thesis, that a high fat diet leads to lipid accumulation in the hypothalamus, highlights a new therapeutic 'target' or area of research that needs to be explored in the hypothalamus during obesity. Specifically, if the link between hypothalamic lipid accumulation and insulin resistance is made, then targeting these signalling pathways that become altered during obesity may serve as a therapeutic target. As such, the findings from this thesis have completely addressed the first component of this question, that lipid accumulation does indeed occur in the hypothalamus during diet-induced obesity.

Another major finding was that the genetically obese, *ob/ob* mouse does not exhibit any lipid accumulation in the hypothalamus compared with lean mice. This suggests that the cause of hypothalamic lipid accumulation in fat fed mice is directly related to the high fat diet itself, and not the increased adiposity. A similar phenomenon has been reported previously, where *ob/ob* mice do not exhibit lipid accumulation or inflammatory events in skeletal muscle (794). Others have demonstrated that the type of lipids that accumulate in skeletal muscle can be directly related to the fatty acid content of the diet consumed (433). Therefore, high fat feeding itself, but not the resulting obesity causes lipid accumulation in the hypothalamus. It would be interesting to see if switching obese, high fat fed mice to a chow diet, or placing *ob/ob* mice on a HFD, would alter the amount of lipid accumulation in the hypothalamus, seeing as how increasing metabolic capacity through exercise training had no effect.

Neuroplastic changes in the hypothalamus, including altered neurochemical phenotype, neuronal activation, synaptic connections, and dendritic growth can be stimulated by dietary factors and physical activity, not only during periods of development, but also during adulthood (83, 316, 412, 638, 826). This is also true of neurogenesis (115, 387, 430,

455, 502), the process by which neurons are born, proliferate, differentiate and integrate into established circuitry. This phenomenon, previously thought to occur only in developing embryos, has now been demonstrated in the adult brains of most animals, including humans (45, 203), and can be influenced by a range of factors including age, sex, olfaction, stress, environment enrichment, and animal species and strain (45, 79, 128, 349, 521, 726). Recently the hypothalamus has been shown to satisfy the criteria for a neurogenic niche in adult mammals, having similar characteristics as other classical neurogenic zones (513, 885). In particular, new neurons can be generated in the hypothalamus, and expresses phenotypic markers of cell proliferation and neuronal fate, including Ki-67, the cell cycle protein, and the uptake of BrdU. Its newly formed cells migrate to the appropriate areas of the parenchyma (869), express a range of neuropeptides and specific markers related to energy metabolism (388, 483), acquire leptin responsiveness (387, 615) and become functionally mature for neuropeptide secretion (387).

Having established a possible mode of dysfunction of the hypothalamus during obesity (lipid accumulation), we then explored a mechanism for improving hypothalamic function through the generation of new neurons with exercise training. We asked whether neurogenesis is a mechanism for improving hypothalamic function following exercise training. This is based on previous studies using growth factors demonstrating the induction of hypothalamic neurogenesis through CNTF treatment, and the sustained weight loss effect (387). A single exercise bout upregulated the expression of a large subset of genes involved in neurogenesis and stem cell activation. Furthermore, seven days of treadmill exercise training was sufficient to induce the proliferation and survival of neurons in chow fed mice, and most importantly, this process was not hindered during obesity. This is in contrast to a number of studies that have shown impaired neurogenesis in obesity (502, 503). It is important to note

that the results of this thesis show that stimulated neurogenesis through exercise, rather than basal neurogenesis, is normal during obesity. The study design did not formally address the question of basal neurogenesis during obesity as we only looked at the proliferation (assessed immediately post BrdU infusion) and not the survival of these newborn cells (which would be assessed at least 28 days post BrdU infusion). Nonetheless, this thesis clearly shows that exercise training induces neurogenesis in the hypothalamus of both lean and obese mice.

While neurogenesis that is induced by growth factor administration modulates metabolic phenotypes (387), the functional relevance of hypothalamic neurogenesis induced by exercise was previously unresolved. To address this question, we utilised the mitotic blocker AraC during four weeks of exercise training in high-fat fed animals. Previous studies examining the role of neurogenesis on metabolic phenotypes are equivocal. On the one hand, blocking neurogenesis within the ARC leads to obesity and glucose intolerance (456), while on the other, blocking neurogenesis in the median eminence leads to resistance to obesity (430). The results of this thesis show that blocking neurogenesis during four weeks of a high fat diet had no effects on body weight regulation or whole-body glucose uptake, a finding that is in agreement with Pierce, et al. (615).

More importantly, this thesis showed that blocking neurogenesis during exercise training induces mild insulin resistance in adipose tissue. Specifically, plasma FFA were increased after insulin treatment and adipose tissue have reduced insulin stimulated glucose uptake in AraC mice, compared with controls. This suggests that exercise-induced neurogenesis may play a role in mediating insulin's action in adipose tissue. This is an interesting finding when considering the beneficial effects of exercise on adipose tissue. Exercise training in both humans and rats leads to increased adipose tissue lipolysis (96, 754)

while swim training in rats leads to increased insulin stimulated glucose uptake into adipose tissue (199).

Given the mild phenotype observed when investigating the functional role of exercise-induced neurogenesis, we next investigated the functional role of pharmacologically (CNTF) stimulated hypothalamic neurogenesis. Pioneering work from Kokoeva, *et al.* (13) demonstrated pronounced neurogenesis in the hypothalamus in response to CNTF, and that this neurogenesis was responsible for the weight loss effects of CNTF treatment. This study has spurred the field of hypothalamic neurogenesis and bodyweight regulation, and therefore, their methodology was replicated in this thesis. CNTF treated mice exhibit a 17-fold greater amount of neurogenesis compared with exercise-induced neurogenesis. The results of this thesis showed that CNTF treatment does not have long lasting effects on body weight regulation or on insulin sensitivity. In fact, rebound weight gain after CNTF treatment resulted in impaired insulin action. This is in contrast to the findings of Kokoeva, et al. (387), where CNTF treatment resulted in sustained weight loss after cessation of treatment. Whilst a plausible reason for the discrepancies between the two studies cannot be found, it is worth noting that no other study has investigated the sustained effects of ICV CNTF treatment, and as such, further studies by independent laboratories are warranted to find a conclusion to the role of CNTF on sustained weight loss.

Taken together, the results of this thesis have added substantial knowledge to the field of hypothalamic neurogenesis. Exercise training is a potent stimulator of hypothalamic neurogenesis in lean mice, and more importantly, in conditions with reduced neurogenesis such as obesity and ageing. Hypothalamic neurogenesis may play a role in mediating insulin

action on adipose tissue after exercise training; however, the functional role of this neurogenesis is still to be ascertained.

Another key defect of obesity is a desensitisation of the hypothalamus to nutrient and hormonal signals (201, 607). This thesis sort to identify whether regular exercise training could improve hypothalamic sensitivity to ghrelin and leptin, two major appetite regulating hormones. Hypothalamic sensitivity to leptin is enhanced immediately post exercise, in both lean and obese rodents (219, 393, 648). We sort to identify the effect that exercise training had on stable changes to hypothalamic leptin and ghrelin sensitivity. The study design precludes signalling events induced by the last exercise bout by measuring sensitivity at least 24 hours after the final exercise session. Exercise training did not improve leptin or ghrelin sensitivity in either lean or obese mice. The results of this thesis indicate that the improved leptin sensitivity seen after exercise training (219, 393, 648) is limited to the period immediately post-exercise, and are most likely due to direct signalling events induced by the exercise itself, rather than stable molecular/structural modifications to the hypothalamus. It also indicates that ghrelin sensitivity is not enhanced after exercise training, something that has not been previously measured in the literature.

There were potential limitations in the aforementioned study, namely that: a) hormone induced food intake and hypothalamic signalling were assessed over a week long period after the end of exercise training; and b) that the obese mice do not exhibit strong leptin or ghrelin resistance. This study design assessed both food intake responses and signalling pathways in the same mouse, meaning that the final assessment was performed 6 days after the final exercise bout. Therefore, there may be a detraining effect and future studies should assess hypothalamic signalling pathways in response to exercise training at least 24 after the final

exercise bout. Also, while the studies described in this thesis did not induce overt leptin or ghrelin resistance, the high fat fed mice exhibit hyperleptinemia and a tread for reduced leptin-induced STAT3 phosphorylation. While obese mice also display reduced c-Fos induction after ghrelin stimulation, although this was not enough to reduce food intake. These results suggest that mild leptin and ghrelin resistance was induced in these mice. Despite the absence of overt resistance in obese mice, exercise training was unable to enhance hypothalamic sensitivity to leptin or ghrelin in chow, or high fat fed mice.

The potential for exercise training to improve hypothalamic function was another key theme explored in this thesis. Despite the ability for exercise training to consistently improve endurance capacity, whole-body glucose tolerance and reduce weight gain in obese mice, exercise training did not reduce lipid accumulation in the hypothalamus, or improve hypothalamic sensitivity to peripheral signals. This may be related to the design of the studies, where mice were first rendered obese, and then commenced their exercise training. Therefore, the results of this thesis show that exercise training is unable to reverse some of the major defects of the CNS seen in obesity. Exercise may be more suited for preventing (rather than reversing) the negative impact of high-fat feeding on the CNS, and as such, studies where high-fat feeding is introduced at the same time as exercise training would investigate this hypothesis.

Collectively, these data demonstrate that the hypothalamus is subjected to metabolic insults in obesity, including the accumulation of different lipid species. This thesis shows that the stimulation of hypothalamic neurogenesis is not hindered during obesity, and is increased to a similar amount as lean mice. This extends upon the previous dogma indicating that hypothalamic neurogenesis is reduced during obesity. Although we could not ascertain a

definitive, functional role for this exercise-induced neurogenesis in insulin action, it may be regulating other metabolic pathways such as lipid metabolism, and this warrants further investigation. Therefore, stimulating neurogenesis through exercise may still be a viable therapeutic target. While the prescription of exercise training as a means of obesity treatment is still a key lifestyle intervention and has many health benefits including improved insulin sensitivity and weight loss, this thesis highlights that it is not sufficient to improve the lipid profile or the hormonal sensitivity of the hypothalamus during obesity.

Chapter Eight: Appendix

Table 8.1 Fatty acid composition of the low-fat and high-fat diets

Fatty acid	LFD	HFD
C12:0 and less	No data	3.20%
Myristic Acid 14:0	No data	0.90%
Palmitic Acid 16:0	0.20%	7.10%
Stearic Acid 18:0	0.10%	9.30%
Arachidic acid 20:0	No data	0.30%
Palmitoleic Acid 16:1	No data	0.10%
Oleic Acid 18:1	2.40%	12.00%
Gadoleic Acid 20:1	trace	0.10%
Linoleic Acid 18:2 n6	0.80%	2.00%
a Linolenic Acid 18:3 n3	0.40%	0.70%
Arachadonic Acid 20:4 n6	No data	No data
EPA 20:5 n3	No data	trace
DHA 22:6 n3	No data	No data
Total n3	0.45%	0.74%
Total n6	0.76%	2.05%
Total Mono Unsaturated Fats	2.46%	12.20%
Total Polyunsaturated Fats	1.21%	2.79%
Total Saturated Fats	0.28%	20.92%

Table 8.2 Internal standards for lipidomics analysis

Standard	Name	Chain	pmol/sample
23 Standard Mix – 10μl per sample			
1	Bis(monoacylglycero)phosphate (BMP)	C14:0/14:0	100
2	Ceramide (Cer)	C17:0	100
3	Ceramide-1-phosphate (Cer-1-P)	C12:0	100
4	Dihydroceramide (dhCer)	C8:0	100
5	Galactosylceramide (GalCer)	C15:0	100
6	Sphingomyelin (SM)	C12:0	200
7	Sphingosine (Sph)	C17:1	100
8	Sphingosine-1-phosphate (Sph-1-P)	C17:1	100
9	Sphinganine (Sphn)	C17:0	100
10	Sphinganine-1-phosphate (Sphn-1-P)	C17:0	100
11	Phosphatidic Acid (PA)	C17:0/17:0	100
12	Phosphatidylcholine (PC)	C13:0/13:0	100
13	Phosphatidylcholine (PC)	C21:0/21:0	100
14	Phosphatidylethanolamine (PE)	C17:0/17:0	100
15	Phosphatidylglycerol (PG)	C17:0/17:0	100
16	Phosphatidylserine (PS)	C17:0/17:0	100
17	Lysophosphatydicacid (LPA)	C17:0	100
18	Lysophosphatidylcholine (LPC)	C13:0	100
19	Diacylglycerol (DAG)	C15:0/15:0	200
20	Triacylglycerol (TAG)	C17:0/17:0/17	100
21	Cholesterol D7		1000
22	Cholesterol Ester	C18:0 d6	1000
23	1-O-AcylCeramide	C18:1	100
GL Standard Mixture – 5μl per sample			
1	Glucosylceramide (GC)/Monohexosylceramide	C16:0 d3	50
2	Lactosylceramide (LC)/Dihexosylceramide	C16:0 d3	50
3	Ceramide trihexoside	C17:0	50

Table 8.3 Differential expression of genes involved in neurogenesis

Symbol	Gene name	Fold change relative to sedenatary	P value
Positive regulator of cell proliferation			
Egf	Epidermal growth factor	1.7 ± 0.2	0.007
Fgf2	Fibroblast growth factor 2	2.1 ± 0.2	0.003
il3	Interleukin 3	1.5 ± 0.3	0.015
Nrp1	Neuropilin 1	1.0 ± 0.1	0.837
Ptn	Pleiotrophin	1.3 ± 0.2	0.156
Vegf	Vascular endothelial growth factor A	3.2 ± 0.5	0.004
Negative regulator of cell proliferation			
Alk	Anaplastic lymphoma kinase	1.0 ± 0.1	0.747
Bai1	Brain-specific angiogenesis inhibitor 1	3.6 ± 0.5	0.002
Cxcl1	Chemokine (C-X-C motif) ligand 1	1.0 ± 0.2	0.904
Ndn	Necdin	0.8 ± 0.1	0.204
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
Odz1	Odd Oz/ten-m homolog 1 (Drosophila)	2.0 ± 0.1	0.003
Other regulators of cell proliferation			
Ache	Acetylcholinesterase	2.0 ± 0.2	0.004
Cdk5r1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	2.2 ± 0.2	0.004
Cdk5rap1	CDK5 regulatory subunit associated protein 2	1.6 ± 0.1	0.005
Cdk5rap3	CDK5 regulatory subunit associated protein 3	3.4 ± 0.7	0.015
Erbb2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	2.0 ± 0.3	0.027
Mdk	Midkine	1.1 ± 0.1	0.243
Ndp	Norrie disease (pseudoglioma) (human)	2.1 ± 0.3	0.007
S100a6	S100 calcium binding protein A6 (calcyclin)	1.1 ± 0.1	0.387
S100b	S100 protein, beta polypeptide, neural	1.4 ± 0.1	0.084
Cell cycle arrest			
Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1	4.0 ± 0.7	0.002
Inhba	Inhibin, beat a	1.8 ± 0.3	0.024
Mii1	Myeloid/lymphoid or mixed-lineage leukemia 1	1.2 ± 0.1	0.080
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
Ep300	E1A binding protein p300	1.5 ± 0.2	0.039
Fgf2	Fibroblast growth factor 2	2.1 ± 0.2	0.003
Hdac4	Histone deacetylase 4	1.3 ± 0.1	0.262
Hdac7	Histone deacetylase 7	1.1 ± 0.1	0.546
Mdk	Midkine	1.1 ± 0.1	0.243
Ndn	Necdin	0.8 ± 0.1	0.204
Pard6b	Partitioning defective 6 homolog beta	1.9 ± 0.2	0.011
S100a6	S100 calcium binding protein A6 (calcyclin)	1.1 ± 0.1	0.387
S100b	S100 protein, beta polypeptide, neural	1.4 ± 0.1	0.084
Cell motility and migration			

Flna	Filamin, alpha	1.1 ± 0.2	0.629
Ntn1	Netrin 1	0.8 ± 0.2	0.600
Pafah1b1	Platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	1.1 ± 0.0	0.455
Pard6b	Partitioning defective 6 homolog beta	1.8 ± 0.2	0.011
Slit2	Slit homolog 2 (Drosophila)	2.3 ± 0.6	0.072
Stat3	Signal transducer and activator of transcription 3	3.6 ± 1.1	0.053
Vegfa	Vascular endothelial growth factor A	3.2 ± 0.5	0.004
Neuronal Differentiation			
Cdk5r1	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	2.2 ± 0.2	0.004
Cdk5rap1	CDK5 regulatory subunit associated protein 1	1.6 ± 0.1	0.005
Cdk5rap2	CDK5 regulatory subunit associated protein 2	1.9 ± 0.3	0.076
Cdk5rap3	CDK5 regulatory subunit associated protein 3	3.4 ± 0.7	0.015
Ywhah	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	2.5 ± 0.5	0.033
Cell fate determination			
Dll1	Delta-like 1 (Drosophila)	4.2 ± 0.7	0.004
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
Shh	Sonic hedgehog	1.3 ± 0.3	0.482
Regulators of cell differentiation			
Ascl1	Achaete-scute complex homolog 1 (Drosophila)	1.9 ± 0.3	0.011
Hdac4	Histone deacetylase 4	1.3 ± 0.1	0.262
Hdac7	Histone deacetylase 7	1.1 ± 0.1	0.564
Inhba	Inhibin, beat a	1.8 ± 0.3	0.024
Mdk	Midkine	1.1 ± 0.1	0.243
Ncoa6	Nuclear receptor coactivator 6	1.8 ± 0.2	0.020
Neurod1	Neurogenic differentiation 1	1.6 ± 0.6	0.385
Nog	Noggin	3.0 ± 0.5	0.005
Nrcam	Neuron-glia-CAM-related cell adhesion molecule	1.5 ± 0.1	0.003
Nrg1	Neuregulin 1	1.5 ± 0.1	0.009
Pax5	Paired box gene 5	1.5 ± 0.2	0.077
Pax6	Paired box gene 6	2.6 ± 0.4	0.012
Growth factors			
Artn	Artemin	2.7 ± 0.4	0.005
Bdnf	Brain derived neurotrophic factor	3.1 ± 0.5	0.004
Bmp15	Bone morphogenetic protein 15	3.4 ± 0.6	0.008
Bmp2	Bone morphogenetic protein 2	2.5 ± 0.3	0.004
Bmp4	Bone morphogenetic protein 4	1.3 ± 0.3	0.374
Bmp8b	Bone morphogenetic protein 8b	1.0 ± 0.1	0.977
Cxcl1	Chemokine (C-X-C motif) ligand 1	1.0 ± 0.2	0.904
Egf	Epidermal growth factor	1.7 ± 0.2	0.007
Fgf13	Fibroblast growth factor 13	1.6 ± 0.1	0.007
Fgf2	Fibroblast growth factor 2	2.1 ± 0.2	0.003
Gdnf	Glial cell line derived neurotrophic factor	1.3 ± 0.1	0.145
Gpi1	Glucose phosphate isomerase 1	1.6 ± 0.1	0.004
Ii3	Interleukin 3	1.8 ± 0.2	0.015
Inhba	Inhibin, beat a	1.8 ± 0.3	0.024

Mdk	Midkine	1.1 ± 0.1	0.243
Ndp	Norrie disease (pseudoglioma) (human)	2.1 ± 0.3	0.007
S100a6	S100 calcium binding protein A6 (calcyclin)	1.1 ± 0.1	0.387
Vegfa	Vascular endothelial growth factor A	3.2 ± 0.5	0.004
Nrg1	Neuregulin 1	1.5 ± 0.2	0.009
Ptn	Pleiotrophin	1.3 ± 0.2	0.156
Cytokines			
Bmp15	Bone morphogenetic protein 15	3.4 ± 0.6	0.008
Bmp2	Bone morphogenetic protein 2	2.5 ± 0.3	0.004
Bmp4	Bone morphogenetic protein 4	1.3 ± 0.3	0.374
Bmp8b	Bone morphogenetic protein 8b	1.0 ± 0.1	0.977
Cxcl1	Chemokine (C-X-C motif) ligand 1	1.0 ± 0.2	0.904
Gpi1	Glucose phosphate isomerase 1	1.6 ± 0.1	0.004
Ii3	Interleukin 3	1.5 ± 0.3	0.015
Inhba	Inhibin, beat a	1.8 ± 0.3	0.024
Mdk	Midkine	1.1 ± 0.1	0.243
Ptn	Pleiotrophin	1.3 ± 0.2	0.156
Synaptic plasticity			
Apoe	Apolipoprotein E	1.7 ± 0.2	0.036
Grin1	Glutamate receptor, ionotropic, NMDA1 (zeta 1)	1.9 ± 0.5	0.103
S100b	S100 protein, beta polypeptide, neural	1.4 ± 0.1	0.084
Ywhah	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	2.5 ± 0.5	0.033
Synaptic transmission			
Apoe	Apolipoprotein E	1.7 ± 0.2	0.036
Chrm2	Cholinergic receptor, muscarinic 2, cardiac	2.2 ± 0.2	0.003
Dlg4	Discs, large homolog 4 (Drosophila)	3.1 ± 0.5	0.005
Drd2	Dopamine receptor D2	3.1 ± 0.5	0.002
Drd5	Dopamine receptor D5	2.1 ± 0.3	0.016
Grin1	Glutamate receptor, ionotropic, NMDA1 (zeta 1)	1.9 ± 0.5	0.103
Nptx1	Neuronal pentraxin 1	1.0 ± 0.1	0.998
Synaptogenesis			
Ache	Acetylcholinesterase	2.0 ± 0.2	0.004
Nrcam	Neuron-glia-CAM-related cell adhesion molecule	1.5 ± 0.1	0.003
Pou4f1	POU domain, class 4, transcription factor 1	9.1 ± 7.8	0.284
Anti-apoptosis			
Gdnf	Glial cell line derived neurotrophic factor	1.3 ± 0.1	0.145
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
Sema4d	Semaphorin-4D	1.5 ± 0.2	0.065
Induction of apoptosis			
Adora1	Adenosine A1 receptor	2.7 ± 0.3	0.005
Apoe	Apolipoprotein E	1.7 ± 0.2	0.036
Inhba	Inhibin, beat a	1.8 ± 0.3	0.024
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
S100b	S100 protein, beta polypeptide, neural	1.4 ± 0.1	0.084
Other genes involved in apoptosis			
Adora2a	Adenosine A2a receptor	2.3 ± 0.5	0.002
Ep300	E1A binding protein p300	1.5 ± 0.2	0.039

Ntn1	Netrin 1	0.8 ± 0.2	0.600
Pax3	Paired box gene 3	1.1 ± 0.5	0.511
Rtn4	Reticulon 4	2.3 ± 0.2	0.001
Vegfa	Vascular endothelial growth factor A	3.2 ± 0.5	0.004
Ywhah	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	2.5 ± 0.5	0.033
Cell adhesion			
Robo1	Roundabout homolog 1 (Drosophila)	1.4 ± 0.1	0.049
Ache	Acetylcholinesterase	2.0 ± 0.2	0.004
Bai1	Brain-specific angiogenesis inhibitor 1	3.6 ± 0.5	0.002
Dll1	Delta-like 1 (Drosophila)	4.2 ± 0.7	0.004
Efnb1	Ephrin B1	2.1 ± 0.2	0.005
Fez1	Fasciculation and elongation protein zeta-1	1.9 ± 0.1	0.007
Nrcam	Neuron-glia-CAM-related cell adhesion molecule	1.5 ± 0.1	0.003
Nrp1	Neuropilin 1	1.0 ± 0.1	0.837
Nrp2	Neuropilin 2	2.0 ± 0.2	0.005
Pard3	Par-3 (partitioning defective 3) homolog (C. elegans)	1.9 ± 0.2	0.006
Rac1	RAS-related C3 botulinum substrate 1	1.0 ± 0.0	0.741
Sema4d	Semaphorin-4D	1.5 ± 0.2	0.065
Slit2	Slit homolog 2 (Drosophila)	2.3 ± 0.6	0.072
Tnr	Tenascin R	4.0 ± 0.9	0.036
Cell signalling			
Dll1	Delta-like 1 (Drosophila)	4.2 ± 0.7	0.004
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
Dvl3	Dishevelled 3, dsh homolog (Drosophila)	2.6 ± 0.3	0.013
Ncoa6	Nuclear receptor coactivator 6	1.8 ± 0.2	0.020
G- protein coupled receptor signalling pathway			
Adora1	Adenosine A1 receptor	2.7 ± 0.3	0.005
Adora2	Adenosine A2 receptor	2.8 ± 0.3	0.002
Bai1	Brain-specific angiogenesis inhibitor 1	3.6 ± 0.5	0.002
Chrm2	Cholinergic receptor, muscarinic 2, cardiac	2.2 ± 0.2	0.003
Cxcl1	Chemokine (C-X-C motif) ligand 1	1.0 ± 0.2	0.904
Drd2	Dopamine receptor D2	3.1 ± 0.5	0.002
Drd5	Dopamine receptor D5	2.1 ± 0.3	0.016
Gnao1	Guanine nucleotide-binding protein G(o) subunit alpha	2.4 ± 0.4	0.016
Regulation of transcription			
Apbb1	Amyloid beta (A4) precursor protein-binding, family B, member 1	4.0 ± 0.7	0.003
Arnt1	Artemin	2.7 ± 0.4	0.005
Ascl1	Achaete-scute complex homolog 1 (Drosophila)	1.9 ± 0.3	0.011
Ep300	E1A binding protein p300	1.5 ± 0.2	0.039
Hdac4	Histone deacetylase 4	1.3 ± 0.1	0.262
Hdac7	Histone deacetylase 7	1.1 ± 0.1	0.546
Hes1	Hairy and enhancer of split 1 (Drosophila)	1.2 ± 0.1	0.114
Hey1	Hairy/enhancer-of-split related with YRPW motif 1	1.2 ± 0.1	0.102
Hey2	Hairy/enhancer-of-split related with YRPW motif 2	1.4 ± 0.7	0.601
Heyl	Hairy/enhancer-of-split related with YRPW motif-	2.8 ± 0.4	0.001

	like		
Mef2c	Myocyte enhancer factor 2C	2.5 ± 0.3	0.001
Mll1	Myeloid/lymphoid or mixed-lineage leukemia 1	1.2 ± 0.1	0.080
Ncoa6	Nuclear receptor coactivator 6	1.7 ± 0.3	0.020
Ndn	Necdin	0.8 ± 0.1	0.204
Neurod1	Neurogenic differentiation 1	1.6 ± 0.6	0.385
Notch2	Notch gene homolog 2 (Drosophila)	2.2 ± 0.3	0.021
Pax3	Paired box gene 3	1.1 ± 0.5	0.511
Pax5	Paired box gene 5	1.5 ± 0.2	0.077
Pax6	Paired box gene 6	2.6 ± 0.4	0.012
Pou3f3	POU domain, class 3, transcription factor 3	2.3 ± 0.2	0.002
Pou4f1	POU domain, class 4, transcription factor 1	9.1 ± 7.8	0.284
Sox3	SRY-box containing gene 3	1.9 ± 0.4	0.051
Stat3	Signal transducer and activator of transcription 3	3.6 ± 1.1	0.053

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