A Novel Role for Brain-Derived Adipokines in the Etiology of Obesity?

by

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Abstract

Obesity is a complex health care issue in industrialized societies and is implicated in the development of diabetes and cardiovascular disease. Fat-derived adipokines, including leptin, resistin, and fasting-induced adipose factor (FIAF), are key obesity hormones that modulate bodyweight and energy homeostasis via hypothalamic-dependent mechanisms. Although they are predominantly derived from adipose tissue, their expression was also detected in the brain and pituitary gland. I hypothesized that adipokines expressed in the central nervous system would impact energy homeostasis and brain function, via paracrine or intracrine-dependent mechanisms. RNA interference (RNAi) was used to silence adipokine expression in several models to investigate their potential roles. Proof-of-principle studies in C6 glioblastoma cells confirmed the effectiveness of using RNAi to silence the expression of brain leptin, and this knockdown increased cell death. RNAi also silenced leptin expression in the rat brain, which induced tissue-dependent changes in resistin (*rstn*) expression. In additional in vitro experiments *rstn* was silenced in mouse N-1 hypothalamic neurons. *Rstn* silencing concomitantly induced the expression of *fiaf* and suppressor of cytokine signaling-3 (*socs-3*), an intracellular inhibitor of leptin signaling, but decreased the activation of AMP-activated protein kinase (AMPK), an enzyme implicated in the control of energy metabolism. Parallel studies revealed that adipokine expression was modified in N-1 neurons and 3T3-L1 adipocytes, and in mice, following treatment with valproic acid (VPA), all-*trans* retinoic acid (ATRA), and rosiglitazone (ROSI), compounds known to induce either weight gain or insulin sensitization. The expression of brain adipokines was also induced by inflammatory stimuli and by two models of brain injury; cerebral hypoxia/ischemia (H/I) and traumatic brain injury (TBI), suggesting their involvement in the pathology of brain injury. In conclusion, these studies implicate centrally-derived adipokines (*cephalokines*) in the control of brain cell survival, signaling, and metabolism. Therefore brain adipokines might be part of a fine 'tuning' mechanism that regulates hypothalamic pathways implicated in the control of bodyweight and appetite, or impact brain repair and function following injury.

List Of Abbreviations Used

αMSH	alpha melanocyte stimulating hormone
aa	amino acid
AAV	adeno-associated virus
ADAR	RNA-specific adenosine deaminase
Ago2	Argonaute 2
AgRP	Agouti-related peptide
AICAR	5-aminoimidazole-4-carboxamide riboside
ANGPTL	angiopoietin-like proteins
ANGPTL4	Angiopoietin-like 4
AMP	adenosine 5'-monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AP-1	activator protein 1
ARC	arcuate nucleus of the hypothalamus
Armi	armitage
ASODN	antisense oligodeoxynucleotides
ATP	adenosine triphosphate
ATRA	all-*trans* retinoic acid
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BMI	Body Mass Index
bp	base pair
bZIP	basic-region leucine zipper
CaMMKβ	calcium/calmodulin-dependent protein kinase beta
cAMP	cyclic adenosine monophosphate

CART	Cocaine and amphetamine related transcript
CCA	common carotid artery
CCK	cholecystokinin
cDNA	complementary DNA
CEBP	CCAAT enhancer binding protein
ChIP	chromatin immuno-precipitation
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
CNTF	ciliary neurotrophic factor
CRF	corticotrophin-releasing factor
CSF	cerebrospinal fluid
C_T	threshold cycle
CTX	cortex
CVO	circumventricular organ
D3V	dorsal third ventricle
DAB	diaminobenzidine
DAPI	4'-6-diamidion-2-phenylindole
DAT	dopamine transporter
dbcAMP	dibutyryl cAMP
DEX	dexamethasone
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DIO	diet-induced obesity
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotides
D-PBS	Dulbeccos PBS

ds	double stranded
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
eIF-2	eukaryotic initiation factor-2
EMSA	electrophoretic mobility shift assay
ER-1	enhanced RNA interference-1
EthD-1	ethidium homodimer-1
FAT	visceral adipose tissue
FBS	Fetal bovine serum
FIAF	Fasting-induced adipose factor
FP	fluid percussion
GAP-43	growth associated protein 43
G-CSF	granulocyte colony-stimulating factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GR	glucocorticoid receptor
HDAC	histone deacetylase
H/I	hypoxia ischemia
HIE	hypoxic/ischemic encephalopathy
HIF-1α	hypoxia inducible factor-1 alpha
HIPPO	hippocampus
HPG	hypothalamic-pituitary-gonadal
HRP	horseradish peroxidase
HYP	hypothalamus
IBMX	3-isobutyl-1-methylxanthine

icv	intracerebroventricular
IFNβ	interferon beta
IGF-1	insulin-like growth factor
IκB	Inhibitor of kappa B
IκK	IκB Kinase
IL-1ra	interleukin-1 receptor antagonist
IL-6	interleukin-6
ir	immunoreactivity
IRS	insulin receptor substrate
ISG	interferon stimulated genes
JAK	Janus kinase
KDa	Kilodalton
KO	knockout
LB	Luria Bertani broth
LH	Lateral Hypothalamus
LIH	Leukemia inhibitory factor
LPL	lipoprotein lipase
LPS	Lipopolysaccharide
LTP	long term potentiation
LV	lateral ventricle
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MBH	microdissected basal hypothalamus
MC3R	melanocortin 3 receptor
MC4R	melanocortin 4 receptor
MCAO	middle cerebral artery occlusion

MCH	melanin concentrating hormone
MON	morpholino oligonucleotide
mRNA	messenger RNA
NFκB	Nuclear factor kappa B
NGF	nerve growth factor
NIDDM	Non insulin-dependent diabetes mellitus
NIH	National Institutes of Health
NMDA	n-methyl-d-aspartate
NPY	Neuropeptide Y
N.S.	non significant
NT	neurotensin
NT 4	neurotrophin 4
NUCB2	nucleobindin2
Ob	Leptin
OBR	Leptin receptor
P2X3	purinergic (P) 2X-ATP receptors
pAMPKα	phosphoryated AMPKα
PAGE	polyacrylamide electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Postnatal Day
PDE	phosphodiesterase
PFA	paraformaldehyde
PGAR	PPARgamma angiopoietin related
PI3K	phosphatidylinositol-3 kinase
PIT	pituitary

PKR	protein kinase response
PMSF	phenylmethylsulphonylfluoride
POMC	Proopiomelanocortin
PPAR	peroxisome proliferator activated receptor
PPRE	PPARγ response element
PTP1B	protein tyrosine phosphatase 1B
PVN	Paraventricular Nucleus
PYY_{3-36}	Polypeptide Y_{3-36}
qPCR	quantitative PCR
RA	retinoic acid
RAR	retinoic acid receptor
rAAV	recombinant adeno-associated virus
RIPA	radioimmunoprecipitation assay buffer
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROSI	rosiglitazone
rstn	resistin
RT	reverse transcription
rTdT	recombinant terminal deoxynucleotidyl transferase
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid X receptor
s.c.	Subcutaneous
SCR	scramble
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SERT	serotonin transporter
SHP-2	src homology 2 domain-containing tyrosine phosphatase 2
shRNA	small hairpin RNA
siRNA	small interfering RNA
SNAP-25	synaptosomal associated protein-25
SOCS-3	suppressor of cytokine signaling-3
Sp1	specificity protein 1
SSC	sodium chloride/sodium citrate
SSRI	serotonin-specific reuptake inhibitor
STAT	signal transducer and activator of transcription
STH	STEALTH siRNA
STZ	streptozotocin
TBE	Tris-borate buffer
TBI	traumatic brain injury
TBS	Tris buffered saline
TLR	toll-like receptor
TNFα	Tumor necrosis factor alpha
TSA	trichostatin A
TTBS	Tween TBS
TUNEL	Terminal deoxyribonucleotidyl transferase mediated d-Uridine 5' triphosphate Nick end labeling
TZD	thiazolidinedione
VEGF	vascular endothelial growth factor
VMH	Ventral medial hypothalamus

VPA	Valproic acid
WAT	white adipose tissue
ZMP	5-aminoimidazole-4-carboxamide 1-β-D-ribofuranotide

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Chapter 1: Introduction

   Adipose tissue has emerged as a key endocrine organ that influences the complex metabolic pathways involved in normal bodyweight regulation. Although the hypothalamus was known to be a key site of bodyweight regulation, it was also hypothesized more than 50 years ago that body fat was producing a metabolic signal that reached these critical brain centers (Kennedy 1953; Olney 1969). However this 'adipostatic' system received minimal attention until the cloning of the ob gene, and identification of leptin, in 1994 (Zhang et al. 1994). In addition plasma leptin concentrations are positively correlated with the degree of adiposity (Schwartz et al. 1996a), and the peripheral or central injection of leptin was shown to reduce food intake and bodyweight via a hypothalamic-dependent mechanism in rodents (Zhang et al. 1994; Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). Thus peripherally-derived leptin was thought to cross the blood brain barrier (BBB) in order to modify the activity of the hypothalamic pathways implicated in the regulation of energy metabolism. However early clinical trials on leptin soon failed, and it was found that human obesity is characterized by a state of leptin resistance since most obese individuals are hyperleptinemic (Schwartz et al. 1996a; Heymsfield et al. 1999). In addition it was also unclear whether peripheral leptin was even capable of efficiently crossing the BBB, especially in obese subjects, or whether leptin stimulated the release of anorexigenic cytokines from the endothelial cells that form the highly impermeable brain capillary system (Banks et al. 1996; Wilkinson et al. 2000; Rivest 2002). Thus it was hypothesized that the brain would also be a site of leptin expression (Morash et al. 1999). Subsequently leptin has been implicated in several other processes, including brain growth and development (Ahima et al. 1999; Steppan and Swick 1999). In addition leptin appears to impact several brain functions such as modulating the development of neuronal pathways, impacting hypothalamic synaptic plasticity and brain cell survival

(Bouret et al. 2004b; Pinto et al. 2004; Russo et al. 2004). Several other hormones have since been identified and cloned from adipose tissue and are collectively defined as adipokines (Trayhurn and Wood 2004). Thus the original hypothesis, that the brain would be a site of leptin expression, was expanded to include other adipokine genes including resistin and fasting-induced adipose factor (FIAF) (Morash et al. 2002; Wiesner et al. 2004). Although these adipokine gene transcripts were readily detected in the rodent brain by RT-PCR and immunohistochemistry, it remained unclear what purpose they served in normal brain function or in central energy homeostasis. Thus the main focus of these studies was to investigate the potential roles of brain-derived adipokines on brain function, both in vitro and in vivo, using a variety of approaches.

I. Obesity And Industrialized Societies

Obesity is one of the greatest health crises facing industrial societies today. There are several hypotheses to account for the ever-expanding waistlines and escalating body mass indices (BMI) in industrialized nations. We can point our fingers at the usual suspects of our current lifestyles, eating oversized portions and lack of physical activity. However some novel culprits have also emerged, including decreases in the amount of time spent sleeping, increased concentrations of industrial endocrine disrupters in our environment, and reductions in the number of smokers, amongst several others (Keith et al. 2006). Within the last 15 years the percentage of obese Canadians has more than doubled, and this trend does not appear to be subsiding (Katzmarzyk 2002). The proportion of overweight and obese children that are being raised in North American families is also on the rise (Prentice 2006). For the first time in the last two centuries population lifetime expectancy is predicted to decrease by 0.33 to 0.75 years (Olshansky et al. 2005). This is due, in part, to the increased risk of developing diabetes, heart disease, cancer, and other co-morbidities associated with carrying excess weight and obesity (Olshansky et al. 2005). Further proof of this

pandemic is the number of children that are now being diagnosed with "adult" diseases such as non insulin-dependent diabetes mellitus (NIDDM). An estimated 10-20% of new pediatric diabetes patients in Canada are now due to type 2 diabetes, compared with one of the first ever reported Canadian cases occurring fifteen years earlier (Pinhas-Hamiel and Zeitler 2005). A recent report suggests that economically priced super sized car seats are now needed in the United States since nearly 300,000 children under the age of 6 exceed the current weight restrictions (Trifiletti et al. 2006). Unfortunately Canadian children probably aren't far behind. In addition to decreased life expectancy, it is estimated that obesity cost Canadians over $4.3 billion in 2001 (Katzmarzyk and Janssen 2004), a figure that is likely much higher today. In summary, these data suggest that major investments in time and money are desperately needed to target the underlying physiological mechanisms that favor weight gain and the development of obesity. One approach is to investigate potential molecular mechanisms that could be used as pharmaceutical targets to promote weight loss, and help maintain healthy bodyweights, in order to squelch this obesity pandemic.

There is a real stigma attached to obesity as a psychological disease, which is associated with a lack of will power and poor eating habits. However there is a large body of evidence suggesting that obesity includes a substantial genetic component (Blundell 1990; Friedman and Leibel 1992). Early obesity studies lead to the hypothesis that humans developed thrifty genes that offered protection during times of famine and low food sources (Neel 1962). Combined with improvements in agriculture leading to an abundant and stable food supply, and reduced daily physical activity (Hill et al. 2003), thrifty genes have rendered us susceptible to excessive weight gain while also inhibiting our efforts to shed those extra pounds. Moreover bodyweights are highly defended during times of weight loss, and there is often a period of refractory weight gain, in both rodents and humans (Kramer et al. 1989; Levin and Keesey 1998; Levin and Dunn-

Meynell 2000). For example food restriction is capable of inducing short term weight loss in rats, however when food is subsequently returned *ad libitum* animals quickly regain weight to match the body masses recorded in control rodents (Harris et al. 1986). Likewise when rats are provided with lower or higher energy density food (2.8 to 1.5 or 5.1 Kcalories/g) they compensate the amount they eat in order to maintain a stable energy intake (Hervey 1969). However it should also be pointed out that under conditions of force feeding subjects also resisted weight gain to a certain extent, suggesting that humans are equipped to maintain body mass within a relatively narrow range (Sims and Horton 1968). Although our bodies aim to preserve a stable BMI, there appears to be a slight bias towards weight gain (Schwartz et al. 2003), leaving most traditional dieting techniques doomed to fail from the start. In this light we should consider that an effective weight loss strategy not only focuses on weight loss, but more importantly maintains a stable weight afterwards.

The central nervous system (CNS), more specifically the hypothalamus, has historically been implicated in the regulation of appetite and energy expenditure, thus exerting a great influence on the control of bodyweight. Many early studies showed that damaging specific hypothalamic nuclei by way of knife cuts, chemical or electrical lesions, can induce various metabolic changes (Kennedy 1953; Olney 1969). For example inducing lesions in the lateral hypothalamus (LH) results in weight loss whereas damaging the ventral medial hypothalamus (VMH) induces hyperphagia and weight gain. Similarly the paraventricular nucleus (PVN) is thought to increase appetite and feeding [reviewed by (Harris 1990; Sainsbury et al. 2002)]. However many of these hypothalamic areas receive input from the arcuate nucleus of the hypothalamus (ARC) in order to influence their output. Within the ARC there are two distinct neuronal populations, orexigenic and anorexigenic neurons. Prototypical orexigenic neurons coexpress neuropeptide Y (NPY)/agouti-related peptide (AgRP) and stimulate feeding.
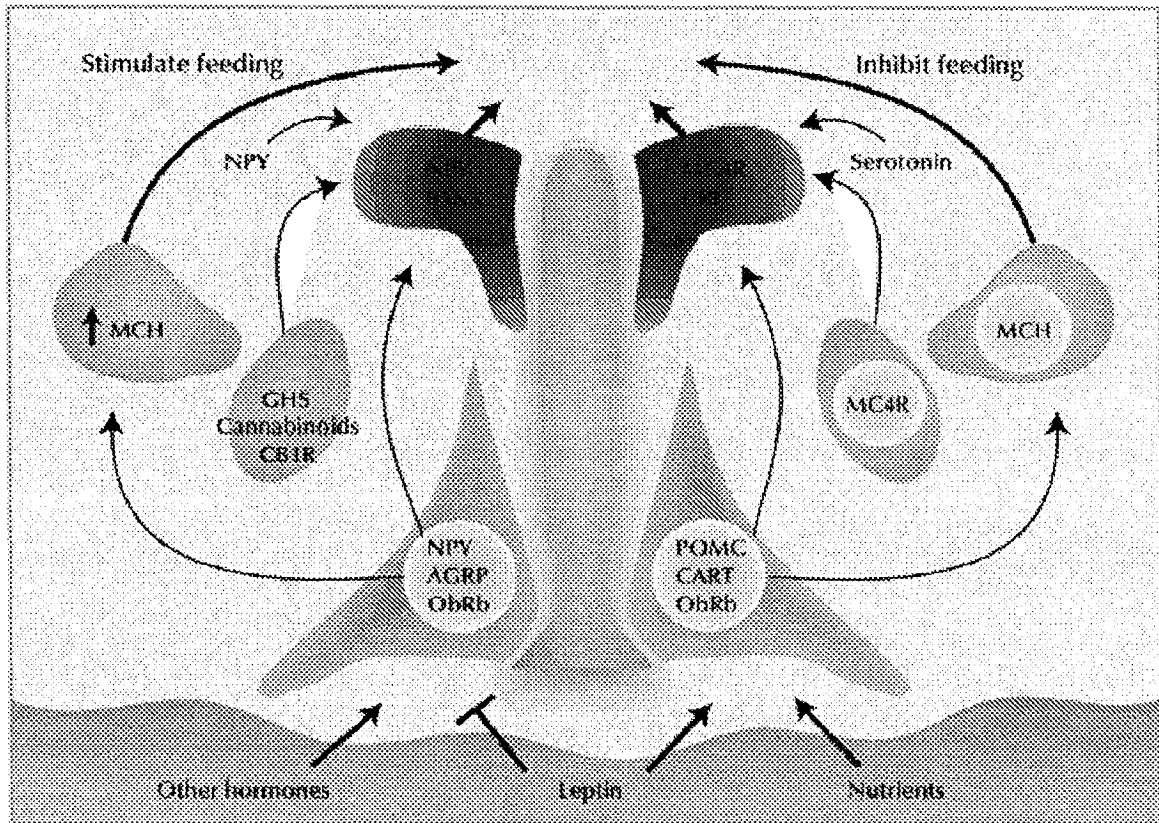
Figure 1: Hypothalamic regulation of energy balance
Two predominant hypothalamic neuronal populations have been implicated in the regulation of feeding and energy metabolism. They are the orexigenic neurons, which co-express NPY and AgRP (left), and the anorexigenic neurons that express POMC and CART (right). The output from these neuronal populations is controlled by metabolic hormones (e.g. leptin) and nutrients that act on down stream targets (e.g. Melanocortin system) to help balance food intake (i.e. appetite) and energy output in order to control bodyweight. Imbalances often result in obesity and lead to further weight gain. (Figure adapted from Friedman 2004)

Conversely the anorexigenic proopiomelanocortin (POMC)/cocaine and amphetamine related transcript (CART)-expressing neurons, are thought to reduce appetite and induce energy expenditure (see Figure 1 [Adapted from (Friedman 2004)]). The balance of these pathways exerts a great influence over weight gain by controlling food intake and energy expenditure (Schwartz et al. 2003; Zigman and Elmquist 2003). The basic principles of thermodynamics dictate that energy intake must match energy expenditure in order to maintain a steady bodyweight. However our brains appear to be 'wired' in a biased manner that appears to favor weight gain (Schwartz et al. 2003). Although other regions of the CNS have also been implicated in the coordinated and adaptive responses to feeding (Berthoud 2006), the ARC has long been considered one of the most critical brain centers (Kennedy 1953; Hervey 1969; Olney 1969).

Various peripheral metabolic inputs have been identified that mediate the output from the orexigenic and anorexigenic neurons of the ARC in order to influence bodyweight regulation (Badman and Flier 2005; Schwartz and Porte 2005). Even more impressive is the fact that our bodies are highly efficient at maintaining body fat stores, which likely involves some coordinated response with the aforementioned hypothalamic regions (Sims and Horton 1968). Hervey (1969) points out that between the ages of 25 and 65 a women will consume over 20 tons of food, whereas she will gain on average 11 Kg during that same period (Hervey 1969). A quick calculation suggests that our bodies are approximately 99.97% efficient at controlling our body mass. Various hypothesis have been put forth as to how bodyweights are so efficiently regulated including: a body temperature-dependent indication of energy stores, a glucostatic system, and the now widely accepted lipostatic theory (Kennedy 1953). In this model increases, or decreases, in body fat are thought to generate a signal that modifies the activity of hypothalamic pathways involved in the control of appetite and energy expenditure (see Figure 2 [Adapted from (Schwartz and Niswender 2004)]).
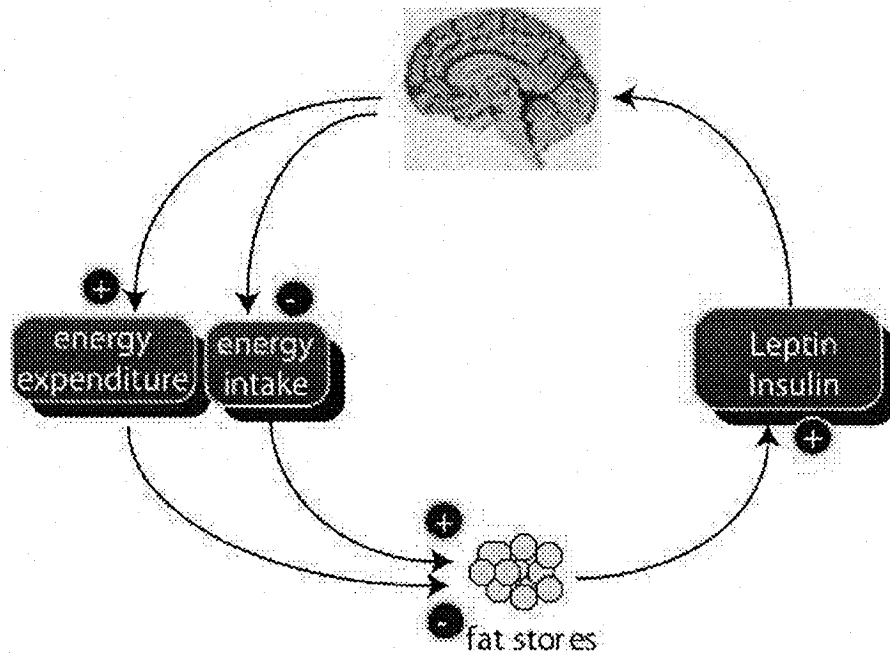
Figure 2: Leptin and the lipostatic loop
The lipostatic loop was hypothesized in the 1950s to control and maintain
a stable bodyweight by sensing the amount of adipose tissue, or energy
reserves, present in the body. Until the cloning of leptin it was still unclear
what adiposity signal(s) was responsible. Now it is thought that increases
in fat mass elevate leptin expression and secretion that signals to the
brain to stop eating, but also increases energy expenditure, in order to
counteract the positive energy balance. Conversely reductions in fat pad
mass had the opposite effect. (Figure adapted from Schwartz and
Niswender 2004)

However the latter was poorly received given that no known circulating factor capable of indicating body fat stores had been identified in the 1950s. Moreover numerous reports have pointed out that a simple lipostatic feedback mechanism would be unable to attain such a high level of efficiency and favor more complex models that include open and closed loops that offer information about metabolic status to the hypothalamus in order to maintain a stable body mass (Hervey 1969; Harris 1990). Therefore other metabolic sensing systems are likely acting in conjunction with the lipostatic loop in order to maintain stable bodyweights.

II. Leptin, The Pleiotropic Hormone

Cloning Of Leptin And Other Adipokines

A singular event in the now prodigious ongoing study of energy balance and bodyweight regulation was the cloning of leptin from adipose tissue by J.M. Friedman's laboratory (Zhang et al. 1994). Subsequent investigations by several research groups revealed that leptin is but one of a large family of factors secreted by adipocytes (*adipokines*: (Koerner et al. 2005; Lafontan 2005; Trayhurn et al. 2006)). These include the familiar (resistin (Steppan et al. 2001); adiponectin (Scherer et al. 1995)), the new (FIAF (fasting-induced adipose factor)(Kersten et al. 2000); visfatin (Fukuhara et al. 2005); vaspin (Hida et al. 2005)) and the unexpected (nerve growth factor (Bullo et al. 2005)). Application of mass spectrometry-based proteomic techniques will certainly enlarge this list of adipocyte secreted proteins (Kratchmarova et al. 2002; Yang et al. 2003b). Many of these targets have been implicated in the regulation of appetite, energy expenditure, insulin sensitivity, and glycemic control, in addition to several other roles. Thus adipokines represent a group of obesity-related drug targets that could potentially be harnessed to treat obesity related illnesses such as cardiovascular disease and diabetes.

The identification of an obesity associated hormone had been predicted over two decades prior to the cloning of leptin. Parabiosis experiments using diabetic db/db, or obese ob/ob mice, provided some insight into the underlying metabolic abnormalities (Figure 3). Both db/db and ob/ob mice of the C57BL6 strain are hyperphagic, hyperglycemic, and hyperinsulinemic which is attributed to genetic abnormalities on chromosome 4 and 6 respectively (Coleman 1973). Like healthy mice, when ob/ob mice were parabiosed with db/db mice they lost significant amounts of weight and eventually died suggesting that the obese mice had intact and functional hypothalamic pathways, but appeared to be deficient in a circulating satiety factor (Coleman 1973). More recently it was shown that this weight loss was almost exclusively from decreases in adipose tissue, while lean tissue was spared (Harris 1999). Although ob/ob partners of mice united with healthy lean mice (+/+) continued to gain weight, it occurred at a slower rate than expected suggesting this weight reducing factor was also secreted in healthy mice, but at a lower level than db/db mice (Figure 3A). In contrast, db/db partners continued to gain weight and appeared totally unaffected by the parabiosis experiment (Coleman 1973). These experiments provided critical evidence suggesting that db/db mice produce an abundance of a factor capable of reducing appetite and weight in lean and ob/ob mice.

Initially the elusive factor was thought to be a humoral product derived from islet cells. The implantation of diffusion chambers containing islet cells, from healthy lean mice, into ob/ob rodents resulted in a pronounced reduction in weight gain and drastic attenuation of plasma glucose levels (Strautz 1970). Moreover this effect was fully reversible by removing the diffusion chambers, suggesting a factor was secreted from the transplanted islets cells derived from lean mice (Strautz 1970). However a notable difference between islet transplantation and ob/ob mice parabiosed with db/db mice is that in the latter studies there is a significant decrease in adipose stores (Harris 1999),
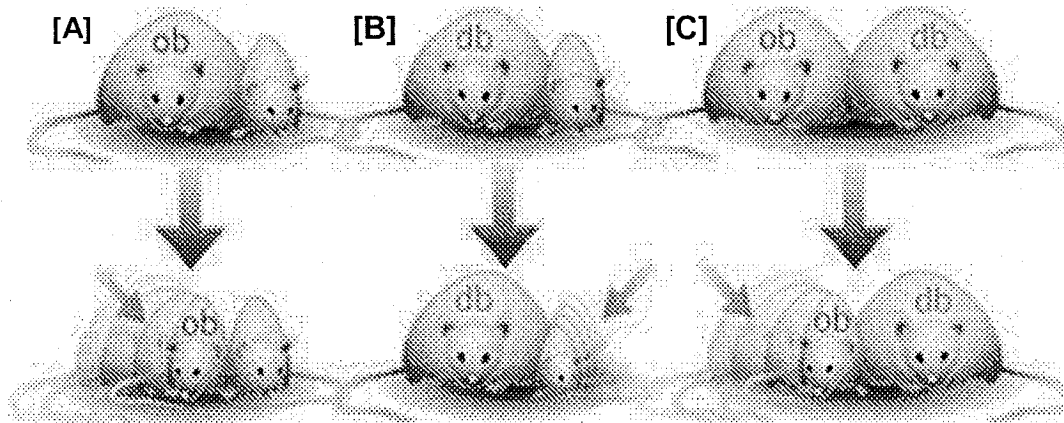
Figure 3: Understanding bodyweight regulation by early parabiosis experiments. Parabiosis experiments suggested that ob/ob mice were deficient in a satiety factor that is produced in lean and db/db mice. [A] When ob/ob mice were sutured together with a lean mouse (ob/ob and lean) it resulted in a modest weight loss. [C] Parabiosis with an obese diabetic mouse (ob/ob and db/db) induced a rapid and significant weight loss in only the ob/ob mouse. In contrast the bodyweights of db/db mice were unaffected by their lean [B] or ob/ob partners [C] suggesting that they were deficient in a functional satiety receptor. (Figure adapted from http://www.scq.ubc.ca/wp-content/uploads/2006/08/parabiosis.jpg)

whereas islet cell transplantation prevented further weight gain and appeared to have no effect on the animals current bodyweight or fat stores (Strautz 1970). This suggests that at least one islet cell-derived factor remains to be identified that may produce beneficial effects on weight maintenance, but is unlikely to be the factor responsible for the drastic weight loss that occurred in ob/ob mice during parabiosis.

It was known for quite some time that the mutation responsible for inducing obesity in ob/ob mice was located on chromosome 6 (Coleman 1973). Using positional cloning techniques Zhang et al. (1994) were able to isolate a single 4.5 Kbp RNA product that appeared to be predominantly, if not exclusively, expressed in adipose tissue of mice (Zhang et al. 1994). Evaluating the amino acid sequences of obese and healthy mice revealed the existence of a premature stop codon at position 105 in ob/ob mice in place of an arginine amino acid residue that is present in lean littermates. Of greater interest leptin appeared highly conserved between species, including humans (Zhang et al. 1994). Moreover given the increased levels of ob mRNA measured in obese mice suggested its potential role as an 'adipostat' factor that somehow modulated, or indicated, adiposity levels thus reviving the hypothetical lipostatic loop model (Kennedy 1953; Zhang et al. 1994; Schwartz et al. 1996a). It soon became apparent that purified recombinant leptin was capable of reducing appetite, increasing energy expenditure, and inducing pronounced reductions in body fat stores in mice (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). Moreover Campfield et al.(1995) were the first to show that the central injection of leptin resulted in rapid reductions in appetite which suggested a role for leptin in central energy homeostasis (Campfield et al. 1995). Further studies have revealed several other physiological roles for leptin including pubertal maturation (Ahima et al. 1997; Chehab et al. 1997), reproduction (Chehab et al. 1996), learning (Oomura et al. 2006), brain development (Ahima et al. 1999; Steppan and Swick 1999), neuroprotection (Dicou et al.

2001) in addition to numerous others. Even today the diverse roles and functions of this pleiotropic hormone are still being identified.

Leptin: Completing The Fat-Brain 'Lipostatic' Loop?

It was more than fifty years ago that Kennedy hypothesized that the brain could sense fat stores in order to control food intake, thus forming an 'lipostatic' system that functions to maintain a relatively stable bodyweight (Kennedy 1953). However it was unclear what adiposity factor(s) were responsible for influencing the central metabolic pathways. The cloning of leptin revived the 'lipostatic' hypothesis since its expression appeared to be correlated with the amount of adipose tissue, i.e. large adipose stores were correlated with increased levels of leptin mRNA in ob/ob mice (Zhang et al. 1994) and adiposity was positively correlated with plasma leptin concentrations (Schwartz et al. 1996a). Given that the expression and release of leptin occurs in response to changes in metabolic status (i.e. high or low energy stores augment or reduce leptin levels respectively) (Becker et al. 1995), and that leptin influences central metabolic pathways (Campfield et al. 1995), it appeared that leptin was the missing link between fat and the brain (see Figure 2). Prior to leptin's inception as an energy gauge, it was already thought that several peripheral metabolic factors were modulating appetite (Kennedy 1953). This has also proven to be correct since several other novel peripheral molecular entities including insulin, ghrelin, polypeptide Y_{3-36} (PYY_{3-36}), cholecystokinin (CCK), have been implicated in the modulation of hypothalamic feeding centers (Badman and Flier 2005; Schwartz and Porte 2005; Fry et al. 2007). However the complexity of this system is not surprising given the high degree of efficiency (~99.97%(Hervey 1969)) with which the brain appears to regulate bodyweight.

Leptin And The Human Physiology Of Weight Regulation

Having established that leptin controls bodyweight by balancing food intake and energy expenditure in rodents, it became possible to consider the therapeutic potential

of leptin as a method to clinically control bodyweight, or treat obesity. Friedman's lab was the first to establish that human leptin possessed biological properties that mimicked those of murine leptin in a rodent system (Halaas et al. 1995). However it was not until 1997, when two severely obese and hyperphagic children were found to have a homozygous frame shift mutation at codon 133 of the human leptin gene, that leptin was fully implicated in the physiology of human bodyweight regulation (Montague et al. 1997; Farooqi and O'Rahilly 2005). Although both patients were cousins and had normal birth weights, they quickly started to gain weight at 4 months and were slowly immobilized by their high body fat content (>50%) (Montague et al. 1997). Leptin-deficient adults were also identified and reported to be hypogonadotropic and obese, but surprisingly adult heterozygotes appeared to be healthy and had normal bodyweights (Strobel et al. 1998). Moreover, like the ob/ob mouse, treating these leptin-deficient children with exogenous leptin lead to drastic weight loss that was mostly attributed to reduced fat mass (Farooqi et al. 1999; Farooqi et al. 2002). These results have also been recapitulated in leptin-deficient adults treated daily with a low dose (10-40 µg/Kg) of recombinant human leptin for a period of 18 months (Licinio et al. 2004). As expected leptin treatment lead to dramatic reduction in food intake and a substantial loss of fat mass, but also activated the reproductive axis in leptin-deficient adults (Licinio et al. 2004). However when it was tested clinically the self administration of leptin failed to induce weight loss in obese patients (Heymsfield et al. 1999), suggesting that most overweight individuals are leptin resistant. Like mice, human leptin deficiency results in a severely obese phenotype which is reversible by leptin treatment and revealed the importance of leptin in the normal physiological regulation of human reproduction and appetite.

Leptin Receptor: Structures And Signaling Pathways

As already noted, parabiosis of lean and db/db, or ob/ob and db/db mice (see Figure 3), suggested that the db/db mice produced an excess of a factor (i.e. leptin)

capable of reducing bodyweights in rodents, but it was unclear why the db/db mice were

unresponsive (Coleman 1973). However it was known that in mice a defect on

chromosome 4 was responsible, leading to the hypothesis that db/db mice were missing

a functional 'lipostatic' receptor (Coleman 1973). The leptin receptor was cloned from the

mouse choroid plexus and it appeared to possess a gp130 signal-transducing

component that is also present in the interleukin-6 receptor (IL-6), granulocyte colony-

stimulating factor receptor (G-CSF) and leukemia inhibitory factor receptor (LIF)

(Tartaglia et al. 1995). This suggested that the leptin receptor, OBR, was a member of

the class I cytokine receptor family, that includes the aforementioned receptors, as well

as interleukin-2, -3, -4, -6, -7, receptors, amongst several others (Hegyi et al. 2004). Six

splice variants of the leptin receptor (OBRa-OBRf) have since been identified that

contain the same extracellular domain, but vary in their intracellular structures (Lee et al.

1996) (see Figure 4). The one exception is the soluble receptor, OBRe, which lacks both

the transmembrane and intracellular domains and appears to act as a circulating leptin

binding protein (Lee et al. 1996). It has been hypothesized that OBRe is a component of

a leptin buffering system that controls the proportion of unbound and active serum leptin,

especially during pregnancy (Gavrilova et al. 1997) and puberty (Quinton et al. 1999).

The long isoform of the leptin receptor, OBRb, is thought of as the functional leptin

receptor since it appears to modulate appetite and energy expenditure. Unlike wildtype

mice, db/db mice possessed a truncated form of OBRb that arises at the

posttranscriptional level by an abnormal splicing event, and appears to be responsible

for the associated obesity and hyperphagia that is characteristic of db/db mice (Lee et al.

1996) (see Figure 5). Moreover this truncated form of the leptin receptor is missing many

of the critical intracellular domains involved in leptin signal transduction (Chen et al.

1996; Lee et al. 1996). Studies in rats revealed a similar mutation in the leptin receptor

of fatty (fa/fa) Zucker rats, that are also characterized by their hyperphagia and obesity
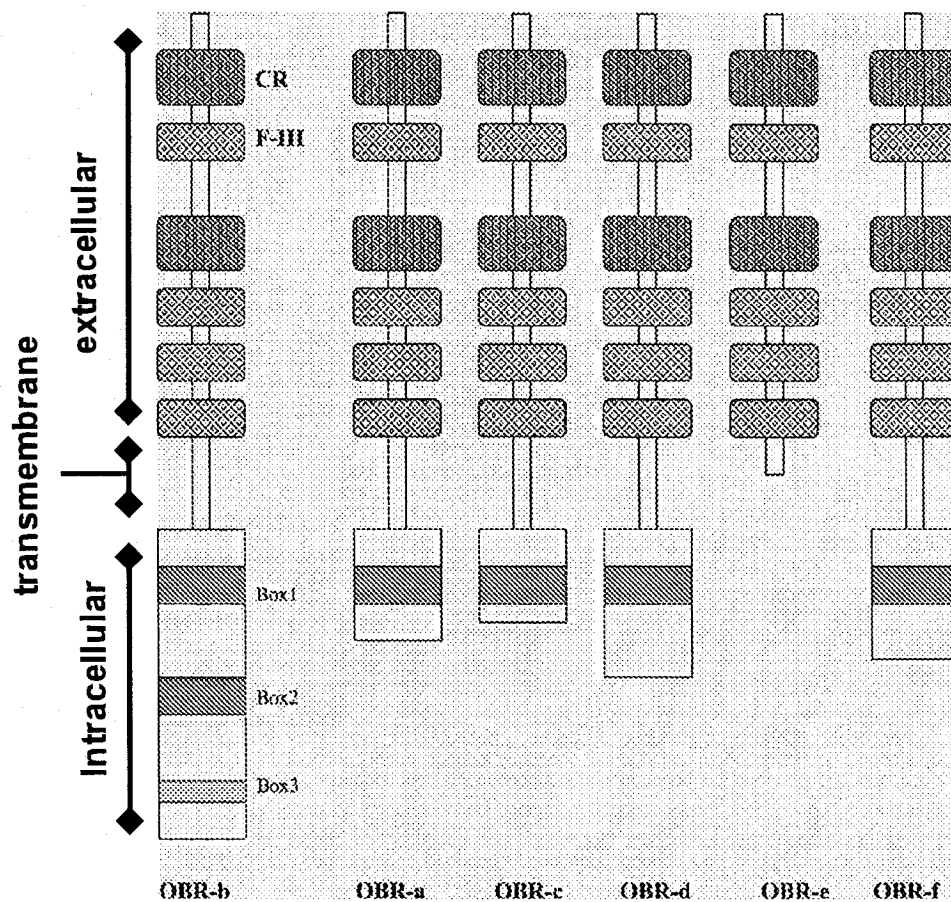
Figure 4: The leptin receptor
Soon after the leptin receptor (OBR) was cloned six splice variants were detected (a-f). Although they all contain the same extracellular domains, the intracellular structures vary greatly in length. Note that OBR-e only contains an extracellular domain and is secreted from cells as a soluble receptor. (Figure adapted from Hegyi et al. 2004)
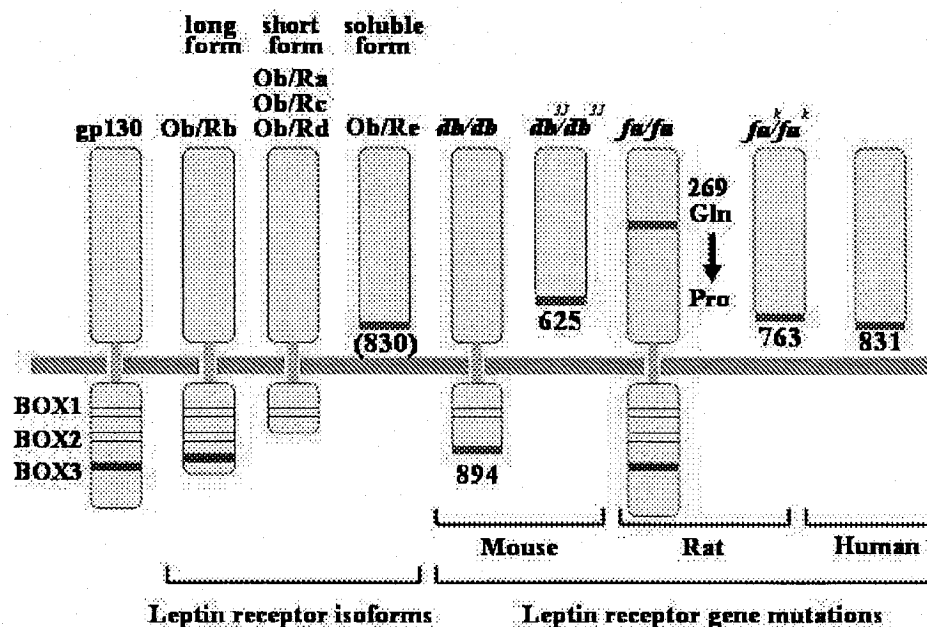
Figure 5: Genetic defects in the leptin receptor
As seen in the previous figure, there are 6 known leptin receptor
isoforms. However various leptin receptor mutations have been
identified in diabetic mice (db/db), fatty Zucker rats (fa/fa) and humans,
that are lacking various functional amino acids or domains that are
normally present in the functional leptin receptor, OBR-b. (Figure
adapted from http://www.tmd.ac.jp/mri/prm/englishi1-10/slide4.JPG)

(Phillips et al. 1996). Clinically leptin receptor mutations are also associated with early onset obesity and lack of pubertal development in humans (Clement et al. 1998; Farooqi et al. 2007). However the phenotype of these individuals appears to be less pronounced than those that are leptin-deficient, suggesting that an unidentified leptin receptor might still exist (Farooqi et al. 2007). In addition, like leptin, leptin receptor mutations in humans are extremely rare and can only account for a handful of obesity cases (Gotoda et al. 1997). Taken together it appears that the leptin receptor is also a critical component involved in normal bodyweight regulation in both rodents and humans.

Even in the absence of ligand, the leptin receptor forms homodimers that undergo the necessary conformational changes that induce a signaling cascade only following the binding of leptin (Devos et al. 1997). Like other members of the cytokine class-1 receptor family, the leptin receptor does not contain intrinsic enzymatic activity and it is dependent on the recruitment and binding of Janus kinase 2 (JAK2) (Tartaglia et al. 1995; Bjorbaek et al. 1997; White et al. 1997). Following leptin binding and receptor activation JAK2 transphosphorylates various tyrosine residues located in its intracellular domain (Bjorbaek et al. 1997; Banks et al. 2000a). These phosphorylation sites serve as docking sites for other proteins, including Src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2), insulin receptor substrate (IRS), and signal transducer and activator of transcription 3 (STAT3) (Hekerman et al. 2005). Although leptin can activate ERK/MAPK and IRS signaling pathways in order to impact bodyweight (see Figure 6), its effects on STAT3 appear to have the greatest effect on central energy metabolism (Ghilardi et al. 1996; Gao et al. 2004; Munzberg et al. 2007). Thus STAT3 binds the intracellular domain of the leptin receptor following the phosphorylation of tyrosine 1138, leading to its subsequent phosphorylation and activation (Hekerman et al. 2005). Phosphorylated STAT-3 is then capable of forming homodimers and translocating to the nucleus where it mediates the expression of several genes including POMC and SOCS-
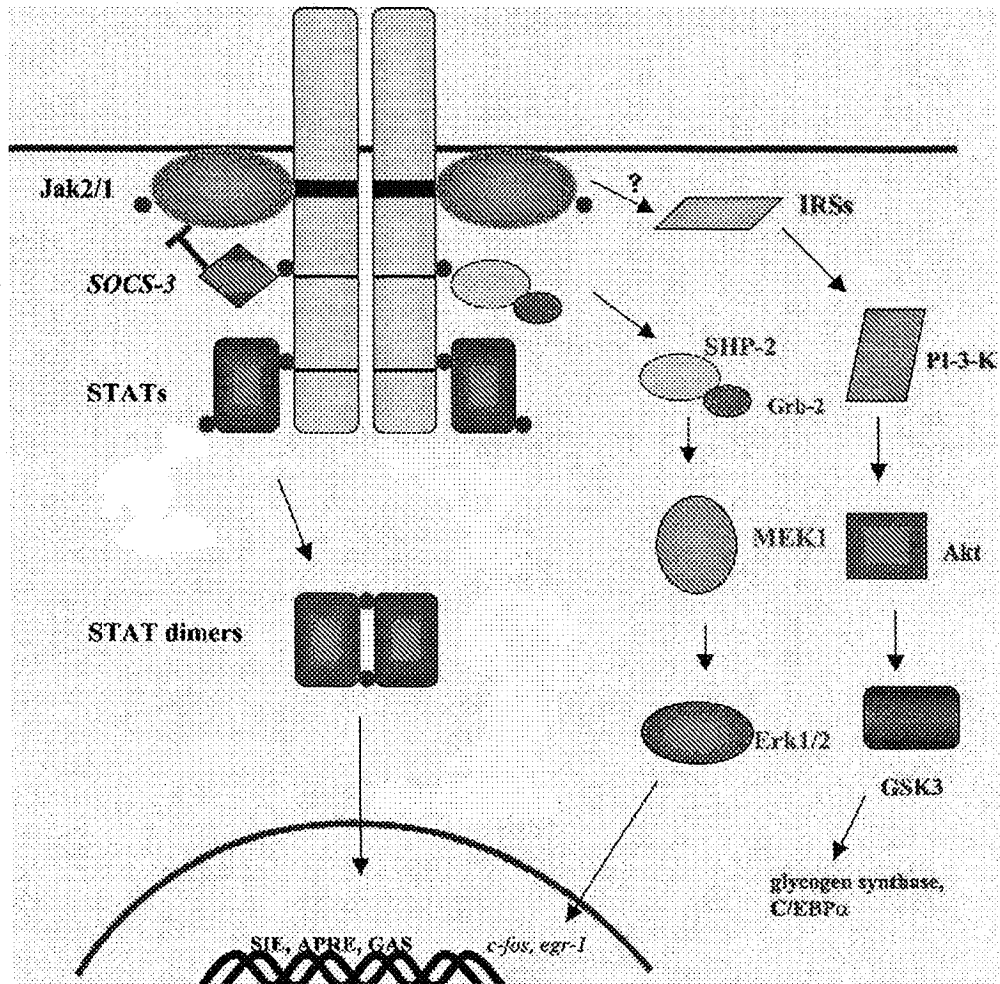
Figure 6: Leptin receptor signaling pathways
Leptin binding induces conformational changes that permit the association, transphosphorylation and activation of JAK2. This leads to the subsequent phosphorylation of tyrosine residues located in the intracellular domain of the receptor that act as docking sites for STAT-3. Once bound, STAT-3 is also activated by phosphorylation which permits its dimerization and nuclear translocations and leads to modifications in gene expression. Similarly the leptin receptor is capable of activating Src homology 2-containing phosphotyrosine phosphatase (SHP-2) which activates a MAPK pathway (i.e. MEK and Erk1/2). The leptin receptor can also induce the activation of an insulin-like signaling cascade (i.e. IRS and PI3-K). (Figure adapted from Hegyi et al. 2004)

3 (Banks et al. 2000a; Bates et al. 2003; Munzberg et al. 2003). Therefore leptin might

control energy homeostasis and bodyweight regulation by inducing multiple signaling

events.

Leptin And The Hypothalamic Metabolic Balance

As noted earlier leptin appears to act directly on the hypothalamus in order to

modify appetite and energy expenditure leading to reductions in bodyweight (see Figure

1) (Campfield et al. 1995). Therefore it was not unexpected when a high level of OBRb

gene expression, the functional leptin receptor, was detected in the hypothalamus of

various species including rats (Schwartz et al. 1996b), mice (Hakansson et al. 1996),

monkeys (Hotta et al. 1998) and humans (Burguera et al. 2000). Moreover, within the

hypothalamus both orexigenic (NPY/AGRP), and anorexigenic (POMC/CART) neurons

express the leptin receptor (Cheung et al. 1997; Baskin et al. 1999). Thus leptin is

capable of inhibiting NPY expression and blocking the depolarization of orexigenic

neurons (Schwartz et al. 1996b; Lee and Morris 1998), suggesting that it mediates at

least part of its actions via an NPY-dependent mechanism. However NPY-knockout mice

have normal bodyweights, and leptin treatment induced a similar bodyweight reduction

as that observed in control littermates, suggesting that leptin also reduces appetite via

other anorexigenic pathways (Erickson et al. 1996). It was subsequently determined that

POMC neurons also co express OBR (Cheung et al. 1997). However unlike NPY

neurons, leptin stimulates anorexigenic POMC neurons and appears to block the

inhibitory inputs from NPY neurons (Schwartz et al. 1997; Cowley et al. 2001). Thus

leptin differentially modulates the depolarization of orexigenic (NPY/AgRP) and

anorexigenic (POMC/CART) neuronal pathways in order to produce adaptive and

coordinated response to changes in adipose tissue and energy stores (recall Figure 1).

However even the slightest imbalances between the input from these opposing

pathways could have a profound impact on the efficiency of bodyweight regulation.

Obesity And Leptin Resistance

The weight lowering effects of leptin in leptin-deficient mice and humans was heralded as the answer to our obesity problem; give overweight people leptin so they eat less, burn more energy, and can achieve healthy bodyweights. In retrospect, it seems illogical to have expected such a simple therapy to cure one of the most complex medical problems facing modern medicine today. Frederich et al. (1995) provided the first evidence that diet-induced obesity (DIO) increased fat mass, and that this was positively correlated with plasma leptin concentrations (Frederich et al. 1995). Thus it appeared that DIO disrupted the lipostatic loop by inducing a state of leptin resistance in mice. In humans plasma leptin concentrations are also strongly correlated with body mass index (BMI) (Schwartz et al. 1996a). Likewise leptin concentrations in cerebrospinal fluid (CSF) are also correlated with adiposity, however the ratio of plasma:CSF leptin was lower in obese individuals (Schwartz et al. 1996a). It is worth noting that the diurnal variations in plasma leptin (25-fold) are much larger than those detected in CSF (2-fold) (Wong et al. 2004). Clinical studies also revealed that the self administration of leptin over a period of 4-24 weeks was ineffective at producing weight loss in obese individuals (Heymsfield et al. 1999). Equally disappointing results were obtained when the timing of leptin injection was varied between morning and night to correct for possible diurnal variations of leptin sensitivity (Zelissen et al. 2005). These data suggest that obesity is further exacerbated by a state of leptin resistance leading to a defective 'adipostat' which leads to further weight gain. There are several putative sites where leptin resistance can occur; at the blood brain barrier, poor receptor binding, or defective receptor signaling (Arch 2005). Although the site of leptin resistance is a contentious issue, it remains clear that finding ways to circumvent this barrier could prove to be an effective means to treat obesity.
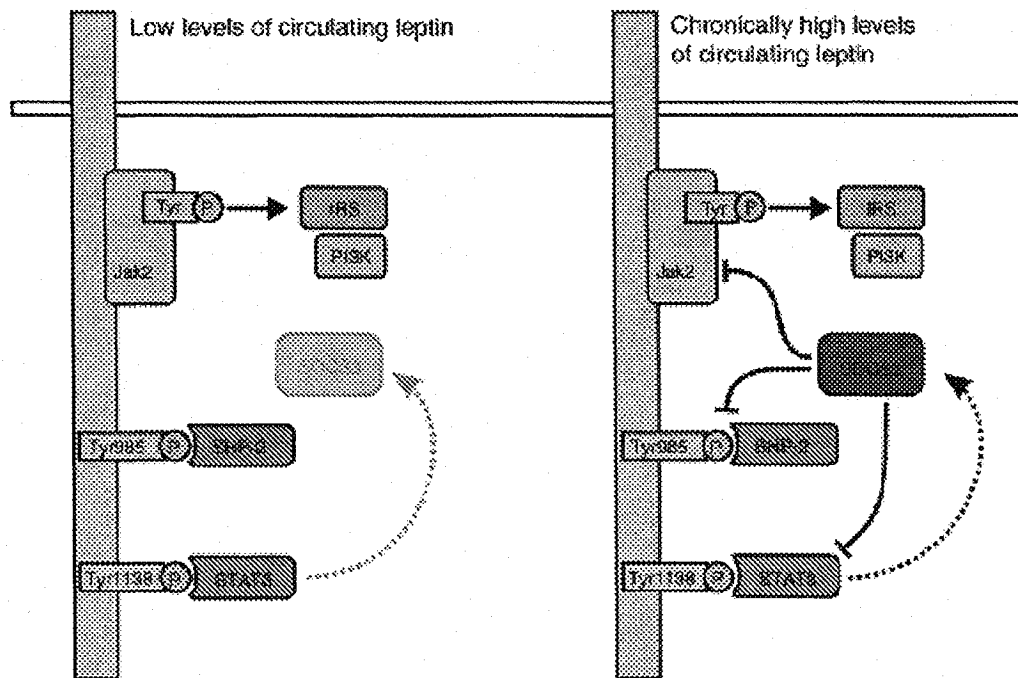
Figure 7: Intracellular leptin resistance
Under normal physiological conditions leptin acutely induces the expression of suppressor of cytokine signaling-3 (*socs-3*). SOCS-3 acts as an autoinhibitory peptide that acutely prevents further leptin receptor signaling by interfering with the interactions that normally occur between the receptor and the various signaling proteins (e.g. STAT-3, SHP-2). However in obesity, a hyperleptinemic state (right), chronic elevations in SOCS-3 appear to block leptin receptor signaling, leading to leptin resistance and an inability to properly control bodyweight. (Figure adapted from Munzberg and Myers 2005)

Defective leptin signaling is one of the most active areas of leptin resistance research. Two major targets have been identified thus far; suppressor of cytokine signaling-3 (SOCS-3) and protein tyrosine phosphatase 1b (PTP1B). SOCS-3 is an immediate early gene that is rapidly induced by signal transducer and activator of transcription 3 (STAT3) following leptin receptor activation. SOCS-3 is capable of binding to the intracellular domains of cytokine receptors, including the leptin receptor, and sequesters key areas that are normally bound by intracellular signaling molecules thus preventing further receptor activation (see Figure 7). Therefore SOCS-3 appears to function as an autoregulatory mechanism that prevents excessive leptin signaling. However in obesity, a state of hyperleptinemia, SOCS-3 expression is chronically elevated in the hypothalamus, more specifically in the ARC (Munzberg et al. 2004), where it appears to attenuate leptin responsiveness by blocking leptin signaling. In contrast SOCS-3 haploinsufficiency, and the neuronal- or POMC-specific deletion of SOCS-3 is highly protective against the development of obesity (Howard et al. 2004; Mori et al. 2004; Kievit et al. 2006). Similarly PTP1B was also found to be a negative regulator of leptin signaling in a hypothalamic cell line since it dephosphorylated JAK-2 in a dose-dependent manner, thus indirectly limiting the downstream phosphorylation and activation of STAT3, leading to ineffective leptin signaling (see above)(Kaszubska et al. 2002; Lund et al. 2005). In contrast, transfecting cells with a dominant negative form of PTP1B was capable of restoring normal leptin signal transduction (Lund et al. 2005). Thus SOCS-3 or PTP1B appear to be major intracellular targets involved in obesity-induced leptin resistance.

III. Adipokines And The Brain

Can Adipokines Cross The Blood Brain Barrier?

Our laboratory began the search for brain-derived adipokines because of our conviction that such large peptides would not readily cross the blood brain barrier (BBB)

to permit binding to their central receptors. In contrast to most tissues, brain capillaries form tight junctions, lack fenestrations, and are surrounded by astrocyte end feet to form the highly impermeable BBB. The unique structure of the BBB limits the transcapillary movement of blood-borne entities, thus protecting the brain against circulating factors such as cytokines and hormones (Fry et al. 2007). Although hydrophobic molecules can easily diffuse across the BBB, hydrophilic molecules including glucose, insulin and other peptides, require the aid of protein transporters (Fry et al. 2007). It was initially unclear how blood borne leptin could reach the ARC, or other key areas involved in bodyweight regulation. Leptin is currently thought to enter the brain via a transport mechanism that is partially saturable over a wide physiological range (Banks et al. 1996; Zlokovic et al. 2000; Kurrimbux et al. 2004; Ahima 2005; Banks 2006b). Although leptin could hypothetically gain access to the ARC by diffusing across the median eminence, a circumventricular organ (CVO) that possesses highly fenestrated capillaries, the data obtained from experiments using radioiodinated leptin failed to generate a diffusion gradient consistent with this model (Maness et al. 1998; Wilkinson et al. 2000; Fry et al. 2007). The issue of leptin transport is further complicated by experimental methods that may be inappropriate. For example, the brain perfusion technique reported by Banks et al. (2000) and Hileman et al. (2002) quantified tissue-bound [^{125}I] leptin as a measure of brain penetration; ie. radioactivity was quantified by direct counting of microdissected brain nuclei (Banks et al. 2000b; Hileman et al. 2002). However measuring total radioactivity fails to distinguish between leptin bound to brain cells, and that which is bound to brain capillaries (Kastin and Pan 2000). Even the more sophisticated technique of brain perfusion with an oxygenated artificial plasma (Kurrimbux et al. 2004) cannot fully represent the situation in the intact animal, since serum leptin is known to form complexes with leptin binding protein (Sinha et al. 1996). This complex of bound leptin prevents leptin from efficiently binding its receptors (Zastrow et al. 2003) and likely

prevents the uptake of leptin. Therefore the brain perfusion technique likely overestimates the amount of leptin crossing into the brain. Doubts also remain as to whether leptin can enter the brain to target neurons directly, or if circulating leptin stimulates the release of anorexigenic cytokines from the BBB that subsequently modify the output from hypothalamic metabolic pathways (Rivest 2002). For example it was recently shown that the overexpression of the interleukin-1 receptor antagonist (IL-1ra) in the hypothalamus blocked the anorectic effects of peripherally administered leptin in rats suggesting that leptin might indirectly mediate its anorectic effects via an IL-1 dependent mechanism (Wisse et al. 2007). It is also plausible that leptin receptors localized in brain microvessels could serve as a 'metabolic' BBB by facilitating the degradation and blocking the entry of leptin into the brain (Wilkinson et al. 2000).

A further troubling issue is the lack of evidence showing that peripherally-derived leptin can directly bind to extra-hypothalamic leptin receptors in order to modify signaling pathways (Rivest 2002; Ahima et al. 2006). For example, we predicted that peripheral injection of leptin should increase *c-fos* expression in basal hypothalamus but not, for example, in hippocampus and cerebral cortex. This is indeed the case, since leptin-induced *c-fos* expression was only observed in the basal hypothalamus (Wilkinson et al. 2000) and these data are largely in agreement with other reports [see eg.,(Kelly et al. 2004)]. However, a *lack* of *c-fos* expression does not necessarily imply an absence of leptin-induced cell activation (Elias et al. 2000). Equally, *c-fos* may not be expressed in all cells that have leptin receptors. Leptin target neurons in brain contain STAT- 3-ir (immunoreactivity) (Hakansson and Meister 1998), and leptin injection activates (i.e. phosphorylates) STAT- 3 in hypothalamus (Vaisse et al. 1996). Our experiments showed that leptin increases STAT-3-ir in hypothalamus but not in hippocampus, cerebral cortex or substantia nigra (see also (Hosoi et al. 2002)), despite STAT-3 being expressed in these brain regions (Stromberg et al. 2000). It could be that these brain regions possess

unique leptin signaling pathways since it was recently reported that peripheral leptin led to increases in phosphorylated ERK1/2, but not STAT-3, in neonatal rats (Walker et al. 2007). However neonatal rats, like neonatal mice, probably lack a fully formed BBB until the third week of life so leptin should be able to reach those brain regions that might otherwise be protected by the BBB in mature rodents (Vorbrodt et al. 1990). In total these data fail to demonstrate whether leptin can cross the intact BBB, especially in extra-hypothalamic brain regions. However there is new evidence that peripheral injection of leptin can facilitate learning and memory performance and long term potentiation (LTP) in mice (Oomura et al. 2006). However it remains uncertain whether the effects of leptin on learning applies to other species, especially since leptin deficient humans have not been reported to display any obvious cognitive defects, and no noticeable improvements were detected following leptin treatment (Montague et al. 1997; Strobel et al. 1998; Licinio et al. 2004).

The detection of leptin in cerebrospinal fluid (CSF) suggests that leptin might reach brain cells from peripheral circulation. However, and as noted for the blood-brain barrier, the transport mechanism of leptin from the blood to CSF is also saturated at physiological concentrations (Wong et al. 2004). Furthermore the concentration of leptin in CSF, at normal plasma levels, appears to be too low to activate the long form of the leptin receptor. Cerebrospinal fluid concentrations of leptin (approx. 1 ng/ml; 0.06 nmol/L; (Wong et al. 2004; Adam et al. 2006)) are significantly below the K_D value of 0.3 nmol/L for the leptin receptor (Uotani et al. 1999). So even if leptin were able to cross the BBB, it appears that it would need to be supplemented with brain-derived leptin, in order to activate central leptin receptors.

Another confounding issue is whether leptin is in fact secreted from the brain. When radioiodinated leptin was injected into the lateral ventricle (LV) of mice there was a detectable increase of radioiodinated leptin in blood which was thought to occur via the

reabsorption of cerebrospinal fluid (CSF) (Maness et al. 1998). Clinical studies also revealed a net efflux of leptin from the human brain, as determined using arterio-venous blood sampling, which was positively correlated with obesity in men (Esler et al. 1998; Wiesner et al. 1999). Moreover women excreted significantly more leptin from their brains than men, and was estimated to contribute upwards of 40% of total serum leptin in lean women, compared with only 13% in lean men (Wiesner et al. 1999). Although lean rats do not appear to secrete significant amounts of leptin from the principle site of brain venous drainage, the superior saggital sinus, these findings recapitulate those reported for healthy men (Reichlin 1999; Wiesner et al. 1999). Perhaps studying rats made obese by feeding a high fat diet would reveal a significant leptin efflux from the brain. Moreover leptin gene expression in the human hypothalamus appears to correlate with the amount of leptin released; leptin excretion was significantly reduced in patients diagnosed with depression and leptin mRNA was undetectable by RT-PCR from samples obtained from cadavers with a similar mental history (Eikelis et al. 2006). In contrast the excretion of brain leptin was elevated in obese subjects, and this was associated with increased hypothalamic leptin gene expression (Eikelis et al. 2007). Thus it appears that the brain is not only an extra-adipocyte source of leptin, but leptin is excreted in the direction of brain to blood and appears to contribute to the total pool of circulating leptin.

Compared to leptin, the case for a brain-derived adiponectin is much stronger: (a) adiponectin does not cross the blood-brain barrier (Pan et al. 2006; Spranger et al. 2006); (b) adiponectin receptors are expressed in brain tissue (Yamauchi et al. 2003; Qi et al. 2004). Therefore the brain has to express adiponectin, or an adiponectin mimetic, in order to activate these central receptors. The former seemed more plausible given that adiponectin mRNA is readily measured in the chicken brain (Maddineni et al. 2005) and we have since confirmed the presence of adiponectin mRNA in rat and mouse brain

by realtime RT-PCR (Wilkinson et al. 2007). Moreover adiponectin and resistin were recently detected in human CSF, although concentrations were 1000- and 100-fold lower than in serum, respectively (Kos et al. 2007). This suggests that the human brain might synthesize other adipokines, in addition to leptin (see below). Like leptin, brain-derived adiponectin requires further study as a putative neuromodulator, particularly since the icv injection of adiponectin reduced bodyweight and stimulated c-fos expression in the mouse brain (Qi et al. 2004). There is also good evidence suggesting that resistin and fiaf are expressed in the CNS, as outlined below.

In summary, we do not contest the fact that limited amounts of leptin do cross the blood brain barrier and gain access to the brain (Wilkinson et al. 2007), however the evidence suggesting there is a net efflux of leptin from the brain must also be considered (Esler et al. 1998; Wiesner et al. 1999; Eikelis et al. 2006; Eikelis et al. 2007). The lack of evidence demonstrating central signalling by peripheral leptin in areas ouside the hypothalamus (Rivest 2002), is puzzling. It is possible that the mouse brain, which does not appear to express leptin, may have a leakier BBB than other species. However in most other species it seems possible that an equilibrium relationship exists between brain and serum leptin pools since leptin appears to cross the BBB bidirectionally from brain to blood, and vice versa. Moreover such a system could help account for the failure of peripherally administered leptin to reduce bodyweight in several clinical trials (Heymsfield et al. 1999; Bell-Anderson and Bryson 2004; Zelissen et al. 2005), whereas leptin-deficient children and adults are highly responsive to leptin treatment (Licinio et al. 2004; Farooqi and O'Rahilly 2005). Such an equilibrium relationship is an extension of Le Chatelier's principle; in the leptin-deficient brain there is no opposing force, or leptin efflux, that would prevent leptin's entry into the brain. In contrast, non-leptin deficient obese individuals appear to have elevated hypothalamic leptin gene expression (Eikelis et al. 2007), therefore the increased endogenous production of brain leptin would be

expected to prevent the uptake of circulating leptin, and instead favor leptin efflux. However it is also possible that the effects of exogenous leptin in leptin–deficiency might be due to compensational changes, or modified leptin receptor signaling.

Adipokines Are Expressed In The Brain

The seminal studies of Pearse and coworkers (Bryant et al. 1976), and others (Dockray 1988), proposed the now readily accepted view that multiple neuropeptides are common to gut and brain. It followed that peptides found in peripheral, eg. gastrointestinal or respiratory, systems could reasonably be predicted to be biosynthesized in the brain and to exert physiological effects there (Polak and Bloom 1986). Ghrelin is the most recent example of this principle. First isolated from the stomach, ghrelin is now known to be expressed in the brain as well (Sato et al. 2005b). With these precedents in mind it is remarkable that adipose-derived peptide hormones such as leptin and resistin have attracted little attention as putative central neurotransmitters or neuromodulators. Substantial evidence has been produced showing that several of these so-called adipose-specific hormones are also expressed in the brain and pituitary gland of numerous species, including man. However it remains unclear whether brain-derived adipokines are of adequate abundance to have any physiological impact on neuroendocrine function.

In the case of leptin, its receptors were reported early on to be widely distributed in the rodent and human brain (Elmquist et al. 1998; Burguera et al. 2000; Funahashi et al. 2003) and pituitary (Cai and Hyde 1999; Jin et al. 1999). The abundance of leptin receptors in brain regions such as cerebellum, hippocampus and cerebral cortex suggested that leptin probably subserves functions in the CNS in addition to, and distinct from, those that control energy homeostasis. Since leptin is a large peptide (16KDa) that may not readily enter the brain, it was hypothesized that many of these receptors, with the exception of those in the basal hypothalamus, would be accessible only to a brain-

derived leptin mimetic or ligand, if not leptin of brain origin. Thus it was subsequently

demonstrated that the rat brain did indeed express leptin mRNA in several distinct

regions (Morash et al. 1999; Wilkinson et al. 2000). Moreover leptin mRNA levels were

enriched in the arcuate nucleus, and expression was regulated by fasting and refeeding

(Wilkinson et al. 2007). Leptin mRNA has since been detected in the brain of many

species, including human, sheep, pig and fish, and in human neuroblastoma and rat

glioblastoma cells (see Table 1). Moreover, as noted already, in vivo investigations by

Esler and coworkers demonstrated that leptin was secreted from the human brain (Esler

et al. 1998; Wiesner et al. 1999; Eikelis and Esler 2005). Intriguingly there have been no

reports of whether the monkey brain is also a locus of leptin expression. The mouse is a

unique case since leptin mRNA was undetectable by Northern blot analysis in whole

brain (Zhang et al. 1994), and we were unable to detect it by RT-PCR in discrete brain

regions, including the arcuate nucleus (Wilkinson et al. 2007). The lack of success in

detecting leptin mRNA in mouse brain has profoundly influenced current thinking of the

physiology of leptin. At present we do not know why leptin expression appears to be

suppressed in the brain of mice. However, an early report by Bennett et al (1996)

included data on leptin expression in C57BL6 mouse *fetal* brain (Bennett et al. 1996).

We were unable to reproduce this in the CD-1 mouse, but we did obtain a clear signal in

neonatal brain, suggesting that the unusual expression of leptin in mouse brain might

occur in a strain- and developmental-dependent fashion (Brown et al; unpublished). This

may reflect the unique energy regulation mechanisms in the mouse detailed by Himms-

Hagen (Himms-Hagen 1999) and by Arner (Arner 2005). Thus all of the central effects of

leptin are assumed to result from circulating peripheral leptin entering the brain, via a

saturable transport mechanism, and binding to leptin receptors. This widely accepted

view is almost solely based on mouse data and neglects the fact that the central

expression of leptin has been detected in several other species, most notably humans.

Table 1. Leptin Gene Expression in Brain

Species	References

Rat	(Morash et al. 1999)
	(Beretta et al. 2002)
	- C6 glioblastoma: (Morash et al. 2000)
	    (Li et al. 2001)
	    (Brown et al. 2005a)

Sheep	(Ehrhardt et al. 2002)
Pig	(Smolinska et al. 2004) (Kaminski et al. 2006)
Mouse	*No expression*: (Zhang et al. 1994)
Monkey	Not known
Human	(Knerr et al. 2001) (Eikelis and Esler 2005) - neuroblastoma cells: (Russo et al. 2004)
Tiger Salamander	(Boswell et al. 2006)
Triturus tadpole	(Buono and Putti 2004)

Moreover the expression of brain-derived leptin suggests it might have neurotransmitter, neuromodulator or neurotrophic properties.

A Dual Adipokine System?

Our evidence that brain cells are also a source of leptin raises an interesting question: why would circulating leptin, and other adipokines, need to gain access to brain regions where they are already expressed? Such a dual system is not without precedent. There are several well-described examples of neurotransmitter/hormonal dualisms. For example catecholamines are synthesized as neurotransmitters within the brain, and as hormones in the periphery, as are gastrointestinal hormones such as somatostatin and cholecystokinin (CCK). The controversy does not apply only to leptin. Pan et al.(2005) reported that " the central nervous system effects of peripheral growth hormone can be attributed to the permeation of the BBB…"(Pan et al. 2005), a claim refuted by the demonstration of growth hormone gene expression, and regulation, in the hippocampus (Donahue et al. 2006). Our evidence, that adipokines are not produced exclusively by adipocytes, suggests they might also be part of a CNS system that operates independently, or in conjunction with, the peripheral systems. This is not to discount the well-described central effects of circulating adipokines (Ahima 2005; Ahima et al. 2006). It is possible that endogenous brain adipokines might modulate the feedback effects of adipose hormone signals originating in the periphery or may provide the brain with an independent adipokine circuitry. For example, leptin-deficient, or leptin insensitive, rodents have impaired hippocampal function (Li et al. 2002). Similarly the direct injection of leptin into the hippocampus improves memory performance in mice and can facilitate long-term potentiation (LTP) via NMDA receptors (Farr et al. 2006; Harvey et al. 2006). There is also new evidence that peripheral injection of leptin can facilitate learning and memory performance (Oomura et al. 2006). However it makes little sense that human cognitive performance should be dependent on the secretion of

leptin from adipose tissue. Plasma leptin concentrations vary widely (e.g. after meals) and may be chronically high in obese individuals, or very low in periods of prolonged food deprivation. A more considered view is that leptin made within the hippocampus, in close proximity to leptin receptors, is responsible for its effects on cognition. On the other hand, in aging obese and diabetic patients, the brain might be more sensitive to changing leptin secretion (Elias et al. 2005). This paradox, of why a leptin target tissue should also be capable of expressing leptin, is demonstrable in the pituitary gland as well (Morash et al. 1999; Lloyd et al. 2001). Here however the pituitary is not protected by the blood brain barrier and is chronically bathed in blood-borne leptin. In this case our hypothesis is that pituitary-derived leptin might 'tune' the leptin signaling pathways to incoming leptin signals from adipose tissue (Wilkinson et al. 2007). In addition, pituitary leptin expression might be maintained under conditions of starvation-induced minimal leptin secretion from adipose tissue.

Development Of Hypothalamic Leptin-Sensing Metabolic Pathways

Neonatal rodents appear fully immune to the anorectic effects of leptin for a period of a couple weeks after birth (Mistry et al. 1999; Proulx et al. 2002). This is not unexpected since the leptin-associated neuronal pathways responsible for the anorectic actions of leptin are not fully intact in rodents at birth (Bouret et al. 2004b). However note that leptin is not totally without effect in the neonatal brain, and is still capable of influencing the expression of neuropeptides associated with appetite and metabolism. For example treating young rats (postnatal day 10; PD10) with leptin reduced NPY mRNA, and stimulated POMC gene expression in the hypothalamus, but failed to modify food intake (Proulx et al. 2002). Although leptin is thought to exert most of its metabolic actions via the ARC nucleus of the hypothalamus, the anorectic actions are highly dependent on projections that originate in the ARC and branch out to other regions of
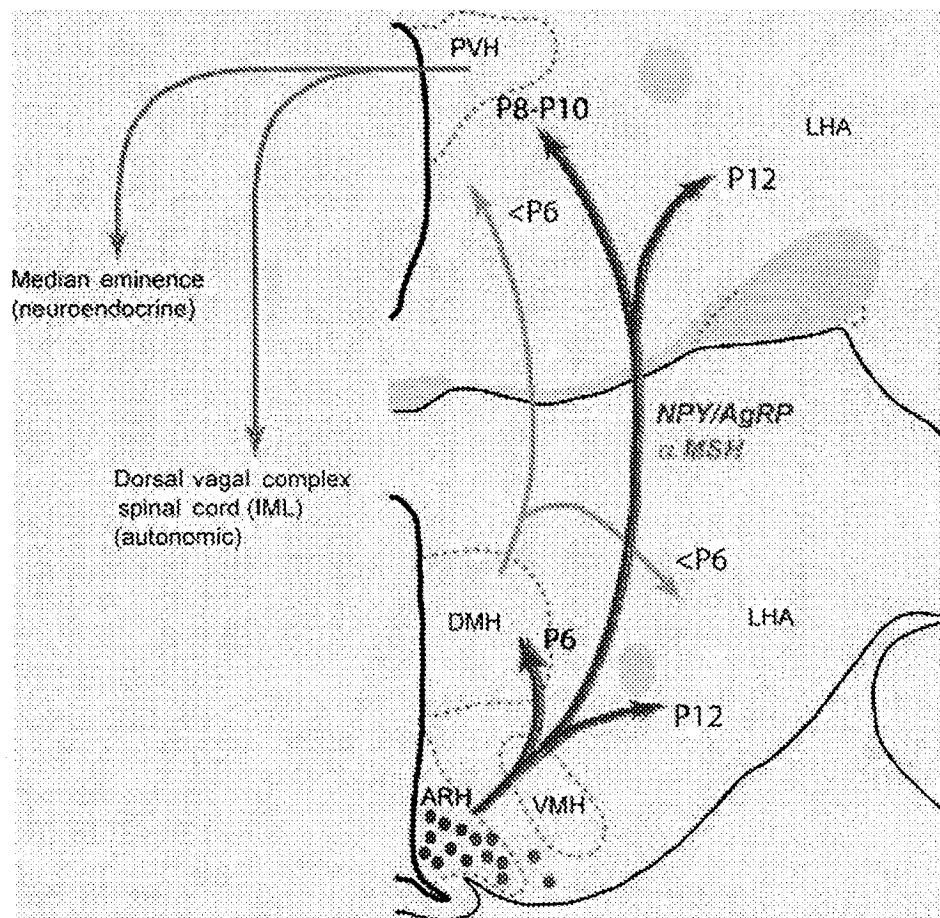
Figure 8: Development of hypothalamic metabolic pathways
Hypothalamic neuronal projections implicated in the control of appetite
and metabolism only develop after birth in rodents. As seen here,
neuronal fibres associated with the anorexigenic effects of leptin project
from the ARC (denoted here as ARH) and begin to innervate other
hypothalamic nuclei between postnatal day 6 (denoted here as P6) and
P12. These projections appear to be absent in the leptin-deficient mouse
(ob/ob), but can be restored with neonatal leptin treatment. However
treating adult ob/ob mice with leptin failed to correct these deficiencies.
Thus leptin appears to act as a trophic factor and facilitates the
development of these critical projections in the immature CNS. (Figure
adapted from Bouret and Simerly 2006)

the hypothalamus, including the LH and PVN, which have also been implicated in the control of appetite (see Figure 8) (Bouret et al. 2004b; Bouret and Simerly 2006). Studies

in mice determined that leptin begins to impose metabolic changes around PD17, and its appetite lowering effects appeared around PD28 (Mistry et al. 1999). This appears somewhat consistent with the timing of neuronal innervation of the LH and PVN from neurons originating in the ARC. Therefore the proper development and organization of these hypothalamic metabolic pathways is a prerequisite for leptin to have any metabolic effect.

In addition to having smaller brains and reduced cell number (Steppan and Swick 1999), the neuroanatomical organization of the ob/ob mouse hypothalamus is significantly altered from that of their lean littermates (Bereiter and Jeanrenaud 1980). Bouret et al.(2004) demonstrated, using the neuronal tracer 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), that there was a remarkable reduction in the number of ARC projections innervating the PVN in the ob/ob mouse, relative to their lean littermates. Although the neurochemical nature of these projections is unknown, they indicate that the feeding circuits of ob/ob mice are poorly developed (Bouret et al. 2004b). In fact daily leptin treatment between PD4 and PD12 induced a significant, but incomplete, restoration of hypothalamic projections reaching the PVN in ob/ob mice (Bouret et al. 2004b). Similarly treating neonatal hypothalamic organotypic cultures with leptin induced a nearly 2-fold increase in neurite outgrowth (Bouret et al. 2004b). In contrast, chronically treating adult ob/ob mice with leptin had little, if any effect, on the neuroanatomical organization of the hypothalamus (Bouret et al. 2004b). However leptin was shown to modify synaptic inputs onto NPY and POMC neurons in adult ob/ob mice that would favor weight loss. In brief treating mature ob/ob mice with leptin decreased the number of stimulatory inputs on NPY neurons, and increased the number of inputs
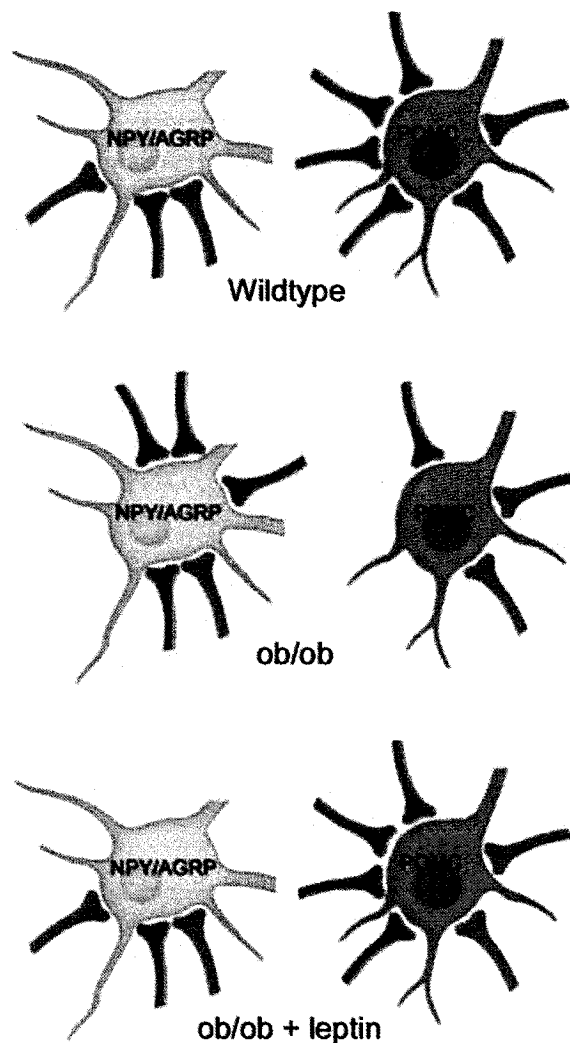
Figure 9: Leptin and hypothalamic synaptic plasticity
In wildtype mice there are an increased number of stimulatory inputs on POMC neurons (red) and a decreased number of inhibitory inputs (blue), relative to those on NPY expressing neurons. In contrast, in the leptin deficient mouse (ob/ob) there are an increased number of stimulatory inputs on the orexigenic neurons (NPY), suggesting that the hypothalamic energy balance is favoring weight gain. However treating adult ob/ob mice with leptin rewired the hypothalamus and resulted in an increased number of stimulatory inputs on POMC neurons, much like wildtype mice. Thus leptin is also implicated in hypothalamic synaptic plasticity and suggests an alternate means through which leptin can modify the hypothalamic energy balance. (Figure adapted from Pinto et al. 2004)

on POMC neurons, thus favouring anorexigenic metabolic pathways (see Figure 9)(Pinto et al. 2004). Leptin was also shown to modify synaptic plasticity in rat hippocampal slices (Shanley et al. 2001),and modified the expression of synaptic proteins, such as syntaxin-1, synaptosomal associated protein-25 (SNAP-25) and Synapsin 2A in adult mice or neonatal rats (PD 10) (Ahima et al. 1999; Walker et al. 2004a). These studies provided two major findings: a) leptin is involved in the formation of hypothalamic neuronal projections, and b) there is a developmental window in which leptin has a profound impact on the architecture of hypothalamic feeding circuits.

Purpose Of The Neonatal Leptin Surge

In humans and rodents there is a well documented association between maternal nutrition and metabolic consequences in their offspring (Myers et al. 2005). Maternal over- or under- nourishment increases the risk for their offspring to become obese adults. This could be mediated in part by the neonatal leptin surge, that has been hypothesized to influence the functioning and development of the neuroendocrine axis (Ahima et al. 1998; Morash et al. 2001a; Walker et al. 2004a). Moreover neonatal leptin treatment induced metabolic changes that manifested later in life. For example Yura et al. (2005) established that fetal under nutrition induces a premature leptin surge that appears to predispose mice to weight gain later in life (Yura et al. 2005). Similarly artificially inducing a premature leptin surge in otherwise healthy mice induced similar metabolic changes, suggesting that the timing of the leptin surge is a critical event in postnatal development (Yura et al. 2005). Likewise feeding lactating dams a high fat diet induced a premature spike in neonatal leptin in rat pups, which was associated with an altered stress response and increased percentage of body fat (Trottier et al. 1998). Although the effects on body composition were no longer detected 15 days after weaning, the altered stress response still remained (Trottier et al. 1998). Similarly leptin appears to enhance glucocorticoid feedback signals in neonatal rats by acting both on

the brain and adrenal glands, suggesting that the increases in neonatal leptin concentrations might be part of the inhibitory mechanism that suppresses the hypothalamic-pituitary-adrenal axis shortly after birth (Proulx et al. 2001; Walker et al. 2004a; Walker et al. 2004b). As with mice, treating neonatal rats with leptin also resulted in profound metabolic changes that became increasingly apparent as they matured (Varma et al. 2004). However rats exposed to high neonatal leptin ate less and had reduced body mass relative to controls, which is the opposite of what was reported in mice (Varma et al. 2004). These rats also suffered from hypercortisonemia, hyperinsulinemia, hyperglycemia and were glucose intolerant and these effects were at least partially attributed to the induction of a lipoatrophic-like state (Varma et al. 2004). Conversely rat offspring of food restricted dams are predisposed to the development of metabolic syndrome as adults, and this was reversible by leptin treatment (Vickers et al. 2005). There is a clear association in humans between birth weight, either high or low, and the future risk of becoming obese (McMillen et al. 2006). Although children do not exhibit a clear leptin surge, leptin levels spike in cord blood during late gestation (Jaquet et al. 1998), and could be impacting brain development and the neuroanatomical organization of the human brain prior to birth. For example the development of human hypothalamic feeding circuits appears to coincide with the increased leptin concentrations measured during the final gestational trimester (Koutcherov et al. 2003). Thus the timing of the leptin surge and development of hypothalamic feeding circuits in humans appears similar to that in rats and mice, except that in humans these events are occurring in utero (Ahima et al. 1998; Bouret et al. 2004b). Similarly leptin might indirectly impact brain development by lowering glucocorticoid concentrations (Walker et al. 2004a). Considering the developmental consequences of leptin in the immature hypothalamus, it seems likely that modulating the timing and magnitude of the neonatal leptin surge could have a significant impact on the development of metabolic pathways

and bodyweight 'set points', thus influencing our propensity to excessive weight gain later in life.

Detecting The Expression Of Other Adipokines In The Brain

As previously stated, multiple adipokines are expressed in the brain and pituitary including leptin, resistin, FIAF and adiponectin. Thus it seems likely that there are many others (Wilkinson et al. 2007). The first evidence for the existence of brain-derived adipokines came from rats which were found to express leptin mRNA in numerous brain sites including the cerebral cortex, cerebellum, hypothalamus, pineal gland, retina, and in the anterior and posterior pituitary (Morash et al. 1999). Leptin was also detected in the rat C6 glioblastoma cell line, and these results were confirmed using two sets of intron-spanning primers (217bp and 495bp) (Morash et al. 2000). As outlined in Table 1 leptin gene expression has been detected in multiple species, with the notable exception of mice. Resistin (*rstn*) and fasting-induced adipose factor (*fiaf*) mRNA were also detected in both the mouse and rat brain, further supporting the hypothesis that the brain is a locus of adipokine gene expression (Wilkinson et al. 2007). As with leptin in the rat brain, *rstn* mRNA was detected throughout the brain of 2 separate mouse strains (C57BL6 and CD-1) using two different sets of intron spanning primers (278 and 330 bp) (Morash et al. 2002). Moreover *rstn* expression was compared in saline perfused and non-perfused tissues to exclude the possibility that blood cells were a contaminating source of *rstn* mRNA, since *rstn* expression has also been detected in human monocytes (Savage et al. 2001). We have since detected *rstn* mRNA in the rat cortex, hypothalamus and pituitary using realtime RT-PCR (Wilkinson et al. 2007). Similarly the expression of *fiaf*, an adipokine that was also identified as angiopoietin-like protein 4 (ANGPTL4) or PPARgamma angiopoietin related (PGAR) (Kim et al. 1999; Kersten et al. 2000; Yoon et al. 2000), was readily detected in the mouse brain and pituitary using RT-PCR (Wiesner et al. 2004). FIAF gene expression has also been reported in human

glioblastoma and oligodendrocyte tissue (Le Jan et al. 2003), in a human glioblastoma cell line (Lal et al. 2001), and again I have confirmed that *fiaf* mRNA is readily detectable in the rat brain using realtime RT-PCR (see Section VI, Chapter 2). It seems likely that several other adipokines may also be expressed in the brains of numerous species where they could be involved in the normal functioning and maintenance of the CNS.

It is obvious from Table1 that the brain of most, if not all, species is capable of expressing the leptin gene. However, as noted above, the mouse appears to be a highly significant exception. Following the discovery of leptin (Zhang et al. 1994), the absence of detectable leptin mRNA in the mouse brain became the accepted view. Thus '...little, if any (leptin) is produced in the CNS..."(Ahima 2005). However it appears that the mouse is an anomaly since this is clearly not the case for most species. Although the expression of brain-derived adipokines, such as leptin and resistin, is relatively weak this alone should not exclude their possible involvement in brain function (Sahu 2003; Steppan and Lazar 2004). As a general princippe, low mRNA abundance does not equate to lack of function. For example leptin receptor mRNA is undetectable by in situ hybridization in neurons that have high levels of receptor protein immunoreactivity (Elmquist et al. 1998; Hakansson et al. 1998). Similarly NT4 (neurotrophin 4) and NGF (nerve growth factor) messenger RNA, genes that are indisputably essential for developmental and adult brain plasticity, are below the level of detection via in situ hybridization (Patz and Wahle 2006). However NGF mRNA was readily quantified by RT-PCR (36 cycles) in cerebral cortex (Patz and Wahle 2006). The number of cycles required to detect leptin gene expression in the rat brain, using semi-quantitative RT-PCR, varied greatly between the various tissues (pituitary, 34 cycles; cortex, 36 cycles; hypothalamus, 39 cycles; fat, 25 cycles) (Morash et al. 2001a). Similar threshold cycle (C_T) numbers were obtained for the detection of leptin using realtime RT-PCR (pituitary, 34 cycles; cortex, 36 cycles; hypothalamus, 38 cycles; fat, 20 cycles) (Wilkinson et al.

2007). The low abundance of central leptin mRNA appears very similar to NT4 and NGF, transcripts that could only detected in the brain using similar RT-PCR conditions. Similarly leptin immunoreactivity is readily visualized in those brain areas where leptin mRNA was detected (Morash et al. 2002), much like what was observed for NGF. Therefore the colocalization of leptin mRNA and immunoreactivity suggests that brain-derived leptin is acting in an autocrine, intracrine or paracrine manner and is regulated in a similar mode to other brain-derived factors (i.e. NT4 and NGF). Although these experiments clearly demonstrate that the brain is a site of adipokine expression, they are inadequate to establish any biological purpose or function.

IV. Role For Brain-Derived Adipokines

Establishing Physiological Roles For Brain-Derived Adipokines

One approach to define a physiological or pathological role for brain leptin is to specifically disrupt or silence central leptin gene expression. The generation of knockout (KO) animals is considered the gold standard in establishing gene function since ablating the expression of target genes permits the identification and study of its potential physiological roles, or changes, that result when it is effectively absent in a whole organism (Davey and MacLean 2006). However these technologies are not widely available to the majority of researchers since they are expensive and require specialized facilities (Wells and Carter 2001; Davey and MacLean 2006). In addition the generation of KO animals is a cumbersome process, and the maintenance of these animals can be equally challenging. Furthermore, this technology is limited to animals from which embryonic stem cells can be readily harvested and that can also undergo the process of homologous recombination (Charreau et al. 1996; Wells and Carter 2001). Thus these prerequisites exclude the use of other common animal models (i.e. rats) and human cell lines that do not undergo this process of genetic rearrangement. In contrast 'knockdown' technologies, such as the use of small interfering RNA (siRNA) or antisense

oligodeoxynucleotides (AS ODN), offer the advantage of transiently silencing gene expression and permits studies on gene function at different developmental points, such as investigating the role of leptin at different points during growth. The recent emergence of RNA interference (RNAi) has permitted researchers to rapidly knockdown target gene expression both in vitro and in vivo in numerous cell lines and tissues, including the CNS (Elbashir et al. 2001a; Xia et al. 2002; Davidson and Paulson 2004). For example the continuous infusion of siRNA into the lateral ventricle (LV) was shown to induce the specific and wide spread knockdown of the dopamine (DAT) and serotonin transporters (SERT) in the CNS of mice (Thakker et al. 2005; Hoyer et al. 2006). Even more impressive is that the RNAi knockdown approach induced an even more pronounced phenotype than animals treated with drugs antagonizing DAT or SERT (Thakker et al. 2005; Hoyer et al. 2006). Therefore the use of knockdown technologies, such as antisense approaches or RNA interference (RNAi), appears to be a highly feasible approach for studying brain-derived adipokines.

Why RNAi Over Other Technologies?

Given the relatively low cost, and efficiency of generating a successful knockdown, it should come as no surprise that RNAi was bestowed as 'the technology of the year' in 2002 by the journal Science (Couzin 2002), and resulted in a Nobel prize in Physiology or Medicine for the co-discoverers Andrew Fire and Craig Mello because of its huge potential to advance medical research (Bernards 2006). Although AS ODN are also capable of attenuating gene expression in vitro and in vivo, only 1 in 8 designed AS ODN are truly effective at inhibiting gene expression (Stein 2001). Moreover in the absence of a chemically modified phosphorothioate backbone, which greatly increases the cost of synthesis, AS ODN are highly susceptible to cleavage by endogenous cellular nucleases (Stein et al. 1988). In contrast there is a 80-90% chance of identifying an effective siRNA, synthesizing siRNA molecules is cheaper, and siRNA duplexes are

more stable than AS ODN molecules (Harborth et al. 2001; Bertrand et al. 2002).

Moreover when the silencing efficiency of siRNA and AS ODN molecules was compared

directly, the siRNA consistently achieved a greater reduction in target gene expression

both in vitro and in vivo, which is likely due to the increased stability of the siRNA duplex

(Bertrand et al. 2002). In total, the advantages of RNAi technologies outweighed

alternative approaches of silencing target gene expression and represented a suitable

means to study the role of brain-derived adipokines in the various animal (mouse and

rat) and cell line models (rat C6 glioblastoma and mouse 3T3-L1 adipocytes or N-1

hypothalamic neurons) that will be used in these studies.

Do Adipogenic Transcription Factors Also Control The Expression Of Brain-Derived Adipokines?

Adipogenesis is a highly regulated and coordinated series of events that involves

the differentiation of fibroblast cells into adipocytes by inducing the expression of several

genes (Green and Kehinde 1975; Cowherd et al. 1999). This is largely attributed to two

main groups of transcription factors; the CCAAT enhancer binding protein (C EBP) and

peroxisome proliferator activated receptor (PPAR) families (Cowherd et al. 1999).

CCAAT enhancer binding protein alpha (CEBPα) and peroxisome proliferator activated

receptor gamma (PPARγ) are two notable members that are known to mediate the

transcription of several adipokine genes. For example resistin promoter analysis

revealed several transcriptional start sites for CEBPα, and the overexpression of CEBPα

in non-adipose tissues was capable of increasing resistin gene expression (Hartman et

al. 2002). Likewise the expression of FIAF, which was also identified as PPARgamma

angiopoietin related (PGAR) peptide, is potently induced by PPARγ ligands including the

thiazolidinedione (TZD) drug family that includes rosiglitazone and pioglitazone (Yoon et

al. 2000). Given the putative roles of adipokines in glucose and lipid metabolism

(Steppan and Lazar 2004; Kersten 2005; Ahima et al. 2006), it is not surprising that

many of these adipogenic transcription factors are exploited as pharmaceutical targets to treat diabetes and obesity-associated conditions. However these 'adipogenic' transcription factor families are also known to be expressed in several non-adipose tissue sites including the brain and pituitary (Williams et al. 1991; Wiesner et al. 2004; Bogazzi et al. 2005). Thus it is not really surprising that the central nervous system is also a locus of adipokine expression. However it remains unclear to what extent these adipogenic transcription factors are regulating brain-derived adipokines, if at all. For example the PPARγ ligand, rosiglitazone, has also been shown to possess neuroprotective and anti-inflammatory properties in the CNS (Garcia-Bueno et al. 2005; Allahtavakoli et al. 2006; Collino et al. 2006). Likewise valproic acid (VPA) is used clinically as an anticonvulsant and antiepileptic drug, but also appears to inhibit CEBPα transcriptional activity (Lagace et al. 2004). However patients treated with VPA often gain weight and become insulin resistant by elusive mechanisms (Pylvanen et al. 2002; El-Khatib et al. 2007). Understanding whether these transcription factors influence, or regulate, centrally-derived adipokines could provide some insight as to how they influence brain function, including their neuroprotective and anti-inflammatory properties, in addition to their ascribed effects on appetite and glucose metabolism.

Neuroinflammation, Brain Injury And Brain-Derived Adipokines

Obesity is now regarded as a chronic low grade inflammatory-like state that is thought to be mediated, in part, by increases in white adipose tissue (WAT) mass and the consequent elevated secretion of circulating interleukin-6 (IL-6) and tumor necrosis factor α (TNFα), although many other adipokines are likely involved (Das 2001; Bullo et al. 2003). Increase in WAT mass is thought to induce a local hypoxic state that increases the expression and secretion of several adipokines implicated in the development of insulin resistance and metabolic syndrome (Trayhurn and Wood 2004; Trayhurn and Wood 2005). The expression of other adipokine targets, including leptin and resistin, is

also induced by inflammatory molecules (Sarraf et al. 1997; Lu et al. 2002; Kaser et al. 2003), but they also possess inflammatory properties themselves (Loffreda et al. 1998; Silswal et al. 2005). The injection of lipopolysaccharide (LPS) is commonly used as a model of inflammation, but also induces anorexia and peripheral insulin resistance (Huang et al. 1999; Ueki et al. 2004). Previous investigations have established that the effects of LPS on appetite are centrally-mediated via the melanocortin system (Huang et al. 1999). I speculated that LPS would induce tissue- and time-dependent changes in adipokine gene expression throughout the body, including the brain, which could be contributing to some of the metabolic abnormalities that result following an inflammatory assault.

As noted already (see section III), leptin has been implicated in brain development (Ahima et al. 1999; Steppan and Swick 1999), but also appears to possess both trophic, synaptic and anti-apoptotic properties (see Figure 8 and 9) (Bouret et al. 2004b; Pinto et al. 2004; Russo et al. 2004). It seems unlikely that leptin is the sole adipokine gene implicated in brain repair and function. Although resistin and FIAF knockout mice have recently been generated (Backhed et al. 2004; Banerjee et al. 2004), I am unaware of any investigations specifically looking at the effects on brain growth or development. If other adipokines, especially from local sources, were involved in normal brain function, maintenance, or repair, then brain injury might modify their expression as part of a protective response. Although several models of brain injury are available, I have chosen to investigate the effects of traumatic brain injury (TBI) in rats, and a cerebral hypoxia/ischemia (H/I) brain injury in neonatal mice, in order to monitor the consequences on the expression of centrally-derived adipokines. TBI is one of the leading causes of injury-related deaths in American children, and these patients are often hypophagic, cachetic, and hyperthermic further compounding the underlying brain injury (Thompson et al. 2003; Suz et al. 2006). TBI also induces a hypoxic/ischemic (H/I)

state that is thought to further exacerbate the initial brain trauma and prolong the associated metabolic consequences (Johan Groeneveld et al. 2002; Dash et al. 2004; Morales et al. 2005). Similarly acute neonatal H/I induced significant and long-term bodyweight reductions in neonatal rats (Fan et al. 2005). I hypothesized that TBI, or H/I, would alter the expression of centrally-derived adipokines that could be involved in neuronal survival and brain repair, as well as reducing appetite and increasing energy expenditure, which are characteristic features of neurotrauma.

V. Research Outline For Studying The Function And Regulation Of Brain-Derived Adipokines

As already described (see above), the role of brain-derived adipokines can now be investigated by RNAi. However this was a high risk approach since it was unknown whether large enough reductions in brain adipokine expression could be achieved, either in vitro or in vivo, to induce physiologically-relevant changes. This is particularly of concern for the application of RNAi to in vivo systems since it is a relatively new technique that has several technological limitations including in vivo delivery. Therefore parallel studies were conducted to investigate the roles of putative adipokine transcription factors on brain adipokine expression since both PPAR and CEBP gene families are also expressed in the CNS (see Figure 10). Moreover ligands of these adipogenic transcription factors, including rosiglitazone and VPA, are known to impact neuronal function in addition to regulating energy metabolism. Similarly adipokines appear to provide a common link between inflammation, obesity and diabetes. I suspected that inducing peripheral inflammation would also impact central adipokine expression, which could be responsible for mediating some of the associated reductions in appetite, or decreased insulin sensitivity, that results following LPS-induced endotoxinemia. Likewise brain damage, as a result of cerebral hypoxia/ischemia or traumatic brain injury (TBI), has long lasting effects on neuronal survival and induces

hypophagia, cachexia and hyperthermia. Again I speculated that these brain injuries would induce changes in adipokine gene expression that could influence patient recovery. In keeping with the hypothesis, that centrally-derived adipokines are impacting neuronal survival and metabolism, I will describe these various approaches to investigate the regulation of central adipokine gene expression.

A further aim of this work was to consider the following question: what is the purpose of having a local brain, or hypothalamic, adipokine system(s)? As stated previously (see section I), the hypothalamic regulation of bodyweight is nearly 99.97% accurate (Hervey 1969). Even slight neurochemical or hormonal imbalances in the hypothalamus, which already appears biased in favor of weight gain (Schwartz et al. 2003), could lead to a slow and steady increase in bodyweight that could become significant after only a few years. Although I do not dispute that there is a clear fat-brain connection that profoundly impacts feeding and energy expenditure (see Figure 2) (Schwartz and Porte 2005), the addition of a local adipokine autocrine loop to this model would provide a much higher degree of accuracy with regards to hypothalamic bodyweight regulation. Moreover the suggestion of adipokine autocrine loops is not unprecedented and has been suggested in several other tissues including the stomach (Bado et al. 1998), salivary glands (De Matteis et al. 2002; Bohlender et al. 2003), and more recently in the rat olfactory mucosa (Baly et al. 2007). If such an autocrine loop did exist we should expect that alterations in local adipokine expression would alter cellular metabolism and impact the sensitivity to afferent peripheral metabolic signals.

A local hypothalamic adipokine regulatory autocrine feeding loop is also not totally without precedent. Although the novel satiety molecule, Nesfatin, has yet to be officially classified as an adipokine, Oh et al. (2006) identified that 3T3-L1 adipocytes and the HTB185 brain medulloblastoma cell line both express nesfatin-1 mRNA (Oh et al. 2006). Moreover 3T3-L1 adipocytes have a higher level of expression than the

medulloblastoma cell line, as detected by Northern blot analysis. In addition, nesfatin is derived by the proteolytic cleavage of nucleobindin2 (NUCB2) prior to secretion, and was readily detected in CSF and hypothalamus of rats (Oh et al. 2006). In rats the icv injection of nesfatin reduced food intake and bodyweight over several days, whereas silencing endogenous nesfatin expression by the continuous infusion of an antisense morpholino oligonucleotide (MON) increased food intake and bodyweight (Oh et al. 2006). Despite being expressed in several peripheral tissues, many, if not all, adipokines are also expressed in the hypothalamus (Morash et al. 1999; Morash et al. 2002; Wiesner et al. 2004). I suspect that many brain adipokines, like leptin, are similar to nesfatin. Like nesfatin, the icv injection of leptin or resistin reduced food intake (Gullicksen et al. 2002; Tovar et al. 2005). Therefore the next logical step to establish the physiological role(s) of brain-derived adipokines is to knockdown their expression.

As outlined in Figure 10, the following studies were designed to investigate the potential roles for brain adipokines using the novel technique of RNAi to specifically interfere with their expression in vitro and in vivo. However this was a high risk approach, thus parallel studies were also undertaken to investigate potential regulatory mechanisms of brain adipokines. In addition I verified whether brain adipokines might also be part of an inflammatory response, and whether they might be implicated in the pathology of brain injury. Using these multiple approaches will hopefully advance our understanding of the regulation and potential roles of brain adipokines in terms of cell survival, intracellular signaling and energy metabolism.

SPECIFIC AIMS

A) Identify potential roles for brain-derived adipokines

B) Elucidate the mechanisms and factors that regulate the expression of centrally-derived adipokines

Proof-of-principle studies:	**Regulatory studies:**
i) Leptin RNAi in vitro	i) Molecular regulation
ii) Leptin RNAi in vivo	ii) LPS and inflammation
iii) *rstn* and *fiaf* RNAi in vitro	iii) Effects of brain injury

Figure 10: Research outline As noted in the Introduction, proof-of-principle studies were carried out in parallel with the regulatory studies.

Chapter 2. Leptin Gene Expression In The Brain

I. Introduction

Adipose Tissue As An Endocrine Organ

The hypothesis that adipose tissue is more than a means to passively store excess energy is not a recent concept. For several decades adipose tissue was thought to be influencing both appetite (Kennedy 1953) and reproduction in humans (Frisch and McArthur 1974). When Kennedy first proposed that a 'lipostatic' loop controlled appetite based on body fat stores, it was unclear how adipose tissue could communicate with the hypothalamus (Kennedy 1953). Similarly Frisch and McArthur (1974) proposed a few decades later that menstrual cycles were highly dependent on having a threshold level of body fat, below which cycling and menstruation ceased to occur (Frisch and McArthur 1974). Although this was thought to be partially associated with the ability to mobilize stored energy required for reproduction and fertility, it did not clearly connect adipose tissue with the reproductive, or hypothalamic-pituitary-gonadal (HPG), axis. The cloning of leptin was a major turning point in adipocyte biology, and provided some of the earliest evidence that adipose tissue is also an endocrine organ that influences bodyweight and fertility (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995; Chehab et al. 1996). Leptin is now regarded as a pleiotropic hormone and has been implicated in a range of physiological processes. Moreover leptin gene expression was also readily detected in numerous sites, in addition to adipose tissue, including placenta (Hoggard et al. 1997), mammary gland (Smith-Kirwin et al. 1998), stomach (Bado et al. 1998), pancreas (Emilsson et al. 2001), bone (Reseland et al. 2001), salivary glands (De Matteis et al. 2002; Bohlender et al. 2003), rat olfactory mucosa (Baly et al. 2007), pituitary (Jin et al. 1999; Morash et al. 1999) and brain (Morash et al. 1999). This ubiquitous pattern of leptin expression suggests that extra-adipocyte leptin might be impacting numerous physiological purposes, in addition to the modulation of

appetite and energy expenditure. A major question is what purpose, or physiological function, does extra-adipocyte leptin serve, especially within the CNS? Similarly is the level of leptin production in these non-adipose stores adequate enough to impact local leptin signaling pathways?

Detection And Regulation Of Leptin mRNA In The Rat Brain

As already stated, leptin mRNA was readily detected and quantified in both the rat brain and C6 glioblastoma cell line (See Table 1). Moreover leptin gene expression was distributed throughout various brain regions, including the cerebral cortex, cerebellum, hypothalamus, pineal gland, retina, and in the posterior and anterior pituitaries, which was confirmed using two sets of intron-spanning primers (217bp and 495bp) (Morash et al. 1999; Wilkinson et al. 2007). The low abundance of leptin mRNA precluded the use of in situ hybridization to determine its localization within the CNS, although signals were detected in sections of cerebellum and anterior pituitary (Wilkinson et al; unpublished observation). Nevertheless tissue microdissection revealed a marked enrichment of *ob* mRNA in the ARC when compared to the complete hypothalamus (Wilkinson et al. 2007). Centrally-derived leptin gene expression also appears to be regulated by age- gender and nutritional status. Striking tissue-dependent developmental changes in *ob* mRNA were detected in the rat cerebral cortex and pituitary, but not in the hypothalamus (Morash et al. 2001a; Morash et al. 2001b). For example *ob* mRNA was increased markedly from birth until puberty in the cerebral cortex of rats, independent of gender (Morash et al. 2001a). Conversely pituitary leptin gene expression was high at birth and steadily declined until post-weaning (Morash et al. 2001a; Morash et al. 2001b). Although hypothalamic gene expression was unaffected by age or gender, fasting adult rats significantly reduced leptin mRNA, which was partially restored when food was returned (Wilkinson et al. 2007). These unique patterns of *ob* gene regulation and distribution are suggestive of alternative, or specific, roles for leptin

in each of these sites.

Leptin, A Neuron-Specific Peptide?

Leptin immunoreactivity (ir) colocalized with the neuron-specific marker, NeuN, in various brain regions including the arcuate nucleus, cortex and hippocampus, as detected using double-label immunofluorescence confocal microscopy (Ur et al. 2002). However leptin-ir was also present in non-neuronal cells in the arcuate nucleus (Morash et al. 2002). Similarly many cells in the paraventricular and supraoptic nuclei were positive for leptin-ir, which appeared to colocalize with oxytocin- and vasopressin-positive cells (Morash et al. 2002). Leptin-ir was also restricted to the nuclear region of some neurons, suggesting that an unidentified nuclear leptin receptor may exist (Morash et al. 2002). This is particularly intriguing since it was recently hypothesized that humans might also possess an unidentified leptin receptor (Farooqi et al. 2007), but this needs to be investigated further.

Detection And Regulation Of Ob mRNA In C6 Glioblastoma Cells

In addition to the brain, leptin mRNA and protein have also been detected in the rat C6 glioblastoma cell line, which has proven to be a valuable model system for studying the regulation of brain leptin (Morash et al. 2000; Li et al. 2001). The C6 cell line was derived from immortalized glial cells, and is known to express the receptors for insulin, glucocorticoids and noradrenaline, purported regulators of adipose-derived leptin expression (Morash et al. 2000). The detection of leptin gene expression in C6 cells, which may not fully represent differentiated glial cells, is consistent with the double-label immunohistochemistry data that suggest leptin is not exclusively expressed in neurons in the CNS (Morash et al. 2002). However since we failed to detect leptin secretion from C6 cells using radioimmunoassay (Imran, Brown, Ur and Wilkinson, unpublished), and since leptin-ir appears to predominantly localize in the nucleus of C6 cells (as in neurons; (Ur et al. 2002)), endogenous leptin might act as an autocrine/intracrine factor

in C6 cells, a concept that might even extend to the CNS. Leptin gene regulation was also unexpectedly the opposite of that reported for adipose tissue. For example, dibutyryl cAMP (dbcAMP), insulin and insulin-like growth factor 1 (IGF1) stimulated *ob* mRNA in C6 cells, whereas *ob* gene expression was inhibited by corticosterone treatment (Slieker et al. 1996; Morash et al. 2000). This tissue-specific pattern of leptin gene regulation, in adipose vs. neuroectodermal tissues, was confirmed using a plasmid based construct that consisted of the rat leptin promoter fused to a luciferase reporter gene (Li et al. 2001). Although additional studies in neuronal cells are now necessary, the C6 glioblastoma model is an important and useful model for studying brain-derived leptin.

Leptin Gene Expression In The Human Brain

As noted in Chapter 1, leptin gene expression has also been detected in the human brain and in a human neuroblastoma cell line (Knerr et al. 2001; Russo et al. 2004; Eikelis and Esler 2005). Knerr et al (2001) used RT-PCR to detect low levels of *ob* mRNA in astrocytomas and glioblastomas, but also detected leptin expression in normal temporal lobe tissue (Knerr et al. 2001). Similarly, leptin mRNA was readily detected by RT-PCR, despite its relatively low abundance, in samples of human hypothalamus (Eikelis and Esler 2005; Eikelis et al. 2006; Eikelis et al. 2007). Thus the rat brain is similar to the human brain in that they both express low levels of leptin. Further evidence that the human brain is also a locus of leptin production includes the detection of increased concentrations of leptin protein in the jugular venous blood at the level of the base of the brain (to exclude the possibility of contamination with facial venous drainage) (Esler et al. 1998; Wiesner et al. 1999; Eikelis et al. 2006; Eikelis et al. 2007). Not only does the brain appear to release leptin, but this extra-adipocyte leptin could constitute as high as 40% of total plasma leptin, depending on age, gender and body weight (Wiesner et al. 1999). In subsequent studies the release of brain-derived leptin appeared to be

decreased in patients suffering from major depressive disorders, relative to healthy controls (-70%, p<0.05) (Eikelis et al. 2006). In contrast, there appears to be increased leptin release in obese individuals (Wiesner et al. 1999). Moreover when *ob* mRNA levels were measured using semi-quantitative RT-PCR they correlated positively with brain leptin excretion. For example post-mortem hypothalamic leptin mRNA levels were lower in the brains of people who had suffered from major-depressive disorders (Eikelis et al. 2006), whereas it was increased in hypothalamic tissues collected from obese cadavers (Eikelis et al. 2007). However it is unclear whether the leptin efflux from the brain is derived entirely from locally produced leptin, or if the brain acts a leptin repository for adipose-derived leptin, or a combination of the two (Reichlin 1999). If the first is true, this would imply that leptin is secreted from brain cells, and possibly neurons. At present we have no evidence from animal studies that this is the case, however leptin secretory granules are present in the anterior pituitary (Vidal et al. 2000), and there are data showing leptin is released from some pituitary tumours (Korbonits et al. 2001).

Detection And Regulation Of Ob mRNA In A Human Neuroblastoma Cell Line (SH-SY5Y)

Like C6 glioblastoma cells, the expression of leptin and its receptors was also detected in a human neuroblastoma cell line (SH-SY5Y) (Russo et al. 2004). Furthermore leptin was capable of stimulating cell proliferation, and inhibited apoptosis, resulting in increased cell number (Russo et al. 2004). These effects of leptin were dependent on intact leptin signaling pathways including the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT), mitogen activated protein kinase (MAPK), and phosphatidylinositol-3 kinase (PI3K) pathways (recall Figure 6). However there was no speculation on whether endogenous leptin is impacting cell survival or whether it is secreted from SH-SY5Y cells in order to activate the receptors located on

the plasma membrane. The SH-SY5Y neuroblastoma cell line exhibits many features of mature sympathetic neurons, including the secretion of noradrenaline (Smith and Hainsworth 1998). It would be interesting to determine whether leptin release could be stimulated in these cells by inducing their depolarization. Collectively these findings suggest that centrally-derived leptin, and potentially other brain-derived adipokines, could play a more complex role in the human brain than initially expected. However further studies are required to unravel the complex interactions of these hormones in order to understand, and elaborate, on their role(s) in the CNS.

II. Study Of Leptin Knockdown In Rat C6 Glioblastoma Cells

Silencing Of Brain Leptin Gene Expression

The studies outlined above clearly suggest that the brain is a locus of leptin expression. Moreover this does not appear to be a unique peculiarity of the rat brain since leptin gene expression, and leptin release, has been detected from the human brain (Eikelis et al. 2006; Eikelis et al. 2007), and leptin mRNA was readily detected in the human neuroblastoma cell line (SH-SY5Y) (Russo et al. 2004). Similarly leptin gene expression has been detected in the CNS of several other species (see Table 1). At minimum, these data emphasize the need for further study of brain-derived leptin. However in order to definitively establish a physiological or pathological role for brain leptin, a specific disruption, or silencing, of central leptin gene expression is necessary. As noted already (see Chapter 1, section IV), the powerful post-transcriptional silencing technique of RNAi offers several advantages over other gene silencing technologies (Makimura et al. 2002; Miller et al. 2005). We hypothesized that RNA interference (RNAi) could selectively silence ob expression in the C6 cell model. These studies were designed as a first step to optimize this technique, but to also elaborate on the possible functions of leptin in vitro (Brown et al. 2005a). Moreover technological advancements have shown that RNAi is a feasible and effective approach for silencing gene expression

in the CNS, although these studies employed targets that had previously been optimized in vitro (Makimura et al. 2002; Thakker et al. 2004; Thakker et al. 2005; Hoyer et al. 2006). Thus the consequences of silencing *ob* expression in intact rat brain will also be investigated (See section VI, Chapter 2).

The Discovery Of RNA Interference

The recent discovery that RNA can interfere with gene expression is not unprecedented. McConnel was amongst the first to show more than three decades ago that there was a transmissible molecule that conferred a learned trait between planarians (flatworms). This factor turned out to be RNA (McConnell 1966), and this was later confirmed in rats (Byrne 1970). Andrew Fire and colleagues took an important further step and showed that long double stranded RNA could specifically reduce, or 'knockdown', endogenous gene expression in Caenorhabditis elegans (*C. elegans*; roundworms) (Fire et al. 1998). It was subsequently reported that long double stranded (ds) RNA silenced gene expression within cultured mammalian neural cells (Gan et al. 2002), and within the rat hypothalamus (Bhargava et al. 2004). However there has been significant concern about the use of long ds RNA since it appears to induce the non-specific global shut down of gene expression in mammalian species by activating endogenous cellular antiviral responses (Elbashir et al. 2001a). More specifically ds RNAs that are greater than 30 base pairs (bp) in length activate the dsRNA-dependent protein kinase response (PKR), which phosphorylates the eukaryotic initiation factor-2 (eIF-2) leading to the universal inhibition of protein synthesis (Manche et al. 1992). Similarly long ds RNA is capable of activating 2',5'-oligoadenylate synthetase, which also induces global mRNA degradation (Minks et al. 1979). Thus it is next to impossible to discern between the effects of knocking down a specific target gene, versus the global responses, that have been reported when long ds RNA is used to silence gene expression in mammalian systems. However long dsRNAs are cleaved by a

ribonuclease III enzyme, Dicer, into short 21-25bp fragments containing 3' nucleotide

overhangs, prior to silencing target gene expression (Bass 2000; Bernstein et al. 2001;

Elbashir et al. 2001b). Thus in order to circumvent the non-specific responses induced

by long dsRNAs Elbashir et al. (2001) introduced small (21 nucleotide) ds RNAs into

mammalian cells, now referred to as small-interfering RNAs (siRNA), that targeted

endogenous or heterologous gene expression (Elbashir et al. 2001a). Substantial

reductions (2-25-fold) in gene expression were achieved using these siRNAs in a variety

of cell lines derived from various tissues and species (Elbashir et al. 2001a). Moreover

single base pair mismatches within the centre of the siRNA abolished its silencing

properties, confirming their stringent ability to specifically target certain mRNA

sequences for degradation (Elbashir et al. 2001c). Thus siRNA molecules offered a

suitable means to circumvent the undesirable side effects associated with long ds RNA

without compromising their ability to reduce target gene expression.

RNAi Mechanism Of Silencing

Although siRNAs were known to possess potent gene silencing effects in

mammalian systems the mechanism by which they attained these reductions were

largely unresolved. Initially Elbashir et al. (2001) offered two possibilities: a hypothetical

post-transcriptional event that modified the relevant genomic DNA regions, or a more

plausible transcriptionally-dependent mechanism whereby the siRNA interfered with

mRNA translation or stability (Elbashir et al. 2001a). Evidence for the latter included data

showing that target mRNA was cleaved prior to translation, and this site was dependent

on only one of the two siRNA strands which spanned the mRNA cut site (Elbashir et al.

2001b; Elbashir et al. 2001c). The resulting mRNA fragments were thought to be rapidly

degraded due to their instability in the cytoplasm since they lacked the protection

normally provided by the combination of the 5' cap and 3' polyadenine tail (Elbashir et al.

2001c). Later evidence suggested that siRNA-dependent cleavage occurred exclusively

in the cytoplasm (Zeng and Cullen 2002), further supporting the hypothesis that siRNA-mediated gene silencing occurs via a post-transcriptional event (Elbashir et al. 2001a; Zamore 2002).

Substantial advancements have been made towards elucidating the RNAi mechanism of gene silencing in mammalian systems, which have aided in the design of more effective siRNA molecules (see Figure 11). Once siRNA reach the cytoplasm of target cells they can be assembled into a RISC (RNA-induced silencing complex) loading complex (RLC) (Tomari et al. 2004b). The protein R2D2 determines the least thermodynamic stable strand of the siRNA molecule based on the 5' positions, which becomes the 'guide' RNA, leaving the other strand to act merely as a 'passenger' (Tomari et al. 2004b). Although the guide RNA strand must contain a 5' phosphate, it is not an absolute requirement for the 'passenger' RNA, although this non-essential phosphate appears to facilitate the high affinity binding of the passenger strand to the R2D2 component of RLC (Tomari et al. 2004b). Once loaded into the RLC, the displacement of the R2D2 component by argonaute 2 (Ago2) triggers the ATP-dependent unwinding of the siRNA by the RNA helicase armitage (Armi) (Nykanen et al. 2001; Tomari et al. 2004a). Ago 2 is an essential component of RISC, and contains two RNA binding domains; PAZ and Piwi that bind the 3' and 5' end of the small guide RNA, respectively (Zamore and Haley 2005). Moreover when the guide RNA is bound by Ago2, the passenger strand of the siRNA is discarded and permits the 'guide' strand to help RISC associate and bind target mRNA. This is followed by the ATP-independent cleavage of a single phosphodiester bond in the target mRNA (Elbashir et al. 2001b). The endoribonuclease activity resides in the Piwi domain of Ago2, and it consistently cleaves target mRNA 10-11bp downstream of the 5' end of the small guide RNA which is also bound by the Piwi domain (Elbashir et al. 2001b; Elbashir et al. 2001c; Zamore and Haley 2005). The cleaved mRNA fragments are then carried to P-bodies, cellular sites
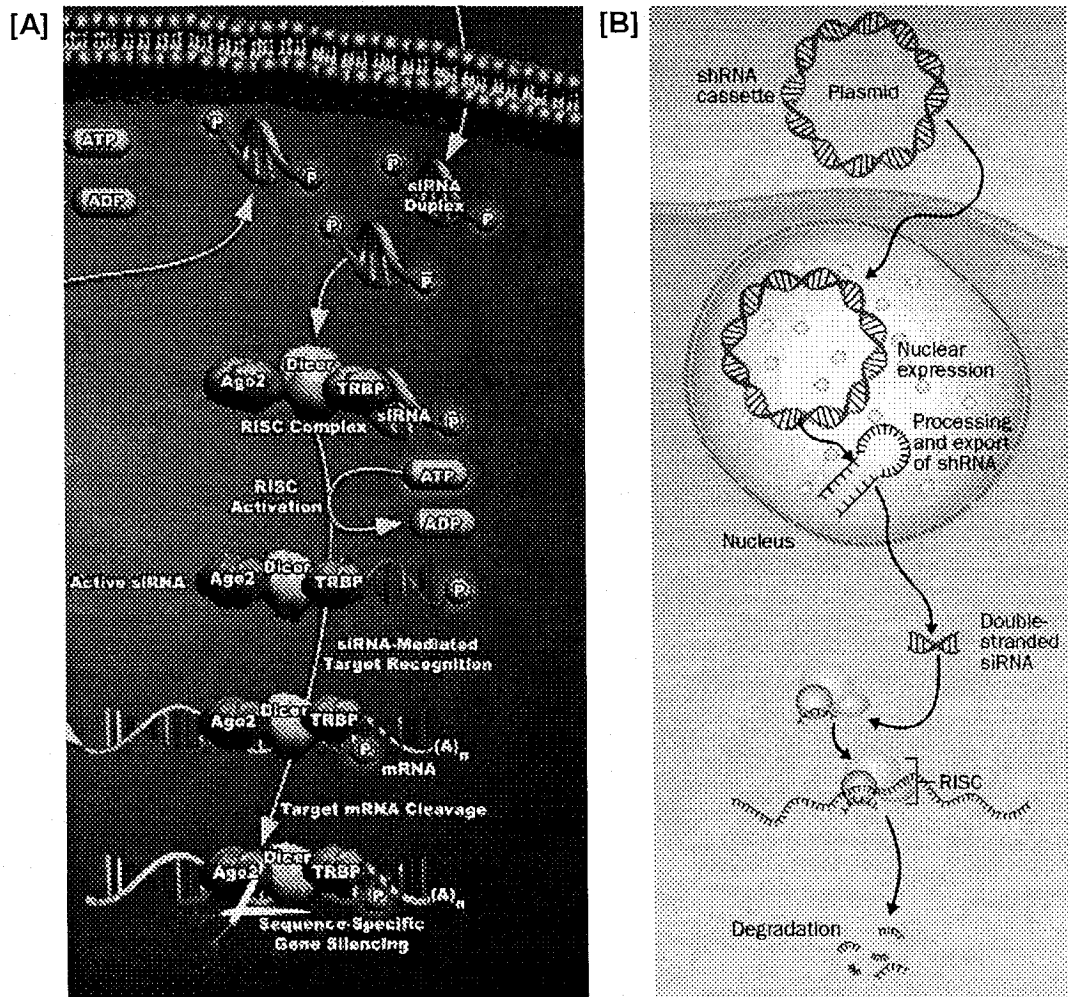
Figure 11: siRNA- and shRNA-mediated silencing
RNAi can be induced in mammalian cells by either [A] siRNA or [B] a shRNA-expressing plasmid. Once cells are transfected the siRNA associates with cytoplasmic proteins to form an RNA-induced silencing complex (RISC). The siRNA then undergoes an ATP-dependent unwinding and the 'passenger' strand of the siRNA is discarded. Subsequently the active siRNA-RISC complex scans mRNAs in order to find its target sequence, and once identified, directs a single cleavage event. The two resulting mRNA strands are then rapidly degraded in P-bodies. [B] Although the shRNA expressing plasmid also have to be transfected into cells, it must reach the nucleus where RNA polymerase III drives the expression of a short RNA sequence. The RNA then folds back on itself and the resulting hairpin loop is exported from the nucleus and cleaved by Dicer. Then, as in [A], the endogenously produced siRNA is integrated into an RISC that directs the cleavage of target mRNA. (Figures adapted from www.ambion.com/tools/pathwaypathway.php?pathway=siRNA%20Pathway and from Davidson and Paulson 2004)

of mRNA decapping and degradation, and the RISC is released from the P-body in the presence of ATP (Zamore and Haley 2005). Thus the RISC complex is free to bind and cleave target mRNA through several cycles (Haley and Zamore 2004). However the recycling of the RISC-guide RNA complex is unlikely to be 100% efficient, and likely contributes to the transient nature of gene silencing associated with RNAi.

The Anatomy And Engineering Of A Functional siRNA

As noted earlier, the first generation of siRNA molecules were modeled after the products derived from the Dicer-mediated cleavage of long double stranded RNA; they were double stranded, 21-23 nucleotides in length, and contained nucleotide overhangs at the 3' position (Elbashir et al. 2001b). In subsequent work Elbashir et al. (2001) tested whether variations in overhang length (1-3 nucleotides), the overall length of the siRNA (18-25 base pairs), or if chemical modifications, impacted silencing efficiency (Elbashir et al. 2001c). Using a luciferase reporter assay they established that the optimal siRNA contained 2 nucleotide overhangs at the 3' position, were 21 nucleotides in length, and chemically modified nucleotides did not reduce silencing efficiency. Single nucleotide mismatches were also determined to abolish the silencing properties of a siRNA (Elbashir et al. 2001c), suggesting that they can only target specific sequences for degradation.

Shortly thereafter a siRNA design 'guide' was generated (Elbashir et al. 2002), and outlined 4 general criteria as summarized below:

1) Obtain the complete mRNA sequence of the target gene, and locate the position ~70-100bp downstream of the translation initiation site.

2) Search for sequences that contain a 5' AA, and the following 19 nucleotides. These sequences should ideally contain approximately a 50% GC content.

3) Search nucleotide databases (i.e. BLAST search, www.ncbi.nlm.nih.gov/BLAST)

4) Design a scramble control that has a similar GC content and sequence structure

However it soon became apparent that target selection should consider other criteria, such as the thermodynamic stability of the siRNA, to determine which strand is incorporated into RISC (Tomari et al. 2004b). Likewise a high-throughput screening of multiple siRNAs revealed the silencing potential varied greatly between various siRNA targets (Huesken et al. 2005). Analysis of those with the greatest, or weakest, silencing properties revealed several possible design criteria, including the conservation of certain mononucleotides at specific positions in the siRNA strands (Huesken et al. 2005). This permitted the development of algorithm-based design programs that are capable of predicting the efficacy of a given siRNA target and revealed the power of computational-based siRNA design (Redmond et al. 2003; Amarzguioui and Prydz 2004; Levenkova et al. 2004; Huesken et al. 2005). Most siRNA suppliers now offer free web-based design tools, such as Invitrogen's BLOCK-iT™ RNAi designer, which employ proprietary algorithms to identify RNAi targets that are guaranteed to identify sequences that will effectively silence target gene expression. However it should be noted that there is still room for improvement in siRNA design. For example considering the stability, modifications and secondary structure of the target mRNA itself could further advance the efficacy of siRNA design (Miyagishi and Taira 2005). However the recent refinements in design and selection of suitable siRNA targets has greatly facilitated and enhanced the success of RNAi-based studies.

Sustainable Knockdown Using Small Hairpin RNA (shRNA)

A major drawback to the use of siRNA is their transient nature of suppressing target gene expression. This can be particularly prohibitive since cellular, or physiological, consequences of knockdown will only occur after protein levels have also been decreased. Thus mRNA levels must be suppressed long enough in order to reduce protein levels, and the time required can vary greatly depending on the half life, or rate of turnover, of the target peptide. This led to the development of plasmid based systems

that continuously synthesizes small hairpin RNAs (shRNA) which permit the long-term

suppression of target gene expression (see Figure 11) (Brummelkamp et al. 2002). The

first shRNA expressing vector, pSUPER, contained an RNA polymerase III promoter that

transcribed small RNA sequences based on the inserted shRNA expression sequence

(Brummelkamp et al. 2002). These short RNAs are thought to fold back on themselves

and form a siRNA-like duplex that contains a small hairpin loop that is approximately 9

base pairs in length. This hairpin loop is subsequently removed by the dsRNA cleavage

enzyme Dicer, resulting in an endogenously produced siRNA molecule. Moreover, as

reported for siRNA, the introduction of mismatches abolished silencing, suggesting that

shRNA-mediated reductions are also target-specific (Brummelkamp et al. 2002).

However shRNA appear to be less effective than siRNA at silencing gene expression

(Yu et al. 2002), and there have been concerns about the variability, reproducibility and

control of dosage when using shRNA-based plasmid systems (Clayton 2004). Likewise it

has been suggested that multiple (up to 20) targets should be screened, since only 2 in

11 shRNA were effective at silencing ataxin-1 when potential shRNA sequences were

screened in HEK 293 cells (Xia et al. 2004). Thus despite the ability of shRNA-based

systems to achieve long-term knockdown, they are not without disadvantages.

Using RNAi To Silence Brain Leptin Gene Expression

Although leptin is readily detectable in the CNS of numerous species (see Table

1), and in various non-adipose tissues (see section I above), the purpose or

physiological function of extra-adipocyte leptin remains uncertain. As noted already,

leptin gene expression was detected in the rat C6 glioblastoma cell line, and leptin is

regulated in a unique manner compared to what has been reported in adipose tissue or

3T3-L1 adipocytes (Morash et al. 2000; Li et al. 2001). Moreover leptin protein appears

to localize in the nuclear region of C6 cells, similar to what was observed in the rat brain

(Morash et al. 2002). Taken together it seems likely that brain-derived leptin plays a

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unique role in the CNS that could be independent of metabolism or appetite. For example leptin clearly impacts brain growth and the development of hypothalamic feeding circuits (Ahima et al. 1999; Steppan and Swick 1999; Bouret and Simerly 2004). Similarly the human neuroblastoma cell line (SH-SY5Y) also expresses leptin, like rat C6 cells, and leptin treatment increased cell survival (Russo et al. 2004). **Thus the following in vitro studies in C6 cells were designed to a) establish the effectiveness of using an RNAi approach to silence the expression of brain leptin and b) investigate the effects of leptin-knockdown on cell survival.**

III. Materials And Methods

Rat C6 Glioblastoma Cell Culture

Rat C6 glioblastoma cells (ATCC # CCL 107; Manassas, Virginia) were maintained in DMEM (Gibco; Burlington, Ont.) supplemented with 10% fetal bovine serum (FBS) in a humid incubator at 37°C in 5 % CO_2 / 95% air. Prior to experiments cells were plated at a density of 30,000 cells per well in 12 well plates (Nunc) and grown for a further 24h. For transfections the normal medium was aspirated and replaced with 500μL/well of the serum free medium, OptiMEM (Invitrogen; Burlington, Ont.) and transfected as outlined below. Following 4-5h of transfection the transfection medium was aspirated and replaced with fresh DMEM containing 10%FBS, unless otherwise stated.

siRNA Transfection Optimization In C6 Glioblastoma

For transfection optimization studies the non-specific scramble siRNA was fluorescently labeled using the Silencer® siRNA Labeling Kit - FAM™ (Ambion; Austin, TX). C6 cells (30,000 cells/well) were plated on glass cover slips in 12 well Nunc plates. Just prior to transfection the cell medium was replaced in each well with 500μL of OptiMEM (Invitrogen). A fixed amount of the FAM-labeled siRNA (50pmol/well; Invitrogen) was diluted in 20μL of optiMEM media (Invitrogen). Various amounts (1, 2,

and 4μL) of two transfection reagents, Oligofectamine™ (Invitrogen) and

NeuroPORTER™ (Gene Therapy Systems; Sandiego, CA.), were diluted to a final

volume of 20μL in OptiMEM. After incubating for 5min at room temperature the FAM-

labeled siRNA (50pmol of FAM-labeled siRNA in 20μL) and transfection complexes

(20μL) were transferred into a single tube and mixed by swirling with the end of a pipette

tip. The solutions were incubated for a further 20min prior to adding onto cells

(40μL/well). Cells were transfected for a period of 4h, after which time they were washed

with Dulbecco's phosphate buffered saline (D-PBS) and fixed for 5min with a 4%

paraformaldehyde (PFA) solution prepared in 0.1M phosphate buffered saline (pH 7.4).

Cells were washed 2 times with D-PBS and then stained for 5min using a 500nM

solution of 4'-6-Diamidino-2-phenylindole (DAPI; 300μL/well, Molecular Probes,

Burlington ON). Cells were washed 3 more times with D-PBS and cover slips were

mounted onto slides using the SlowFade® Antifade kit (Molecular Probes). Slides were

photographed using a Retiga 1300 (QImaging; ON, Canada) digital camera attached to

a Leitz Laborlux S microscope. Transfection efficiency was confirmed by visualizing

uptake of the label (for images see Figure 12).

Small Interfering RNA (siRNA) Design:

The complete rat leptin mRNA sequence (nm_013076) was obtained from the

NCBI website (http://www.ncbi.nlm.nih.gov/entrez), and following instructions from

DHARMACON research inc. (Lafayette, CO) and

[http://www.mpibc.gwdg.de/abteilungen/100/105/sirna.html] which is provided by T.

Tuschl and colleagues, we identified several potential siRNA target sequences

contained in the rat leptin mRNA transcript. These criteria included identifying

sequences located at least 70 nucleotides downstream of the translational start site

which contains an AA dimer followed by 19 nucleotides. Sequences with a GC content

that did not fall between 30-70%, or showed significant similarity with other mRNA

sequences based on a BLAST search (NCBI), were eliminated. A non-specific scramble sequence was also generated by randomly arranging the nucleotides contained in the target sequence, 5' AAGTCACTGTGACAGTCTCGT 3', which served as a control molecule (for siRNA sequences please see Table 3). Multiple leptin siRNA targets were synthesized using the *silencer* siRNA construction kit (Ambion; Austin TX.), along with a non-specific control molecule, according to the manufacturer's instructions (for sequences refer to Table 3). Preliminary studies (see below) were carried out to test the multiple targets in an attempt to find the siRNA with the greatest silencing ability. Subsequently the selected target, and the scramble control, were chemically synthesized in both the unmodified (siRNA L7 and siRNA SCR), and the stability-enhanced forms (siSTABLE L7 and siSTABLE SCR) (Dharmacon, Lafayette, CO.) for all subsequent experiments. Molecules were ordered in the annealed format, and sequences were dissolved in sterile water and stored at - 20°C in stock concentrations of 20μM or 50μM for in vitro and in vivo studies respectively.

Blocking The dbcAMP Induction Of Leptin Gene Expression

To assess whether siRNA L7, or siSTABLE L7, could block the stimulatory effects of dbcAMP on leptin gene expression (Morash et al. 2000), C6 cells were plated in 12 well plates as described above. Twenty four hours later medium was replaced with OptiMEM (Gibco) and cells were propagated for a further 18h before being transfected for 4h with 100nM of either the unmodified siRNAs (siRNA L7 or siRNA SCR) or the stability enhanced molecules (siSTABLE L7 or siSTABLE SCR). Leptin gene expression was stimulated in C6 cells by the addition of 1μM dbcAMP (Sigma; St. Louis, MO) and total RNA was isolated 20h later.

siRNA And siSTABLE Dose Response

Cells were plated in 12 well Nunc plates as described above. Subsequently cells were transfected one day later with various doses (25, 50, 100, 200nM) of siRNA L7 and

siRNA SCR, or the stability enhanced equivalents (siSTABLE L7 and siSTABLE SCR).
After 4h medium was replaced with fresh DMEM containing 10% FBS until total RNA
was isolated 24h later for RT-PCR analysis.

Time Course Of Silencing With siRNA L7 And siSTABLE L7

C6 cells were transfected with 100nM of the unmodified molecule, siRNA L7, and
RNA was isolated 20, 30, 46, and 55h later, whereas control cells were transfected with
100nM of the siRNA SCR and cultured for 55h. Similarly following transfection using
100nM siSTABLE L7, cells were propagated for 24, 48, 72 and 96h prior to RNA
isolation for RT-PCR analysis. Again control cells were transfected with the siSTABLE
SCR and cultured for 96h. Similarly the silencing capacities of both siRNA L7 and
siSTABLE L7 were tested when C6 cell proliferation was reduced, by culturing cells in
reduced serum DMEM (2% vs. 10%) for 72h after transfection.

RNA Isolation From C6 Cells

RNA was isolated from cells using the RNEasy mini kit (Qiagen; Mississauga,
ON) according to the manufacturer's instructions. In brief, after treatment the medium
was aspirated from cells and they were lysed and scraped in 350µL of lysis buffer.
Lysates were transferred to QIAshredder columns and centrifuged for 2min at 13000g.
The supernatant was then diluted with 1 volume of ice cold 70% ethanol and then
transferred to the nucleic acid binding column. After centrifugation (1min, 13000g)
columns were washed with 350µL of RW1. After another brief centrifugation (15s,
13000g) 80µL of a prepared DNase solution (10µL of DNase stock and 70µL of RDD
buffer; Qiagen) was added onto the column and incubated for 25min at room
temperature. Columns were centrifuged, and then washed again with 350µL of RW1.
RNA binding columns were transferred into new collection tubes and 500µL of RPE was
added and columns were briefly centrifuged (15s, 13000g). The flow thru was discarded
and the RPE wash was repeated. Columns were dried by centrifugation (1min; 13000g)

before transferring into RNAse-free Eppendorf tubes. RNA was eluted from the column by adding 25µL of RNAse-free water and centrifuging for 1min (13000g). RNA concentrations were determined by using duplicate samples that were diluted in water (1:70) and measuring absorbance (260nm) by spectrophotometry. Absorbances were corrected by taking background measurements for water, and nucleic acid concentrations were calculated using Beers law [i.e Conc (µg/µL) = Absorbance corrected for background x 2.8]. All RNA samples were stored at -70°C until subsequent use.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA (1.5µg) was heat denatured for 5 minutes at 65°C and reverse transcribed (RT) using Omniscript reverse transcriptase (Qiagen) for 1hour at 37°C in a total volume of 30µL containing: 1X buffer, 3µL 0.5mM dNTP (provided), 3µL of 50nM OligodT (Pharmacia; Mississauga, ON), 1.5µl of RNasin (10U/µl, Promega; Mississauga, ON), and 1.5µL OmnIscript reverse transcriptase. Reactions were terminated by a final incubation at 93°C for 5 minutes. For semi-quantitative RT-PCR I used previously published leptin primers directed at a 217 base pair region spanning exons 2 and 3 of the ob gene (Morash et al. 1999), and cyclophilin (Ma et al. 1994) (for primer sequences please see Table 2). PCR reactions were composed of the following: 3-5µL cDNA, 1X reaction buffer, 2µL of 0.2mM dNTP, 25pmol each forward and reverse primer, 2.5U HotStar *Taq* DNA polymerase (Qiagen) in a total volume of 95µL. Samples were initially heated to 95°C for 15minutes to activate the HotStar *Taq* DNA polymerase and amplified as follows: denaturation at 94°C for 45s, annealing at 58°C for 45s and extension for 1min at 72°C for predetermined cycle numbers (leptin, 34-35 cycles; cyclophilin, 18-19 cycles; see Figure 13) that were determined based on liner range values for C6 cells grown under basal conditions (untreated and in DMEM supplemented with 10%FBS).

Similarly a negative control was used to verify that no contamination of the samples occurred during the RT-PCR procedure. Amplification products were separated by electrophoresis using 1.5% agarose gels made using Tris-Borate buffer (TBE). Amplicons were visualized using ethidium bromide staining, and photographs were taken using a Polaroid camera. Product yield was determined using NIH (National Institutes of Health) Image (version1.60) software and data reactions were normalised to the level of expression of cyclophilin. Thus data are expressed in arbitrary units (Leptin/cyclophilin) as a ratio of leptin mRNA relative to cyclophilin.

Western Blot Analysis

For Western blot analysis C6 cells (70,000/well) were propagated in 6 well Nunc plates and transfected 24h later using 100nM siSTABLE L7, or the siSTABLE SCR control, as described above. After transfection cells were propagated in DMEM containing 2%FBS for a further 72h. Cells were washed twice using 2mL of D-PBS/well, and then scraped into Eppendorf tubes using 1mL of D-PBS. Cells were centrifuged (5min; 5000g) and then excess D-PBS was aspirated. Cellular pellets were lysed in 50μL of radioimmunoprecipitation assay buffer (RIPA: 150mM NaCl, 50mM Tris, 50mM Sodium Phosphate, 6mM Sodium Deoxycholate, 0.1% Igepal, 1mM sodium fluoride, 1mM sodium orthovanadate) that was supplemented with 1mM phenylmethylsulphonylfluoride (PMSF), 10μg/mL antipain, 10μg/mL leupeptin, 10μg/mL pepstatin, 5mM EDTA (ethylenediaminetetraacetic acid) and 5mM EGTA (ethylene glycol tetraacetic acid) on day of lysis. Cells were homogenized using a 23 gauge needle and syringe, and homogenates were centrifuged for 10min at 10000g. Supernatants, or cellular lysates, were transferred to a clean Eppendorf tube and protein concentration was determined using the DC protein assay kit (BioRAD; Mississauga, ON). Cell lysates (30μg) were diluted to a final volume of 16μL using RIPA buffer, and then supplemented with 8μL of a 3x SDS-PAGE sample buffer (0.2M Tris-HCl, 30% glycerol, 6% sodium

dodecyl sulphate (SDS), 5% 2-mercaptoethanol and 0.1% bromophenol blue). Samples were separated on a 15% SDS-PAGE gel and electrically transferred for 2h onto HYBOND-C nitrocellulose membrane (100volts; Amersham Bioscience; Baie d'Urfé, QC). Membranes were blocked overnight at 4°C in TTBS containing 10% milk powder and then probed with a leptin-specific antibody (1:1000; Y-20;SC-843; Santa Cruz Biotechnology; Santa Cruz, CA) for 2h at room temperature. Blots were washed 3 times in TTBS and incubated for 1h using an anti-rabbit horseradish peroxidase (HRP) conjugated antibody (1:5000; Amersham Bioscience; Baie d'Urfé, QC). Bands were detected using SuperSignal™ West Pico Chemiluminescent substrate (BD Biosciences; Brockville, ON). Blots were exposed for 1-3min and films developed in a KODAK automated developer. Bands were quantified using NIH Image (v1.60), and data are expressed as a percentage of control. Membranes were stained with Ponceau S to confirm equal loading and transfer between samples.

C6 Cell Death Analysis Using Ethidium Homodimer-1

C6 cells were plated at 25,000 cells/ well in 12 well plates (Nunc) on glass cover slips 24h prior to transfecting for 4h with either siSTABLE L7 or siSTABLE SCR (100nM) as described above. Cells were propagated in DMEM containing 2% FBS for a further 72h. Medium was aspirated and cells were stained for 45min at room temperature using an optimized concentration of Ethidium homodimer-1 solution that was prepared in D-PBS (4μM, EthD-1, Molecular Probes; Eugene, OR). Cover slips were mounted onto glass slides using the SlowFade® Antifade kit (Molecular probes). Slides were viewed using a Leitz Laborlux S fluorescent microscope and 4 images were captured per coverslip in equally spaced quadrants using a Retiga 1300 digital camera and OpenLab Software (Quantum Technologies, ON Canada). Dead cells were counted by hand in each of the 4 images to obtain a reproducible cell death 'profile', and data are expressed as a percentage of control +/- SEM.

Terminal Deoxyribonucleotidyl Transferase Mediated D-Uridine 5' Triphosphate Nick End Labeling (TUNEL) Staining

Cells were treated the same as above, however 72h posttransfection cells were stained using the DeadEnd™ Colorimetric TUNEL System (Promega; Madison WI) according to the manufacturers instructions. In brief, 72h following transfection with siSTABLE L7, or the siSTABLE SCR control, cells were fixed with 4% PFA. Coverslips were washed twice with D-PBS, and then cells were permeabilized for 5min with a 0.2% Triton X-100 solution prepared in PBS. Cells were washed 2 more times with D-PBS and then cells were equilibrated for 10min using 100µL of the provided equilibration buffer. Coverslips were dried by touching edges on Kimwipes, and then 100µL of the recombinant terminal deoxynucleotidyl transferase (rTdT) reaction mix was added and reactions were incubated for 60min at 37°C. Cells were washed with 2x SSC (sodium chloride/Sodium Citrate) for 15min at room temperature, and then washed twice with D-PBS. Endogenous peroxidases were blocked by treating slides for 5min with 0.3% hydrogen peroxide prepared in D-PBS, and then washed twice more with D-PBS. The Streptavidin antibody solution was diluted 1:500 in D-PBS, and 100µL was added to each coverslip for 30min at room temperature. Cells were washed 3 times with D-PBS, and 100µL of the provided diaminobenzidine (DAB) solution was added to cells for 10-12min, and reactions were terminated by rinsing cells several times in deionized water. Coverslips were mounted using glycerol, and fixed onto slides with clear nail polish. Slides were viewed using a Leitz Laborlux S fluorescent microscope and 4 images per slide were captured with a Retiga 1300 digital camera and OpenLab Software (Quantum Technologies, ON Canada). Dead cells were counted by hand, as outlined above, and cell death is expressed as a percentage of the control +/- SEM.

shRNA And pSILENCER 2.1-U6 Hygro Plasmid

A small hairpin RNA sequence targeting leptin was derived using the target mRNA sequence for siRNA L7 as described by the manufacturer. Sense (5'- GAT CCC GCG TGC TGC AGA TAG CTC ATT TCA AGA GAA TGA GCT ATC TGC AGC ACG TTT TTT GGA AA -3') and antisense (5'- AGC TTT TCC AAA AAA CGT GCT GCA GAT AGC TCA TTC TCT TGA AAT GAG CTA TCT GCA GCA CGC GG-3') oligonucleotides were ordered from UCDNA services and purified by polyacrylamide electrophoresis (PAGE; University of Calgary; Calgary, AB). Nucleotide sequences were annealed by heating equimolar amounts to 95°C in thin walled PCR tubes, and then cooled slowly (2°C/min), using a PCR thermal cycler. Annealed inserts were added into reactions containing linearized pSilencer 2.1-U6 hygro plasmid (Ambion; Austin, TX), and ligated according to the manufacturer's protocol using T4 DNA ligase (Invitrogen). Ligated plasmids (1μL) were diluted and then used to transform competent E. coli (50μL, DH5α; Invitrogen) using a brief heat shock (42°C; 2min). The transformed E. coli was diluted with 950μL of S.O.C. medium (Invitrogen) and incubated at 37°C for 90min while gently rocking. Various volumes of E. coli (1μL, 10 μL and 100 μL) were plated on Luria Agar (Invitrogen) containing 100μg/mL ampicillin (Sigma). To prevent drying out plates were sealed with parafilm, inverted, and incubated overnight at 37°C. The next morning plates were inspected and the number of individual colonies was noted to estimate transformation efficiency. Individual colonies were selected to inoculate 2mL of Luria Bertani (LB; Invitrogen) nutrient broth containing 100μg/mL ampicillin. The primary inoculate (2mL) was incubated for 8h at 37°C while shaking vigorously on a platform shaker. Afterwards the primary inoculate (25μL) was used to inoculate a 250mL flask containing 25mL of LB broth supplemented with 100μg/mL ampicillin and fitted with a sterile gauze plug. Flasks were placed in an incubator containing a platform shaker and vigorously agitated (200rpm) overnight at 37°C. The next morning the secondary

inoculate was divided into two 15mL conical tubes and centrifuged at 5000g for 15min. The supernatant was aspirated and neutralized with bleach, and the pellets were frozen at -70°C until the plasmid prep. Plasmids were isolated and purified using Qiagen MIDI plasmid kit according to the manufacturer's protocol (Qiagen; Burlingtion, ON), and the sequence of the insert was confirmed by DNA sequencing (Cortec; Kingston, ON).

Plasmid Transfection Optimization

C6 cells were plated on glass coverslips as outlined above, except 50,000cells/well were used in 12 well Nunc plates because of cytotoxicity associated with these transfection procedures. Just prior to transfection medium was replaced with 500µL of serum-free DMEM. Plasmid transfection optimization was performed using an enhanced green fluorescent protein (EGFP) expressing plasmid which was chosen based on its similar size to the pSilencer vector that was to be used in subsequent experiments. The EGFP expressing plasmid was obtained from Dr. Paul Linsdell (Department of Physiology and Biophysics) and propagated and purified as outlined above. A fixed amount of plasmid (2.4µg/ well) was diluted to 75µL in OptiMEM. As above varying amounts of lipofectamine 2000 (2, 3, 4 and 6µL) were diluted in OptiMEM to a final volume of 75µL, and incubated at room temperature for 5 min. The plasmid (75µL) and transfection complexes (75µL) were placed into a single tube and mixed by swirling with a pipette tip and incubated for a further 25min. Cells were transfected for 4h using 140µL of the above mixture, and then the medium was aspirated and replaced with DMEM containing 10%FBS. Cells were propagated for a further 24h and then washed with D-PBS and fixed for 5min using 4% PFA. Cells were washed twice and then stained for a further 5min with DAPI (500nM). Coverslips were washed again prior to mounting onto slides using SlowFade® Antifade kit (Molecular Probes), and viewed using a Leitz Laborlux S microscope. Transfection efficiency was confirmed by visualizing EGFP expression within cells (pictures not shown).

Leptin knockdown with shRNA L7

For leptin knockdown studies C6 cells were plated at 150,000 cells/well in 6 well Nunc plates 24h prior to transfection. Medium was changed to 1500µL of OptiMEM and the transfection complexes were prepared as above using the optimized ratios (7.5µL Lipofectamine 2000: 7.2µg/plasmid per each well in a total volume of 450µL). Cells were transfected for 5h, and then propagated for a further 20h prior to isolating total RNA. However unlike above, leptin gene expression was assessed using realtime RT-PCR as described below (please see section VII. Materials and Methods)

Effects Of shRNA L7 On C6 Cell Death

As above C6 cells were plated at 50,000 cells/well in 12 well Nunc plates 24h prior to transfection. Medium was changed to 500µL of OptiMEM and the transfection complexes were prepared as above (2.5µL Lipofectamine 2000: 2.4µg/plasmid per each well in a total volume of 450µL). Cells were transfected for 5h, and then propagated for a further 72h in DMEM containing 2% FBS prior to monitoring cell death using Ethd-1 as outlined above.

Statistics

Data were analyzed using ANOVA and the Newman-Keuls post hoc test, or using the Student's *t-test* where appropriate using graphpad PRISM v4.0a. Significance was set at $p < 0.05$.

IV. Results

Transfection Optimization In C6 Cells Using FAM-Labeled siRNA

As seen in Figure 12A, the nuclei of untransfected C6 cells (DAPI-stained) contain no endogenous green fluorescence. Moreover Oligofectamine (Fig12C) appears to be a much more effective reagent to transfect C6 cells, relative to NeuroPORTER (12B), based on the greater amount of intracellular FAM-labeled siRNA (green

fluorescence) that was visibly detected. In addition the transfection efficiency was dependent on the ratio of Oligofectamine:siRNA, and for C6 cells the optimal ratio of lipid:siRNA was determined to be 2μL Oligofectamine:50pmol FAM-labeled siRNA (data not shown). It is also worth noting that in C6 cells the FAM-labeled siRNA localized in the perinuclear region (Fig 12C).

Semi-Quantitative RT-PCR Optimization In C6 Glioblastoma

The log-linear phase of amplification was determined for leptin and cyclophilin using standard semi-quantitative reaction parameters, and cDNA generated from total RNA isolated from unstimulated C6 cells. As seen in Figure 13A leptin was undetectable until 30 cycles, and amplified linearly until cycle 35 when further increases in the amplicon signal were no longer detectable. Cyclophilin was analyzed in a similar manner except that it was detected as early 15 cycles, and reached its maximum by around 21 cycles (Figure 13B). In all subsequent studies in C6 cells I used 34-35 cycles or 18-19 cycles to amplify leptin and cyclophilin mRNA respectively.

Target Optimization

In preliminary studies four potential rat leptin siRNA targets were tested for their ability to reduce leptin gene expression in C6 cells (for sequences see Table 3). As seen in Figure 14 only siRNA L7 was capable of significantly reducing leptin gene expression (50%; $P<0.01$). In contrast, the other targets showed little or no silencing effect relative to the control, siRNA SCR.

Figure 12: C6 glioblastoma cell transfection optimization
Following transfection with a FAM-labeled siRNA (green) C6 cells were fixed using 4% PFA, and stained with DAPI to visualize the nuclei (blue). Images were overlaid in Adobe PHOTOSHOP (V7.0) to visualize the relative location of the fluorescently labeled siRNA. [A] Untransfected C6 cells contain no endogenous green fluorescence. [B] NeuroPORTER was a relatively poor transfection reagent and failed to deliver a significant amount of siRNA into C6 cells. [C] Cells transfected with Oligofectamine contain larger amounts of FAM-labeled siRNA that localized to the perinuclear regions of cells as indicated by the arrows. (Magnification 400X)

74
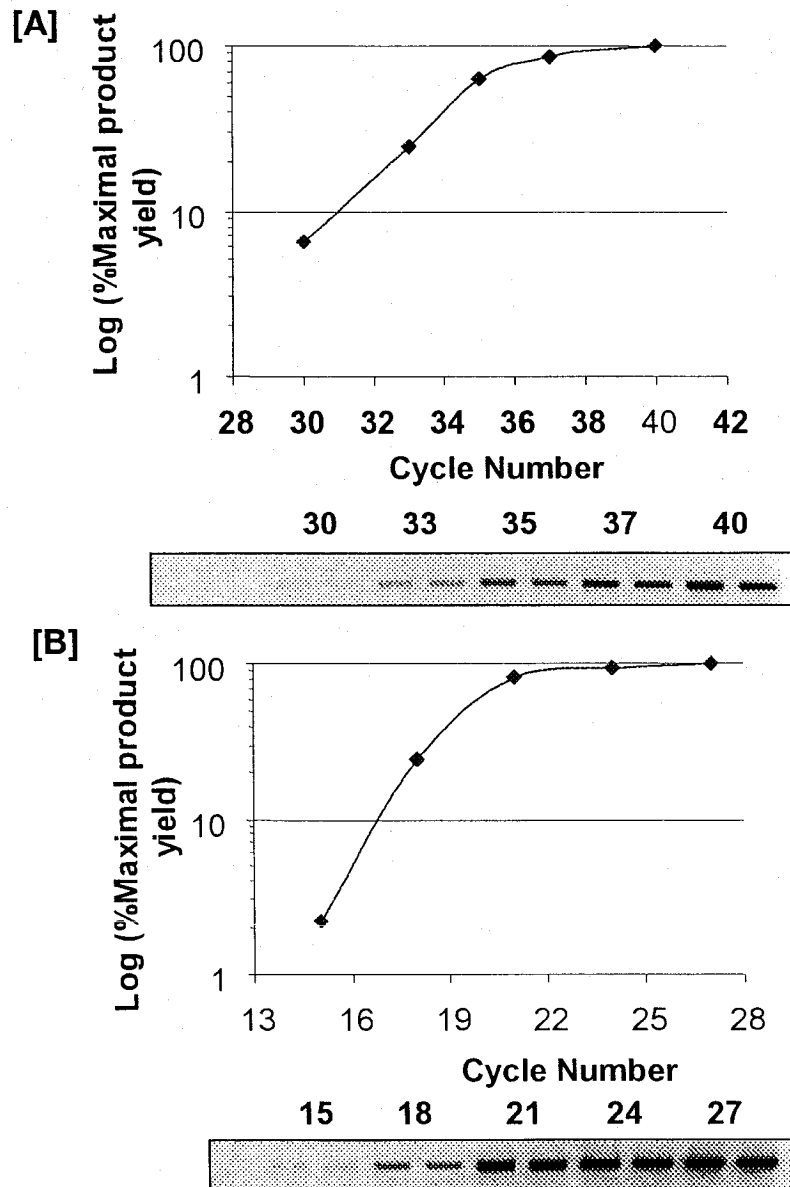
Figure 13: Linear ranges for semi-quantitative RT-PCR in C6 glioblastoma cells

Total RNA was isolated from unstimulated C6 cells and was reverse transcribed (1.5µg in 30µL) using OligodT and amplified by PCR for various cycle numbers using primers specific for rat leptin or cyclophilin. Samples were separated on 1.5% agarose gels stained with ethidium bromide and band intensity was quantified using NIH image (V1.60). Data are expressed as a percentage of the maximal product yield and plotted on a logarithmic scale. As seen in [A], leptin was linearly amplified between 30-35 cycles, which is also seen in the representative agarose gel. [B] Similarly cyclophilin was linearly amplified between 15-21 PCR cycles, and a representative agarose gel is shown.
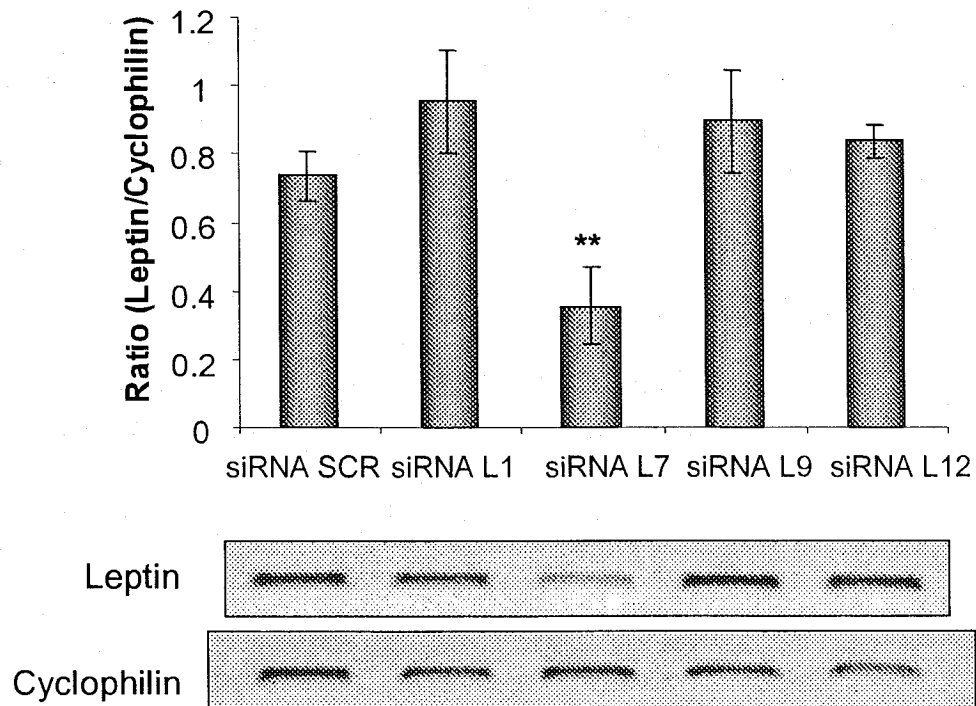
Figure 14: Leptin siRNA target optimization
Various siRNA targets were designed against leptin using the criteria set out by Elbashir *et al.* (2002). Twenty-four hours after transfecting C6 cells (100nM) RNA was analysed by semi-quantitative RT-PCR and relative leptin gene expression is shown as a ratio (leptin/cyclophilin) ± SEM. Representative agarose gels for leptin and cyclophlin are shown. Only siRNAL7 was able to significantly reduce leptin mRNA (greater than 50%; **p<0.01) relative to the scramble control (n = 4 for all)

IVa. Stability Enhanced Vs. Unmodified siRNA

Blocking The dbcAMP Induction Of Leptin Gene Expression In C6 Cells

It has previously been reported that leptin gene expression is stimulated using dbcAMP in C6 glioblastoma (Morash et al. 2000). As seen in Figure 15A the non-specific control, siRNA SCR or siSTABLE SCR, did not prevent 1 μM dbcAMP from inducing a 5-fold increase in leptin gene expression relative to basal levels. In contrast using cells previously transfected with siRNA L7 (Fig15A), or siSTABLE L7 (Fig 15B), the stimulatory effects of dbcAMP were significantly attenuated by 60% (p<0.001) relative to cells transfected with the non-specific controls using both the unmodified, and stability-enhanced, siRNA. However it should be noted that these molecules were not 100% efficient at blocking the stimulation of leptin gene expression since a 2-fold increase was detected (p<0.01 for both) relative to control cells grown in OptiMEM alone.

siRNA Dose Response In C6 Cells

Having established the silencing potential of siRNA L7 and siSTABLE L7, dose response studies were undertaken to optimize the amount of siRNA required to achieve the greatest silencing effect. Leptin gene expression was attenuated in a dose-dependent manner in C6 cells using unmodified siRNA L7, with the 50nM dose achieving the maximal silencing effect, whereas expression began to increase with higher doses (Fig 16A). Similarly the stability-enhanced molecule, siSTABLE L7, dose-dependently silenced leptin gene expression, however the maximal silencing effect was achieved with the 100nM dose (Fig 16B), and unlike the unmodified molecules, a higher concentration (200nM) maintained these silencing effects.

RNAi Time Course In C6 Cells

Using the unmodified siRNA L7 we achieved a maximal knockdown 30h post-transfection, with near normal gene expression resuming by 55h (Fig 17A). Although the stability-enhanced siRNA, siSTABLE L7, achieved maximal silencing (24h; p<0.01) at

77
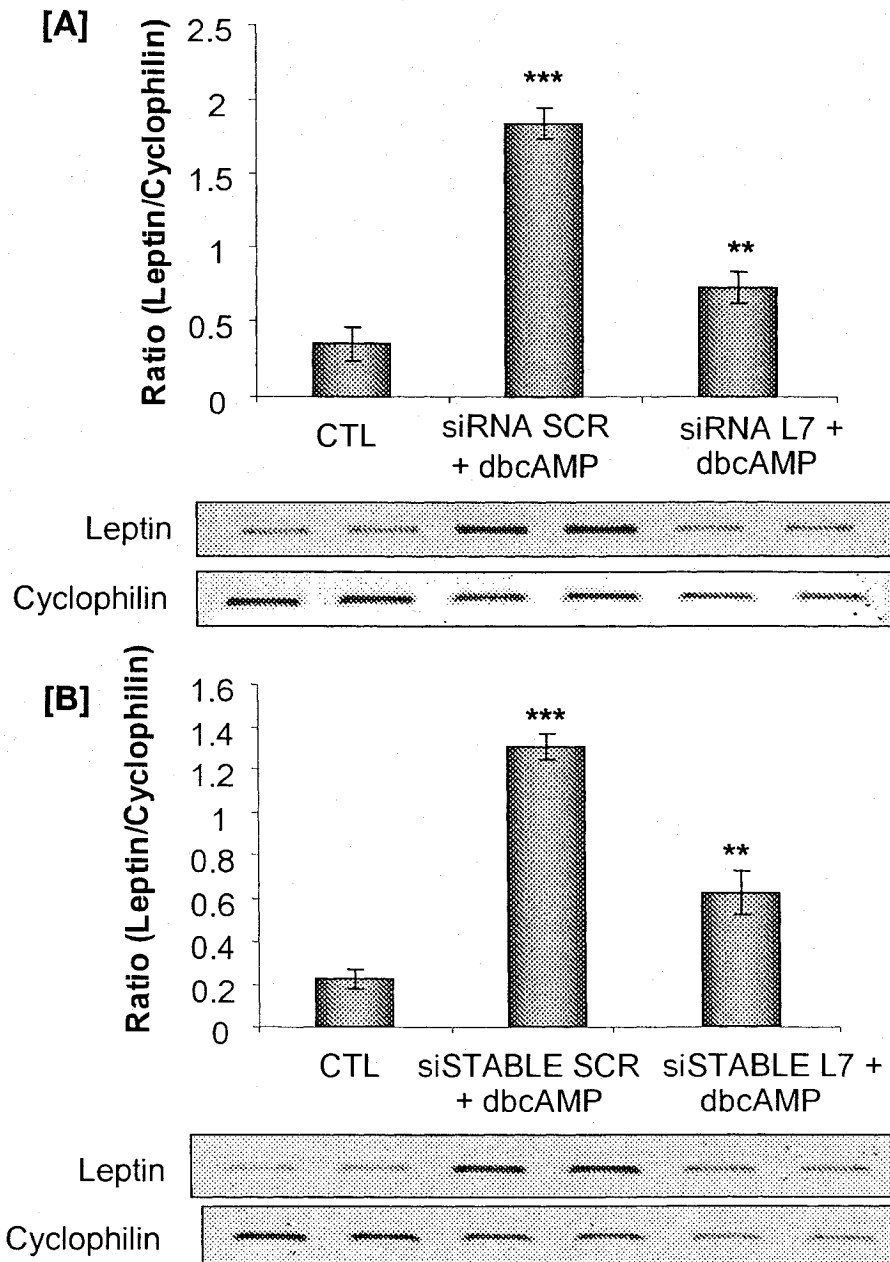
Figure 15: Blocking the dbcAMP-induction of leptin gene expression in C6 cells. Transfecting C6 cells with the non-specific control RNAi molecules, either the unmodified siRNA SCR (100nM) [A] or the stability-enhanced siSTABLE SCR (100nM) [B], failed to block the dbcAMP-mediated induction of leptin mRNA as detected by semi-quantitative RT-PCR. Although neither of the leptin-specific RNAi molecules completely blocked the stimulation of leptin expression, siRNA L7 [A] and siSTABLE L7 [B] were equally effective at attenuating its induction. All values are expressed as a relative ratio (leptin/cyclophilin) ± SEM (n=6 for each condition). Representative agarose gels are shown for leptin and cyclophlin. (**p<0.01, ***p<0.001)
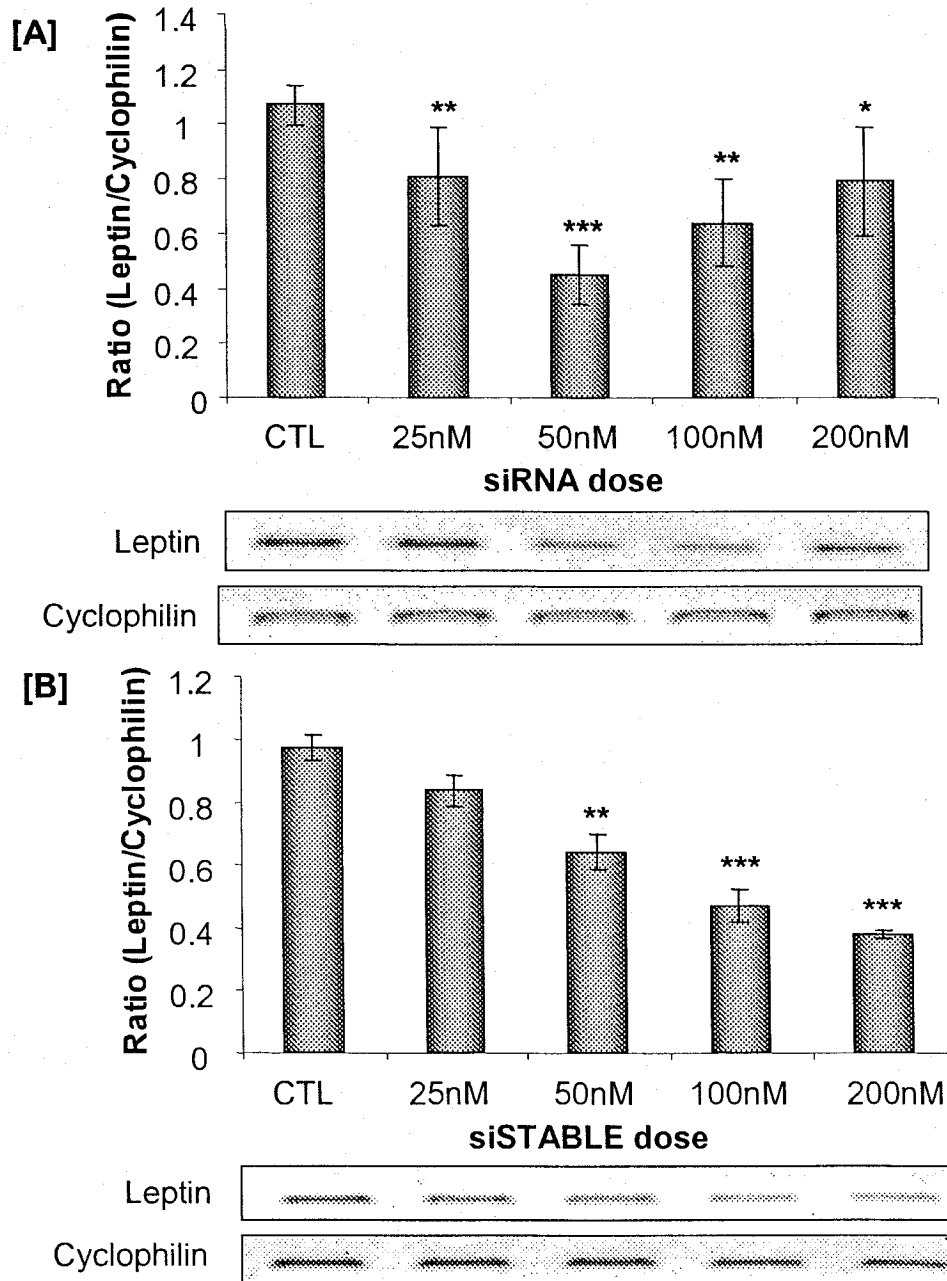
Figure 16: Dose-dependent silencing of leptin gene expression in C6 cells. C6 cells were transfected for 24h with various doses of the unmodified siRNA L7 and leptin gene expression was analysed by semi-quantitative RT-PCR. As seen in [A] the silencing effect was biphasic, and the maximal silencing effect occurred with the 50nM dose. [B] siSTABLE L7 dose-dependently inhibited leptin gene expression with maximal silencing occurring with the 100 and 200nM doses. Values are expressed as a relative ratio (leptin/cyclophilin) ± SEM and representative agarose gels are shown for leptin and cyclophilin in both experiments (n=3-4 for each dose). ($p<0.05$, $p<0.01$, $p<0.001$)

[A]

[B]
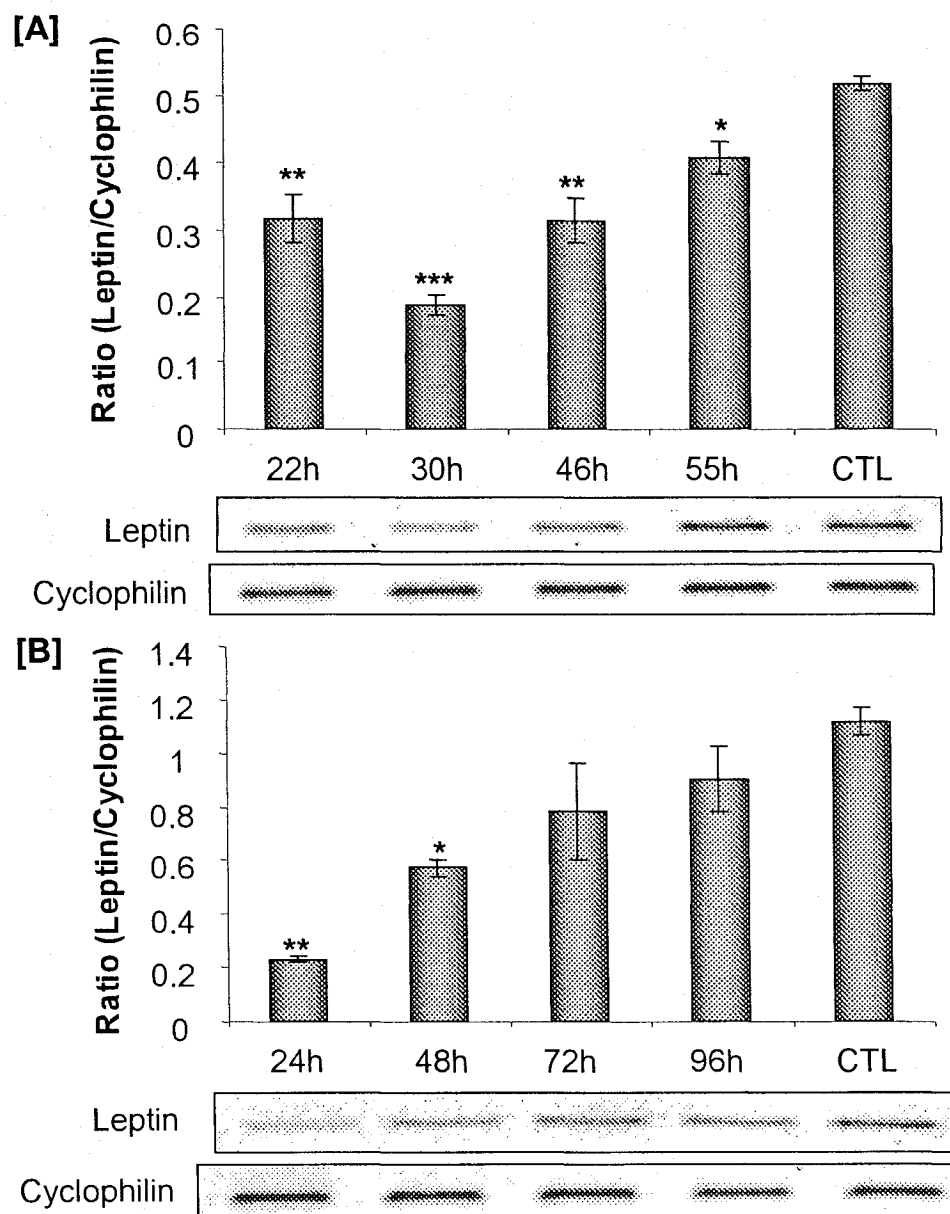
Figure 17: Time-dependent silencing of leptin gene expression in C6 cells.
Leptin and cyclophilin gene expression were analysed at various time points following transfection with siRNA L7 [A] or siSTABLE L7 [B] by semi-quantitative RT-PCR. [A] Maximal gene silencing was detected 30h after cells were transfected with siRNA L7 (100nM), but remained slightly lower 55h later. [B] Leptin gene expression was significantly attenuated for up to 48h following transfection with siSTABLE L7 (100nM) relative to the control. Values are expressed as a relative ratio (leptin/cyclophilin) ± SEM (n=3-4 for each time-point). Representative agarose gels are shown for leptin and cyclophilin in both experiments (*p<0.05, **p<0.01, ***p<0.001)
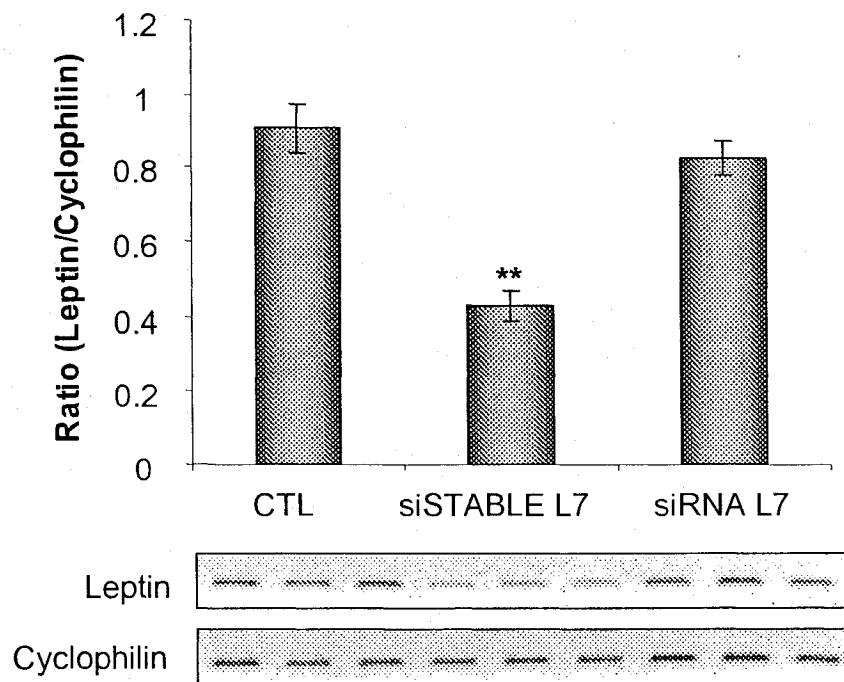
Figure 18: Long-term leptin knockdown in C6 cells; siSTABLE L7 vs. siRNA L7

To prolong the silencing effects C6 cells were cultured in reduced serum conditions (2% vs. 10% FBS) following transfection with either a non-specific control, siSTABLE L7 or siRNA L7 (100nM). Leptin gene expression was analysed by semi-quantitative RT-PCR 72h after transfection. As seen in the graph, only siSTABLE L7 was capable of significantly silencing leptin gene expression over this time frame. Values are expressed as a relative ratio (leptin/cyclophilin) ± SEM and representative agarose gels are shown for leptin and cyclophilin (n=6 for each). (**p<0.01)

about the same time point as siRNA L7, leptin gene expression was significantly

reduced (~50%; p<0.05) for more than 48h (Fig 17B). In addition when C6 cell

proliferation was slowed, by propagating cells in DMEM containing 2% FBS for 72h,

siSTABLE L7 was capable of attenuating leptin gene expression by more than 50%

(p<0.01), whereas leptin mRNA had returned to control values in cells transfected with

siRNA L7 (Fig 18).

Decreased Leptin Protein In C6 Glioblastoma

As above C6 cells were transfected with siSTABLE L7 and propagated for a

further 72 in reduced serum conditions (DMEM containing 2% FBS), and cell lysates

were analyzed by Western blot analysis (Fig 19). Using the pre-optimized conditions we

observed a 55% reduction in leptin protein levels in cells treated with siSTABLE L7,

relative to the scramble control (p<0.0001). Moreover Ponceau S staining confirmed

equal protein loading between samples (not shown).

Silencing Leptin Increases C6 Cell Death

Following leptin knockdown cell death was assessed using a nucleic acid dye,

EthD-1, which can only access the nuclear region of dead cells. As seen in Figure 20A,

reducing leptin gene expression in C6 cells using siSTABLE L7, led to a two-fold

increase in cell death relative to cells transfected with the siSTABLE SCR control

(p<0.001). Likewise when cells were analysed using a TUNEL assay, 72h after being

transfected with siSTABLE L7, a 2.5-fold increase in cell death was detected (Fig 20B;

p<0.001).

Small Hairpin RNA (shRNA) Targeting Leptin

A shRNA targeting leptin was cloned into pSilencer 2.1-U6 hygro plasmid as an

alternate approach to silencing leptin for prolonged periods. When C6 cells were

transfected with the shRNA L7 expressing plasmid we achieved a 45% (p<0.001)

reduction in leptin relative to cells transfecetd with a non-specific hairpin insert, as
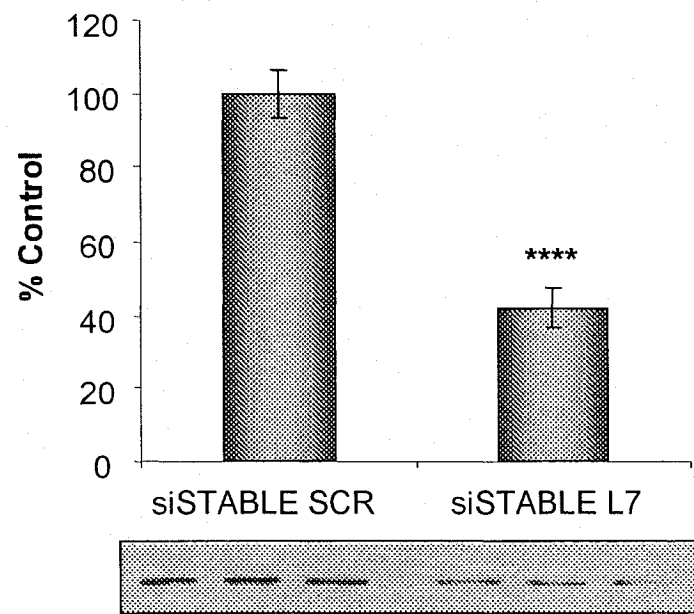
Figure 19: RNAi-mediated reduction in leptin protein
Cells were transfected with siSTABLE L7 (100nM), or the control, and cultured for a further 72h in reduced serum conditions. Cellular lysates were analysed by Western blot and revealed that leptin protein was reduced by 55% following transfection with siSTABLE L7 (*p<0.0001, n=6). Blots were stained with ponceau S to confirm equal loading and transfer occurred between samples. A representative blot is shown for leptin following a knockdown experiment.
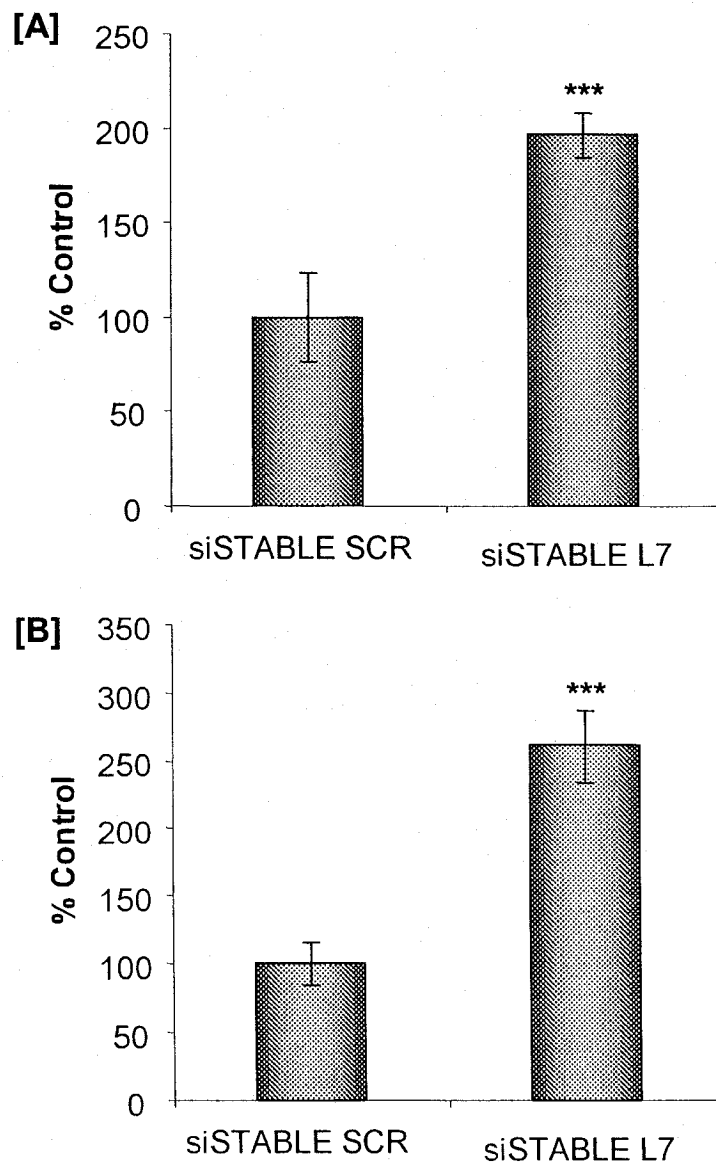
Figure 20: Leptin silencing increased C6 cell death
[A] C6 Cells stained with ethidium homodimer-1 72h after transfection with siSTABLE L7 (100nM) revealed a 2-fold increase in cell death relative to those transfected with the control molecule (siSTABLE SCR). [B] In identical experiments TUNEL staining C6 cells revealed a 2.5-fold increase in cell death. Data were collected from duplicate experiments and values are expressed as a percentage of the control ± S.E.M. (n = 7-8). (***p<0.001)
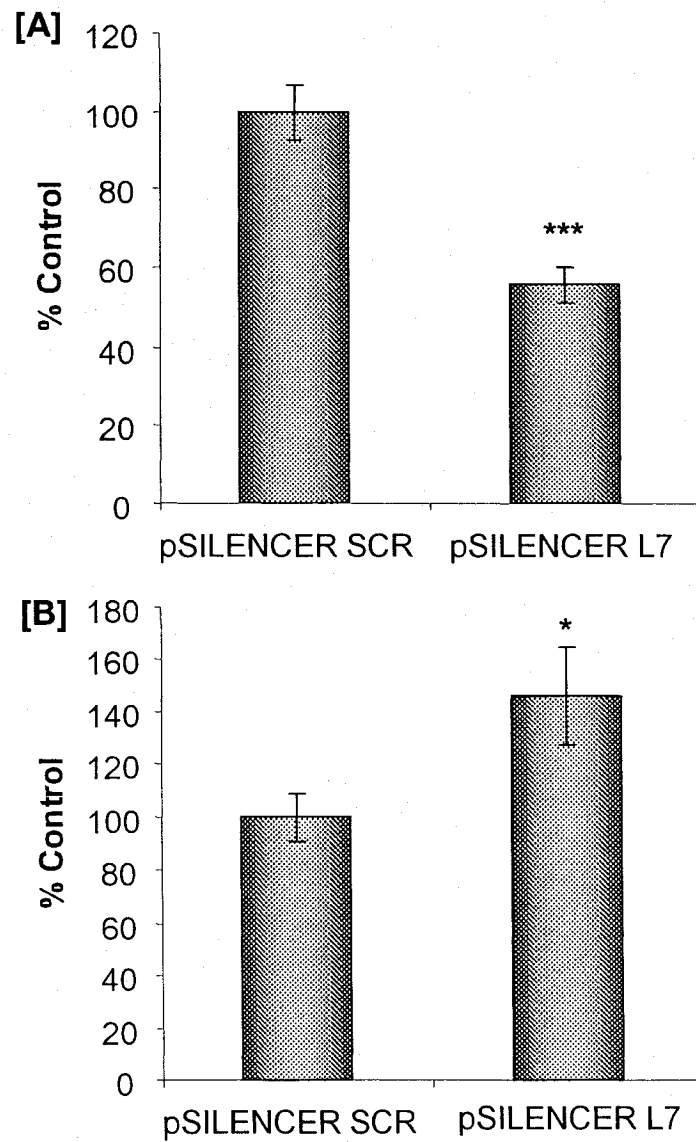
Figure 21: Using shRNA L7 to silence leptin in C6 cells
Transfecting C6 cells with a shRNA L7 expressing plasmid leptin gene expression was reduced by 45% 24h later when measured using realtime RT-PCR (n=4). [B] When C6 cells were transfected with shRNA L7 expressing plasmid and cultured for a further 72h in reduced serum conditions a 45% increase in cell death was detected by staining with EthD-1 (n=11). Values are expressed as a percentage of the control ± SEM. (p<0.05, p<0.001)

detected using realtime RT-PCR (Fig 21A; please see section VII). Moreover when C6 cells were transfected with the shRNA L7 expressing plasmid we were able to detect a 46% (p<0.05) *increase* in cell death 72h after transfection, as assessed using EthD-1, relative to the controls (Fig 21B).

V. Discussion

These data provide the first evidence that leptin mRNA and protein can be significantly decreased in C6 glioblastoma using the RNAi approach. Although I only identified one effective target against leptin, siRNAL7, it was capable of blocking the dbcAMP-dependent stimulation of leptin gene expression in C6 glioblastoma cells. Moreover I established that its silencing properties were dose- and time- dependent. In parallel experiments I employed a stability-enhanced siRNA, siSTABLE L7, which contains chemically modified nucleotides. Like siRNAL7, transfecting C6 cells with siSTABLE L7 was equally effective at blocking the dbcAMP-mediated stimulation of leptin gene expression in C6 cells. Additionally its silencing properties were also time and dose-dependent, however unlike the unmodified siRNA L7, siSTABLE L7 silenced leptin over longer periods and did not appear to induce a 'repelling' silencing effect in the dose response studies. Similarly only siSTABLE L7 was capable of significantly reducing leptin mRNA for up to 72h after transfection, and this effect was further confirmed by western blot analysis. In addition a significant increase in C6 cell death (>2-fold) was detected following leptin knockdown, as measured using ethidium-homodimer-1 and TUNEL staining. An alternate approach to prolong leptin silencing was to generate a shRNA-expressing plasmid that produced shRNA L7 against rat leptin. Although shRNA L7 was capable of silencing leptin, it appeared to be slightly less effective than siSTABLE L7. Also the effects of shRNAL7 on C6 cell death were less prominent than those detected following transfection with siSTABLE L7. Taken together these data suggest that leptin can be silenced in an in vitro model of brain leptin expression, C6

glioblastoma cells, and that the use of siSTABLE L7 was more effective than the unmodified molecule, siRNA L7, or the shRNA L7 expression system.

Although RNAi-mediated silencing of gene expression is transient, this appears to be dependent on the stability of the siRNA molecule and the rate of cellular division. As seen in the time course studies, the stability enhanced molecule reduced leptin over greater periods of time, but reductions in leptin gene expression were still only transient. As explained above (see section II), following target mRNA cleavage and the ATP-dependent release of the resulting fragments, the RISC-siRNA complex becomes free to bind further leptin mRNA. However it is unlikely that this process of recycling is 100% efficient and some siRNA are likely lost and degraded with each cycle. Similarly by culturing C6 cells in serum-deprived medium, to reduce cell proliferation, the transient nature of gene silencing was affected. Under 'normal' conditions (i.e. 10% FBS) leptin gene expression was reduced by 30% 72h after transfecting with siSTABLE L7 (Fig 17B), whereas under 'serum-deprived' conditions (i.e. 2% FBS) leptin remained suppressed by more than 50% relative to controls (Fig 18). This is not unexpected since siRNA-mediated gene silencing appears to persist longer in non-dividing cells. For example RNAi only reduced gene expression for 3-7 days in undifferentiated P19 cells, a proliferating mammalian cell line (Omi et al. 2004). In contrast a long-term knockdown (>3 weeks) was achieved in two in vitro models of non-dividing mammalian neurons that were derived either by the differentiation of mouse embryonic carcinoma P19 or from mouse primary hippocampal neurons, when the same siRNA was employed (Omi et al. 2004). Thus enhanced siRNA stability, and reducing cell proliferation appear to prolong the transient nature of gene silencing. However there are likely other mechanisms also responsible for the transient nature of RNAi-mediated gene silencing.

Early studies on siRNA revealed their tolerance for chemical modification, without compromising their silencing properties. The application of chemically modified siRNA

offers several advantages over other approaches such as enhanced stability, as demonstrated here, and improved target recognition and specificity (Braasch et al. 2003; Layzer et al. 2004). This has become particularly important since the initial enthusiasm for the high specificity of siRNA and shRNA (Elbashir et al. 2001c; Brummelkamp et al. 2002) was soon met with reports of RNAi molecules inducing off target, or non-specific, responses (Jackson et al. 2003; Couzin 2004). Included amongst these off-target responses is the induction of interferon pathways that were thought to involve Toll-Like receptors (TLR), as well as the stimulation of the protein kinase response (PKR) pathway that has been associated with the use of long ds RNA (Sledz et al. 2003; Agrawal and Kandimalla 2004; Jackson and Linsley 2004). The use of chemically modified siRNA appears to be one method to circumvent these unwanted side effects since this approach appears to enhance target recognition (Braasch et al. 2003; Agrawal and Kandimalla 2004). Similarly it is thought that the selective incorporation of modified residues (i.e. 2'-O-methyl guanosine or uridine) disrupts the binding of siRNA to the endogenous RNA receptors implicated in these off-target pathways, without compromising the silencing properties of the siRNA (Judge et al. 2006). As advocated by Stein (2001), we confirmed that leptin was decreased both at the mRNA and protein level in order to limit the possibility that reductions in leptin was the result of activating non-specific pathways (Stein 2001). In addition the three other leptin targets (siRNA L1, L9 and L12), and the scramble control (siRNA SCR), had no effect on leptin expression further suggesting that siRNA L7-mediated leptin knockdown was specific and unlikely to be the consequence of a non-specific RNAi mechanism. Similarly cell death resulted using two different RNAi molecules, siSTABLE L7 and shRNA L7, further suggesting that this effect on cell survival was specific. However investigating the use of alternative targets on leptin gene expression, and C6 cell death, remains an important goal (Jackson and Linsley 2004).

In my hands the siRNA-mediated silencing of leptin was dose-dependent. The unmodified siRNA induced a biphasic response, with the largest reduction in leptin mRNA occurring with the 50nM dose. Although siSTABLE L7 also induced a dose-dependent reduction in leptin, maximal silencing was achieved with the 100 nM dose and there appeared to be no rebound, or 'repelling', effects when cells were transfected with a higher dose. This repelling effect has also been observed in Chinese Hamster Ovary cells (CHO), where higher doses of siRNA induced a more rapid decrease in the length of gene silencing (Hong et al. 2005). It was hypothesized that the higher doses of siRNA increase the expression of endogenous endonucleases such as RNA-specific adenosine deaminase (ADAR) or the exonuclease ER-1 (enhanced RNA interference), leading to a more rapid attenuation of gene silencing both in vitro and in vivo (Hong et al. 2005). Perhaps the reduced silencing effects of shRNA as seen here, and reported by others (Yu et al. 2002), could result from the continuous expression of shRNA that induce these endogenous exonucleases and activate this 'repelling' response. Similarly the chemical modifications associated with siSTABLE L7 may help prevent it from binding these RNA sensing receptors, as well as increase its resistance to these endogenous exonucleases, allowing it to elude the RNAi-attenuating mechanism.

A functional consequence of this inhibition was a marked induction of cell death in C6 cells, which is consistent with the recent demonstration that leptin treatment increased cell proliferation, by inhibiting apoptosis, in the human neuroblastoma cell line, SH-SY5Y (Russo et al. 2004). Although this was an opposite approach, by decreasing local leptin expression I was able to detect the anticipated increases in C6 cell death. A two-fold increase in cell death was detected in cells transfected with siSTABLE L7, relative to control-treated cells (Fig 20A; $p < 0.001$), when cells were assessed using EthD-1, a nuclear stain that can only enter dead cells with compromised plasma membranes (Levesque et al. 1995). In similar experiments a 2.5-fold increase in cell

death was detected using TUNEL staining (Fig 20B; p<0.001), indicating that DNA

fragmentation had also occurred (Spanova et al. 1997). Thus it seems likely that

silencing of leptin expression induces apoptosis in C6 glioblastoma cells, although this

will require further confirmatory studies (Ishimaru et al. 1999). However as noted

already, leptin reduced apoptosis in human neuroblastoma cells (Russo et al. 2004), and

this effect was at least partially dependant on the long form of the leptin receptor

(OBRb). Although C6 cells do not express OBRb, they do express the short form of the

leptin receptor, OBRa (Morash et al. 2000). This implies that the OBRa might also be

capable of mediating the anti-apoptotic effects of leptin. However, comparable studies in

vivo indicate that the absence of leptin (in ob/ob mice) or OBRb (db/db mice) results in

neurodegeneration and smaller brains compared to control mice (Ahima et al. 1999).

Since the db/db mouse has a normal brain complement of OBRa (Ahima and Flier

2000), this receptor is clearly not sufficient for cell survival in the presence of leptin. We

conclude that the anti-apoptotic effect of leptin in C6 cells may not be mediated via

OBRb or OBRa receptors, further suggesting an alternative leptin receptor may exist.

In these in vitro studies I established that RNAi is a feasible approach to study

the function of brain leptin. Although three RNAi approaches were tested (i.e. unmodified

siRNA, a shRNA expressing vector, and a stability-enhanced molecule, siSTABLE) the

chemically modified molecule appeared to offer the greatest advantages, most notably

maximal leptin knockdown over an adequate time span to achieve a physiological effect.

In addition these knockdown studies suggest that endogenous leptin is involved in C6

cell survival. In conclusion these data suggest that RNAi is a reasonable approach to

study the physiological function of leptin in the in vivo CNS.

The following section describes experiments designed to acutely block the

induction of leptin gene expression in the intact rat brain by injecting siSTABLE

L7 into either the lateral ventricle (LV) or dorsal third ventricle (D3V). Similarly

siSTABLE L7 was continuously infused into the lateral ventricle for a period of 14 days to verify whether 'naked' siRNA could suppress leptin gene expression, and to determine whether significant changes in bodyweight occurred. In addition to monitoring bodyweight and food intake, leptin, resistin (*rstn*) and *socs-3* gene expression were measured using realtime RT-PCR.

VI. Study Of Leptin Knockdown In The Intact Rat Brain

The Use Of RNAi To Study Central Metabolic And Appetite Regulatory Pathways

Using RNAi to investigate brain-derived adipokines is a logical next step since it will permit the distinction between the effects of peripheral vs. centrally-derived ligands, and does not require complex and expensive genetic manipulations that are required with standard knockout (KO) procedures. Moreover this approach has been used successfully by others to study neuropeptides implicated in hypothalamic energy balance. For example Makimura et al. (2002) first used siRNA, and a shRNA-expressing system, to silence hypothalamic AgRP expression which led to modest reductions in the bodyweights of mice (Makimura et al. 2002). Subsequently others have used viral-mediated RNAi systems in rats in order to silence leptin receptor expression in the CNS (Hommel et al. 2006), whereas non-viral RNAi approaches have also been used to successfully block the induction of STAT-3 expression (Satriotomo et al. 2006), a critcal component of the leptin signaling cascade. Although various RNAi approaches have successfully been used in the CNS, (extensively reviewed by (Fountaine et al. 2005; Thakker et al. 2006)), I will discuss them briefly in order to justify my choice for using a non-viral RNAi approach for these investigations.

These experiments are designed to test the hypothesis that the silencing of central leptin, using our siSTABLE L7 target, will induce detectable changes in body weight and food intake. Similarly other adipokines that have been implicated in hypothalamic energy metabolism are also readily detected in the rat brain

[A]

| CTX | PIT | HYP | FAT |

rstn

[B] **fiaf**

| CTX | PIT | HYP | FAT |
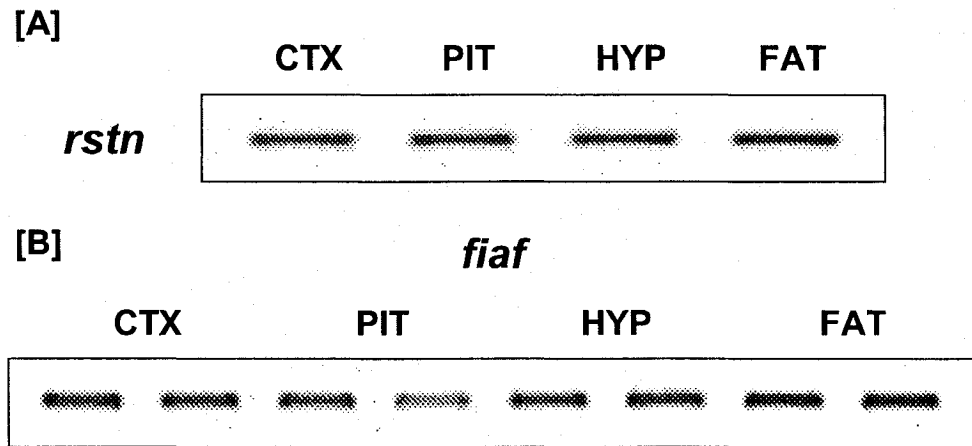
Figure 22: Detecting rstn and fiaf in the rat brain and pituitary using RT-PCR

[A] Resistin (rstn) and [B] FIAF (fiaf) gene expression were readily detectable by RT-PCR in various rat tissues collected from male adult rats. As shown here, amplicons of the expected size were detected in all tissues analysed when PCR products were separated on 2% agarose gels stained with ethidium bromide.

including resistin (*rstn*), fasting-induced adipose factor *(fiaf)* (see Figure 22), as well as suppressor of cytokine signaling-3 (*socs-3*) (further detail on these targets is provided in Chapter 3). The expression of these other related brain adipokines will also be investigated to verify whether adipokine crosstalk could be occurring in the intact rat CNS.

In Vivo Silencing Using siRNA

Although RNAi appears to be a highly effective means to silence gene expression in non-mammalian species, most notably C. elegans, or roundworms (Fire et al. 1998), its application to mammalian species had been limited because of the non-specific responses associated with the introduction of long ds RNA into mammalian cells. Despite the effectiveness of siRNA to overcome these specificity issues *in vitro* (Elbashir et al. 2001a), initially it was unclear whether siRNA were capable of reducing target gene expression *in vivo*. One of the earliest reports revealed that siRNA could silence gene expression in the mouse liver (McCaffrey et al. 2002), and the *in vivo* silencing potential of siRNA was soon confirmed in a variety of tissues including the kidney, spleen, lung, liver and pancreas (Lewis et al. 2002). In addition the injection of 'naked' siRNA induced the expected reductions in target gene expression in mice without activating the interferon response pathways which have been associated with off-target RNAi effects (Heidel et al. 2004; Jackson and Linsley 2004). Moreover the profile of siRNA-responsive tissues is consistent with the localization pattern obtained when a radioiodinated siRNA was administered peripherally, with the largest signal being detected in the liver and kidneys (Braasch et al. 2004). However little if any radioactivity, was detected in the CNS suggesting that siRNAs do not cross the highly impermeable BBB (Braasch et al. 2004). In contrast when a siRNA targeting agouti-related peptide (AgRP) was bilaterally injected into the mouse brain a 50% reduction in AgRP mRNA was detected (Makimura et al. 2002). Similarly a reduction in AgRP, as

seen with immunohistochemistry, was also detected following the intracerebroventricular

(icv) injection of a pSUPER shRNA-expressing plasmid that was designed to target

AgRP (Makimura et al. 2002). Thus RNAi appeared to be an attractive option to silence

gene expression in vivo, more specifically within the CNS.

Viral Based Delivery

One of the largest obstacles facing the application of siRNA to in vivo systems is

the route of delivery (McCaffrey et al. 2002; Paroo and Corey 2004). This led others to

develop viral based systems whose greatest advantage is their ability to efficiently

deliver shRNA expressing cassettes into a variety of tissues, including the brain (Xia et

al. 2002; Hommel et al. 2003). Viral-mediated gene delivery systems have been in use

for a few years, and these vectors are highly efficient at transducing a variety of dividing

and non-dividing cell types, including neurons (Davidson and Breakefield 2003).

Although viral-based systems have become the fashionable, or preferred, method of

delivering shRNA expressing cassettes into the CNS, they are not without

disadvantages. For example despite the ability of lentiviruses to efficiently transduce

neurons in order to deliver genes, precautions must be taken to eliminate possible

immunogenic and oncogenic effects that may occur since these viruses encode foreign

proteins and are prone to integrating into the target cell genome which could potentially

disrupt the expression of tumor suppressor genes (Kaiser 2003; Thomas et al. 2003). In

contrast, recombinant adeno-associated viruses (rAAV) do not appear to integrate into

the host genome, nor are they prone to the immunogenic effects associated with some

of the earlier viral vectors (Fountaine et al. 2005). In addition shRNA appear to be less

effective at silencing gene expression than siRNA (Yu et al. 2002), and the constitutive

expression of shRNA might induce the expression of endogenous endonucleases that

have been reported when cells were treated with high doses of siRNA (Hong et al.

2005). Similarly the AAV-mediated delivery of a shRNA expressing vector proved fatal in

mice, which was attributed to the saturation of the endogenous RNAi pathways in mice (Grimm et al. 2006). In addition, the generation of shRNA expressing viral vectors is time consuming, especially since alternate non-viral RNAi approaches appear equally effective for studying gene function in the CNS.

Non-Viral Mediated Silencing Of Gene Expression In The Brain

To reiterate, one of the greatest challenges facing the application of RNAi based technology is the efficient mode of intracellular delivery, either in vitro or in vivo (McCaffrey et al. 2002; Paroo and Corey 2004). To improve in vivo delivery chemically modified siRNA molecules have been used, such as siRNAs conjugated with a 3' cholesterol group, in order to facilitate cellular uptake (Rossi 2004; Soutschek et al. 2004). Others have overcome this issue by using electroporation (Akaneya et al. 2005), or employing lipid-based transfection reagents such as JetSI™ (Hassani et al. 2005; Guissouma et al. 2006) or Oligofectamine™ (Baker-Herman et al. 2004; Satriotomo et al. 2006). However the continuous infusion of 'naked', or unmodified siRNA, has also been shown to successfully reduce the expression of purinergic (P)2X-ATP receptors (P2X3) in the dorsal horn (Dorn et al. 2004). Likewise the continuous infusion of a GFP-specific siRNA into the lateral ventricle (LV) of EGFP transgenic mice over a period of 14 days was shown to induce widespread knockdown throughout the CNS (Thakker et al. 2004). This was accomplished using an osmotic minipump that was attached to a catheter tube and brain infusion cannula that delivered a continuous supply of siRNA into the lateral ventricle (see Figure 23) (Thakker et al. 2006). Although knockdown appeared to be mostly localized to proximal tissues near the site of infusion, reductions were also detected in more distal brain regions. This localized pattern of knockdown is consistent with the evidence suggesting that siRNA can pass between closely associated neurons (Zhao et al. 2006). Similarly these reductions in EGFP expression did not appear to be an indirect consequence of neurotoxicity (Thakker et al. 2004; Hoyer et al. 2006). Not
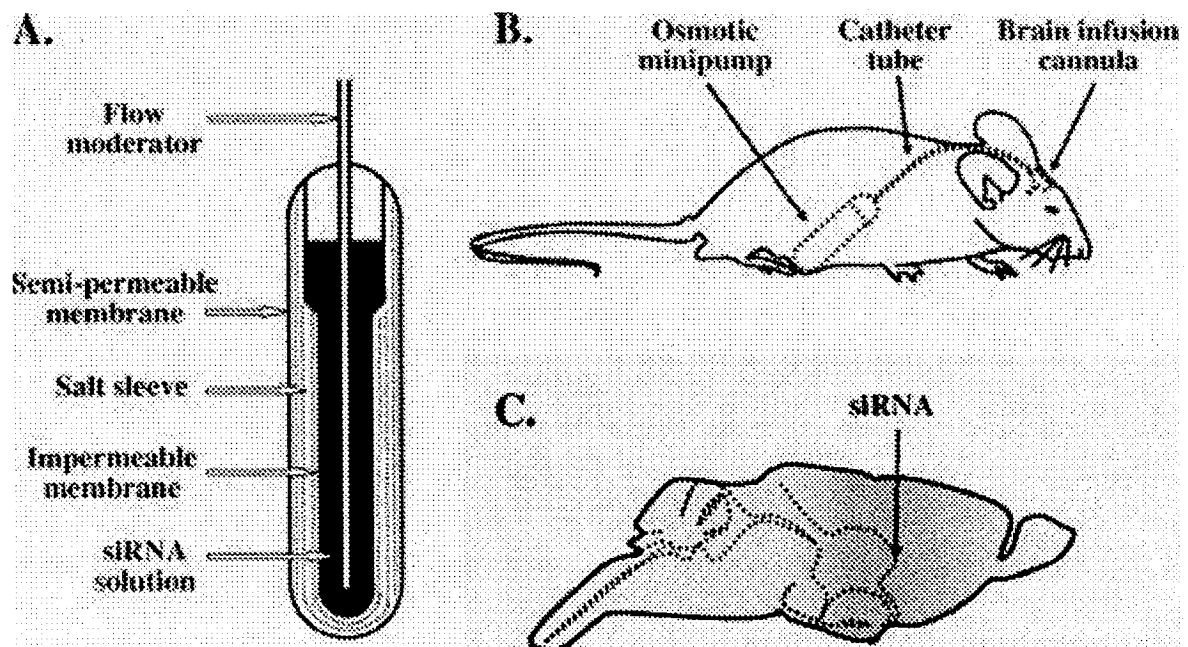
Figure 23: Non-viral RNAi in the intact rodent CNS
Recent advancements in RNAi have shown that the central infusion of 'naked' siRNA is capable of inducing widespread gene silencing in the rodent CNS. [A] Osmotic minipumps are filled with an isotonic siRNA solution that are capable of releasing a continuous flow of siRNA over a period of 14 days. [B] Pumps are implanted behind the neck and are connected by catheter tubing to a brain infusion cannula that is affixed in the lateral ventricle. [C] Although gene knockdown is reported to be widespread, the greatest reductions, as indicated by the intensity of shading, were detected in close approximation to the site of infusion. (Figure adapted from Thakker et al. 2006)

only did this approach successfully silence the expression of the serotonin transporter (SERT), but the behavioral effects observed in these knockdown animals were similar to those obtained from animals treated with serotonin-specific reuptake inhibitors (SSRI) (Hoyer et al. 2006). Thus the infusion of 'naked' siRNA molecules appears to be an attractive option since it does not require the generation, and screening, of viral vectors that express shRNA. Moreover this approach appears capable of inducing wide-spread knockdown in the CNS, whereas viral vectors appear more limited to their site of injection (Thakker et al. 2006). Also no precautions need to be taken to control for potential oncogenic or immunogenic effects that can occur when using a viral based delivery approach (Kaiser 2003; Thomas et al. 2003).

Acute Gene Silencing In The Intact CNS

In contrast to the long-term infusion, when 'naked' siRNA were acutely (1-3days) infused into the brain they appeared ineffective at transducing brain cells or silencing target gene expression (Isacson et al. 2003; Senn et al. 2005). In a similar study a highly effective siRNA against BDNF that was identified in vitro appeared devoid of silencing properties when injected into the spinal cord (Baker-Herman et al. 2004). Alternatively, this same target efficiently blocked the hypoxia-mediated induction of BDNF when it was complexed with Oligofectamine™ in order to facilitate intracellular delivery (Baker-Herman et al. 2004). Similar results were obtained when an siRNA targeting STAT-3 was used to block increases in its gene expression that normally occur following transient focal ischemia in the cerebral cortex (Satriotomo et al. 2006). Thus despite the limited ability of these siRNA to acutely silence basal levels of gene expression, they appear highly effective at attenuating the induction of gene expression in the CNS.

Although I have shown that RNAi is an effective means to silence brain leptin in vitro, it remains unclear whether this approach will work in vivo. The first step will be to test the effectiveness of siSTABLE L7 to acutely block the induction of leptin gene

expression in the rat brain. Previously our laboratory has shown that hypothalamic leptin gene expression is nutritionally-sensitive in the rat; it is decreased by an overnight fast and is partially restored by refeeding (Wilkinson et al. 2007). Thus fasted rats, with low hypothalamic leptin expression, will be injected with siSTABLE L7 icv into the LV or D3V, or the non-specific control molecule, as described previously (Baker-Herman et al. 2004; Satriotomo et al. 2006). Leptin expression will subsequently be induced by refeeding rats for 24h prior to collecting tissues for gene expression analysis. Similarly I will test whether the continuous infusion of siSTABLE L7 into the lateral ventricle over a period of 14days, as done previously for mice (Thakker et al. 2004; Thakker et al. 2005), is capable of reducing bodyweights or food intake in adult male rats. Tissues will also be collected on the final day for measuring changes in leptin gene expression, but the expression of another brain-derived adipokine, resistin, and *socs-3*, a negative feedback inhibitor of leptin gene expression (Munzberg and Myers 2005), will be analysed. **These experiments will verify whether the non-viral RNAi approach is an efficient method for silencing leptin gene expression in vivo. In addition these experiments will attempt to clarify whether brain-derived leptin serves any physiological purpose, or impacts the expression of other brain-derived transcripts, despite its relatively low abundance within the rat central nervous system.**

VII. Materials And Methods

Rats

Male Sprague-Dawley rats (150-175g) were obtained from Charles River Breeding Farms (St. Constant, QC) and were provided with Purina rat chow and drinking water ad libitum. Animals were housed in pairs in clear plastic cages under a 12h light: 12h dark photoperiod and were permitted to acclimatize for 7-9 days prior to experimentation. On the day of experimentation animals were moved together into the surgical suite, and following procedures, were returned to the rat room for observations.

ICV Injection Of siRNA Into The Lateral Ventricle Or Dorsal Third Ventricle

Both groups of animals (siSTABLE L7 and siSTABLE SCR) were deprived of food overnight prior to the icv injection of siRNA. Rats were anesthetized using isoflurane USP (Abbott laboratories, Montreal, QC) administered using a vetroson® non breathing anesthetic machine (Summit hill laboratories; Nevesink, NJ). Just after falling asleep rats were fixed in place using a Stoelting stereotaxic frame (Harvard Apparatus; Montreal QC) and unconsciousness was maintained throughout the experiment by providing a 3.5-4% mixture of isoflurane gas delivered using an oxygen gas flow. An ocular lubricant (Refresh® Lacri-Lube®; Allergan; Markham ON) was applied to their eyes to prevent drying out. After shaving, the scalp was washed with 70% ethanol and the surgical field was sterilized using an iodine solution. A 1.5cm rostral-caudal incision was made using a 10 blade in order to expose bregma. The Hamilton needle and syringe (10µL; Hamilton; Reno, Nevada) was fixed onto the manipulator arm of the stereotaxic frame (Harvard Apparatus) and brought to bregma, and coordinates were noted. Membranes were then removed by gently scraping the skull with the scalpel blade, and the field was dried using sterile gauze pads. Stereotaxic coordinates for locating the appropriate site of injection were obtained from a rat brain atlas (see Figure 24) (Paxinos and Watson 1986). For injections into the lateral ventricle (LV) the manipulator arm was placed -1.0mm caudal of bregma, 1.5mm lateral to the midline, whereas for the dorsal third ventricle (D3V) the needle was placed -1.3mm caudal to bregma, and 0.4mm lateral to the midline. After determining and marking the site of injection a small hole was made in the skull using a rotary tool attached with a flexible shaft and a stainless steel burr (Fine Science Tools; North Vancouver, B.C.). The needle was filled with 10µL of transfection solution (8.5µL of a 50µM siSTABLE L7 that had been mixed with 1.5µL of Oligofectamine™ 15min earlier as described by others (Baker-Herman et al. 2004; Satriotomo et al. 2006)), and then slowly lowered into place (-4.3
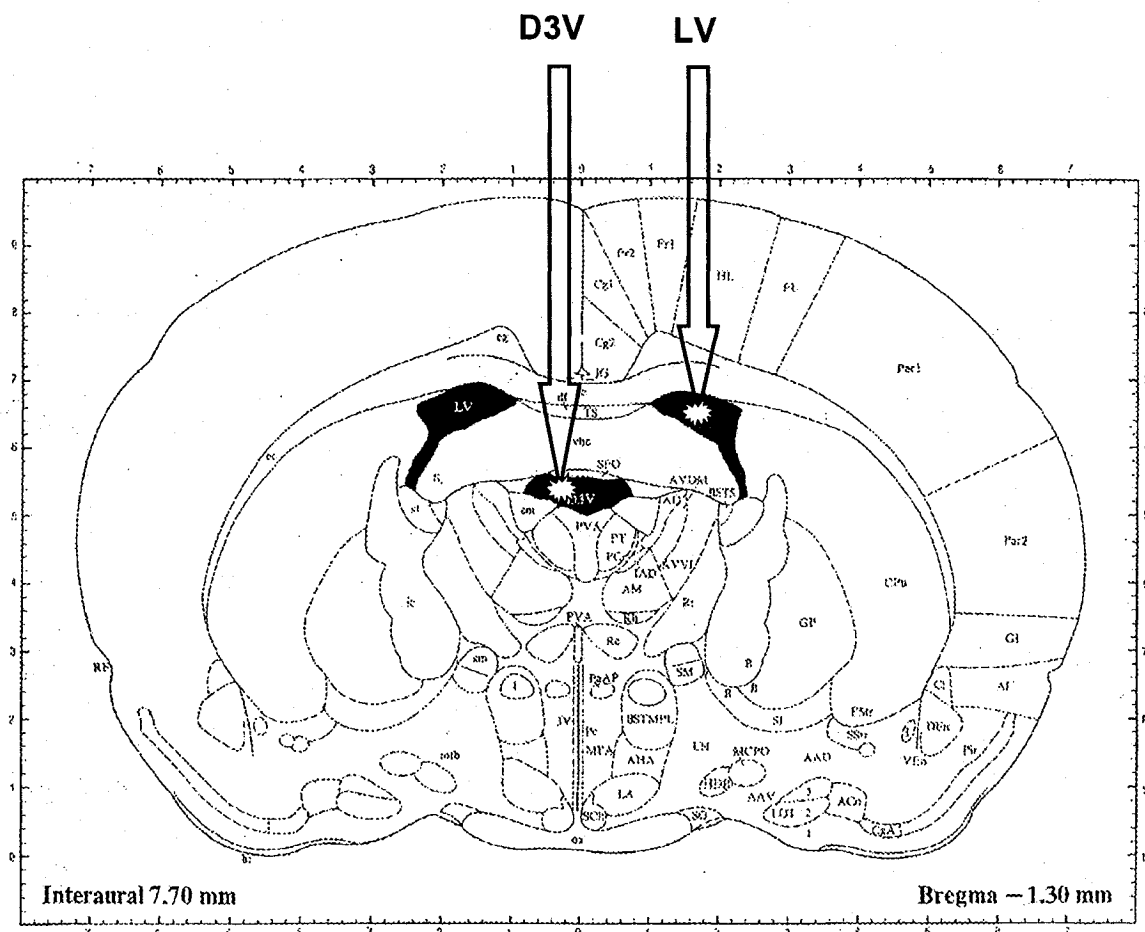
Figure 24: Injecting into the rat ventricular system
The leptin-specific siSTABLE L7, or the control molecule, were injected either into the dorsal third ventricle (D3V), or the lateral ventricle (LV), as indicated by the arrows using predetermined stereotaxic coordinates. (Figure adapted from Paxinos and Watson 1986)

mm for the LV, or -4.6mm for the D3V). The solution was slowly injected over a period of 1-2 min and the needle was left in place for a further 60-90s. The needle was slowly retracted and the scalp was closed using wound clips. Incisions were covered with a 2% xylocaine jelly (Astra Pharma INC; Mississauga, ON) and rats were injected with ketoprofen (Merial; Baie d'Urfé, QC, 5 mg/Kg), a non-steroidal anti inflammatory analgesic. Rodents were weighed and then allowed to recover individually for 15 min prior to returning to their home cage where they were housed in pairs. An excess of food was measured out for each cage just after surgery, and then weighed again in the morning. The difference was used to calculate the total overnight food intake per rat. Animals were closely monitored for the next couple of hours to ensure that wounds were properly closed and there were no adverse effects from the surgery. Approximately 24h post-injection rats were reweighed, sacrificed by decapitation and tissues were dissected out (cortex (CTX) is parietal cortex 2x2 mm on either side of the midline and is 1-2mm thick; and mediobasal hypothalamus (HYP) was isolated by using 2 coronal cuts, rostral to the mamillary bodies and caudal to the chiasm, and 2 saggital cuts 1mm on either side of the mid line and about 2mm thick) and snap frozen on dry ice and stored at -70°C until processing.

ICV Injection Of Leptin Into The Lateral Ventricle

Rats that were fed ad libitum were injected exactly as above except that they received a single injection of recombinant human leptin (Sigma; 7.5µg in 7.5µL) into the lateral ventricle. Animals were allowed to recover individually for a brief period (30min) prior to sacrifice. Following decapitation tissues were collected (CTX and HYP) and snap frozen on dry ice and stored at -70°C until processing.

Preparation Of The Osmotic Minipumps And ICV Cannula

Alzet® osmotic minipumps (model # 1002; 0.25µL/h) and brain infusion kits were obtained from Durect (Cupertino, CA). All the filling procedures were carried out in a

laminar flow hood. Pumps and brain infusion kits were opened and placed in sterile 60mm culture dishes. Catheter tubing was cut to length (2-2.5inches for a 200 g rat) and connected to the brain infusion cannula, and the other end to the flow regulator. Extra tubing (1cm) was added on to the flow regulator to facilitate filling (see Figure 25). The pumps were loaded with a 50µM siRNA solution (siSTABLE L7 or siSTABLE SCR) according to the manufacturer's instructions until brimming. Catheter tubing was held so that the ends of the brain infusion cannula and flow regulator were at an even height to prevent leakage, and was filled carefully using a syringe and the provided needle to avoid the introduction of air bubbles. The extra tubing was carefully removed so that the flow regulator was also brimming and then it was carefully inserted into the mini pump to avoid generating, or introducing, any air bubbles into the catheter tubing. Pumps, and the connected cannula, were placed back into their respective culture dishes, and then 5mL of sterile saline was added. Dishes were sealed with parafilm and incubated in a humid incubator overnight at 37°C in order to activate the pumps.

Minipump Implantation

As above rats were anesthetized, placed into the stereotaxic frame and their scalps were shaved prior to making an incision. After noting the coordinates of bregma a pouch was formed under the skin just above the shoulders by inserting sterile blunt end scissors through the incision made in the scalp. Membranes were then gently cleared from the skull using a 10 blade, and the skull was dried using sterile gauze pads. Using the recorded coordinates for bregma, the location of the lateral ventricle was found (-1.5mm lateral and -1mm caudal of bregma (Paxinos and Watson 1986) (see Figure 24) and earmarked on the skull using an extra brain infusion cannula that was fixed in a pinvise attached to the manipulator arm. The cannula was slightly retracted so a hole could be drilled through the skull using a rotary tool attached with a flexible shaft and a stainless steel burr (Fine Science Tools). The extra 'dummy' cannula was removed from

[A]

[B]
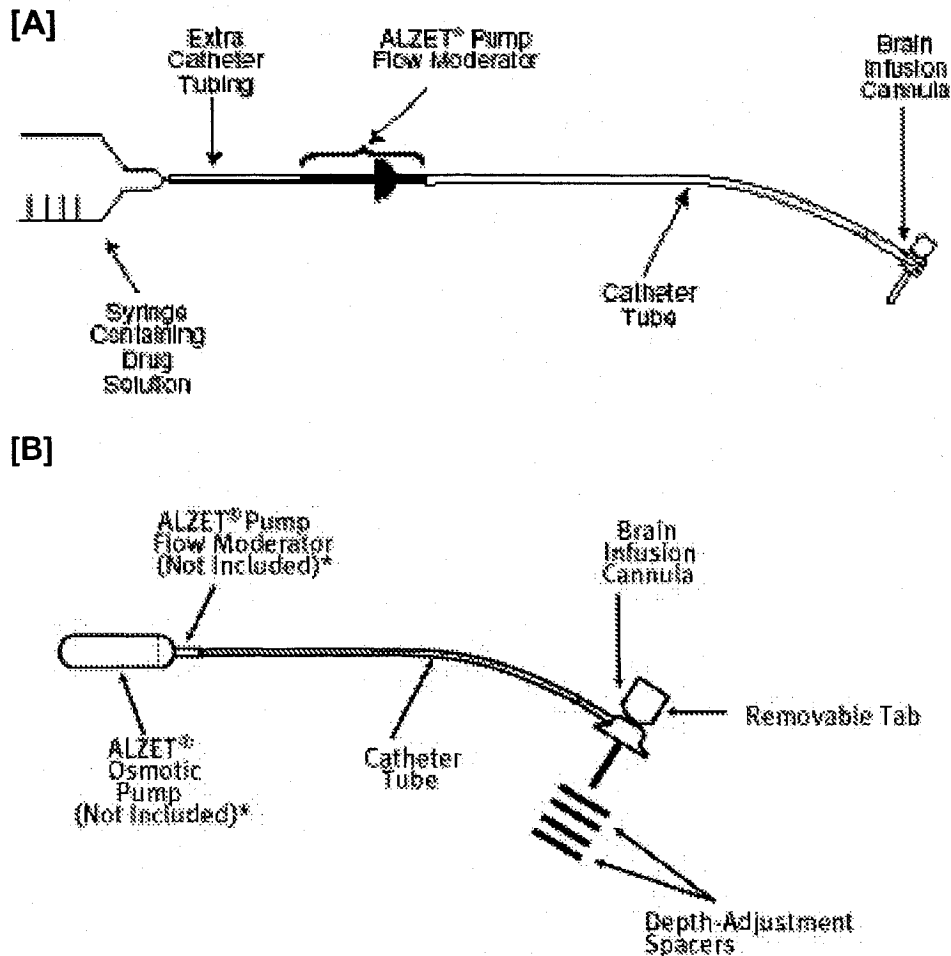
Figure 25: Filling and implanting osmotic mini pumps

[A] After connecting the flow modulator to the brain infusion cannula with catheter tubing, the assembly was carefully filled using a needle and syringe with the same siSTABLE solution that was used to fill the minipumps. Note that the flow modulator and infusion cannula were held at the same height during filling. [B] Subsequently the flow modulator was carefully inserted into a pre-filled osmotic minipump in order to avoid the introduction of any air bubbles. Pumps were activated by an overnight incubation at 37°C in sterile saline. (Figures were obtained, and adapted, from www.alzet.com)

the pinvise and replaced with the sterile brain infusion cannula so that the pump could be inserted into the pouch made under the skin via the incision made in the scalp. The skull was dried again just prior to adding instant adhesive (Loctite; Mississauga, ON) to the bottom of the brain infusion cannula as it was lowered onto the skull. After drying for 1min, the plastic holder on the ICV cannula was cut off and the incision was closed using wound clips. Wounds were covered with a 2% xylocaine jelly (Astra Pharma INC) and rats were injected with 5mg/Kg of ketoprofen (Merial). Rats were allowed to recover individually for 15 min prior to returning to their home cage where they were housed in pairs. Animals were closely monitored for the next couple of hours to ensure that their wounds were properly closed and there were no adverse effects from the surgery. Body weights were recorded daily for the 14 days following surgery between 11:00-14:00h. On the final day food was measured just before lights out and then weighed again in the morning, and the difference was used to calculate the total overnight food intake per rat. On the last day rats were removed individually into a separate room prior to sacrificing by decapitation. Tissues (CTX; HYP; whole pituitary (PIT; anterior and posterior lobes) and epididymal visceral adipose tissue, (FAT)) were collected and snap frozen on dry ice and stored at -70°C until processing.

Total RNA Isolation From Tissues

For hypothalamic, pituitary and cortex tissues total RNA was isolated using the RNEasy mini kit (Qiagen) as outlined above following the DNase protocol (see section III). However one notable exception is that during lysis tissues were homogenized with an 18 gauge needle and 1mL syringe prior to loading lysates onto the Qiashredder column. For adipose tissue total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. In brief, frozen samples were homogenized in 1.5 mL of Trizol reagent for 30s using a tissue homogenizer. The homogenate (1mL) was transferred to a sterile Eppendorf tube and samples were centrifuged for 4min at

12000g to remove any large pieces of intact tissue or membranes that remained. The supernatant (1mL) was then transferred into Eppendorf tubes containing 200µL of chloroform, and samples were mixed 5-6 times by inversion. Samples were centrifuged for 15min at 12000g in order to obtain 3 distinct layers. The top clear layer (350µL) was removed and added to an equal volume of isopropanol, samples were mixed by inversion and were left at room temperature for 20min to permit the isolated RNA to precipitate. Samples were subsequently centrifuged (15min, 12000g) in order to obtain RNA pellets. The supernatant was discarded, and pellets were washed with 500µL of ice cold 70% ethanol. After another centrifugation (5min, 12000g) the ethanol wash was discarded, and this step repeated. Following the removal of the ethanol wash samples were centrifuged for a final 5min (12000g), and the residual ethanol was careful removed using a 100µL sterile pipette tip. Samples were dried for 5min by placing in a laminar flow hood. Isolated pellets were dissolved in 30-50µL of autoclaved water depending on the size of the starting sample. Total RNA concentrations were determined using spectrometry as outlined in section III.

Realtime PCR Primer Design

Primers and dual-labeled probes were designed for target genes using the PrimerQuest design program available online from Integrated DNA technologies (www.idtdna.com/Scitools/Applications/PrimerQuest/Default.aspx/; Coralville, IA). In brief, the mRNA target sequence of interest is copied and pasted into the program, and the parameters for realtime primer and probe design are selected. The program automatically generates 5 sets of primer/probe combinations, which can be further narrowed by i) selecting those that have a product <150bp, ii) do not form hairpins at high temperatures (<40°C), iii) are specific for your gene of interest based on an NCBI blast search for short nearly exact matches (as performed for the siRNA target sequences). If possible sets that were intron-spanning were selected since they further

reduce the chances of amplifying genomic DNA. Please see Table 2 for all PCR primer and probe sequences. For our studies all primers and dual-labeled probes were obtained from Eurogentec in the standard PAGE (primers) or HPLC format (dual-labeled probes) (San Diego, CA).

Realtime RT-PCR

Total RNA (2 µg) was reverse transcribed in a total volume of 40µL and PCR amplified using SuperScript™ III RT enzyme master mix (Invitrogen) according to the manufacturer's protocol. In brief, RNA was diluted to 16µL and then heat denatured for 5min at 70°C. Samples were returned to ice prior to the addition of 20µL of the 2x reverse transcription master mix and 4µL of the SuperScript™ III RT enzyme master mix (Invitrogen). The RT reaction consisted of a 10min incubation at 25°C, 45min incubation at 42°C, followed by a 5min 85°C termination step, and the resulting complementary DNA (cDNA; 40µL) was stored at -20°C. For PCR amplification of *leptin, rstn, fiaf,* and *socs-3* samples were amplified in triplicate and only 1 gene was analysed per reaction. Reactions were prepared using Platinum® quantitative PCR SuperMix-UDG and following the supplied guidelines. They consisted of: 17.5 uL of the 2 X Platinum® Quantitative PCR SuperMix-UDG (Invitrogen), 7 pmol of forward and reverse primers (engineered as described above; see Table 2) 1 pmol of dual-labeled probe, 2-3µL of cDNA in a total volume of 35 µL using Platinum® quantitative PCR SuperMix-UDG, and contained 3µL cDNA, and were amplified according to the manufacturer's protocol. In brief samples were incubated for 10min at 95°C, followed by 60 amplification cycles of 95°C for 20s and 60°C for 60s in a BioRad thermal cycler and an iQ realtime PCR detection system. A standard curve, that was prepared using a serial dilution of a reference sample, was included in each realtime run to correct for possible variations in product amplification. Relative copy numbers were obtained based on standard curve values, and were normalized to the values obtained for the house keeping gene,

cyclophilin. Data are expressed as a percentage of the control +/-SEM. It should be noted that no significant variations in our house keeping gene were observed between groups when evaluated using either the threshold cycle (C_T), or using the relative levels of expression, and this is consistent with previous reports (Bond et al. 2002).

Statistics

For all the in vivo studies data were analyzed using the Student's *t-test* since values were only being compared directly between the two groups of animals used in each experiment (siSTABLE L7 or siSTABLE SCR). Significance was set at $p < 0.05$.

VIII. Results

VIIIa. Acute Leptin Knockdown In Vivo: *The Effects Of A Single siRNA Injection Into The Lateral Ventricle Vs. Dorsal Third Ventricle*

Body Weight And Food Intake

As seen in Figure 26, Injecting siSTABLE L7 in either the lateral ventricle (LV), or the dorsal third ventricle (D3V), of fasted male rats (200g) induced a small, but significant, increase in overnight bodyweight gain (20% and 16% respectively, $p < 0.05$) relative to animals injected with the non-specific control, siSTABLE SCR. In contrast, food intake was unchanged between groups, regardless of the site of the injection.

Blocking The Induction Of Leptin Gene Expression Using siSTABLE L7 In Fasted Rats

Following an overnight fast, rats were injected icv with siSTABLE L7 into the LV and food was returned. A 32% reduction in feeding-induced leptin gene expression was detected in the cortex ($p < 0.05$) of animals injected with siSTABLE L7 relative to those receiving the non-specific control molecule, however no changes in hypothalamic leptin mRNA were detected (Fig 27A). In contrast, injecting siSTABLE L7 into the D3V of fasted rats induced a 45% ($p < 0.01$) reduction in food-induced cortical leptin expression, and reduced hypothalamic leptin expression by more than 70% ($p < 0.01$), relative to animals injected with siSTABLE SCR (Fig 27B).
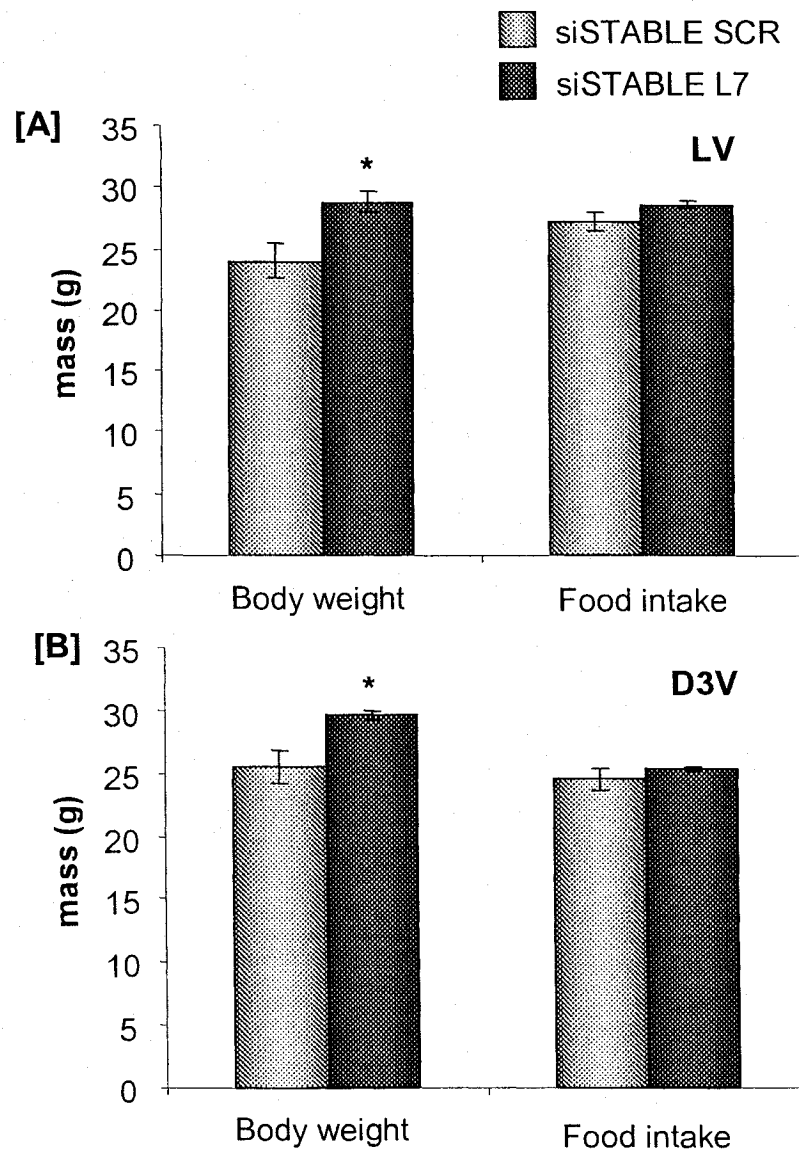
Figure 26: Effects of leptin knockdown on overnight bodyweight and food intake

Rats and the provided food were weighed just following injection and then again prior to sacrifice. The weight differences were used to calculate weight gain (g) (left) and overnight food intake (g) (right). Injecting siSTABLE L7 into the lateral ventricle (LV) [A], or into the dorsal third ventricle (D3V) [B], induced a significant increase in overnight weight gain, but had no effect on food intake, relative to animals injected with the non-specific control molecule, siSTABLE SCR (n=4-5 for each group). (*p<0.05)
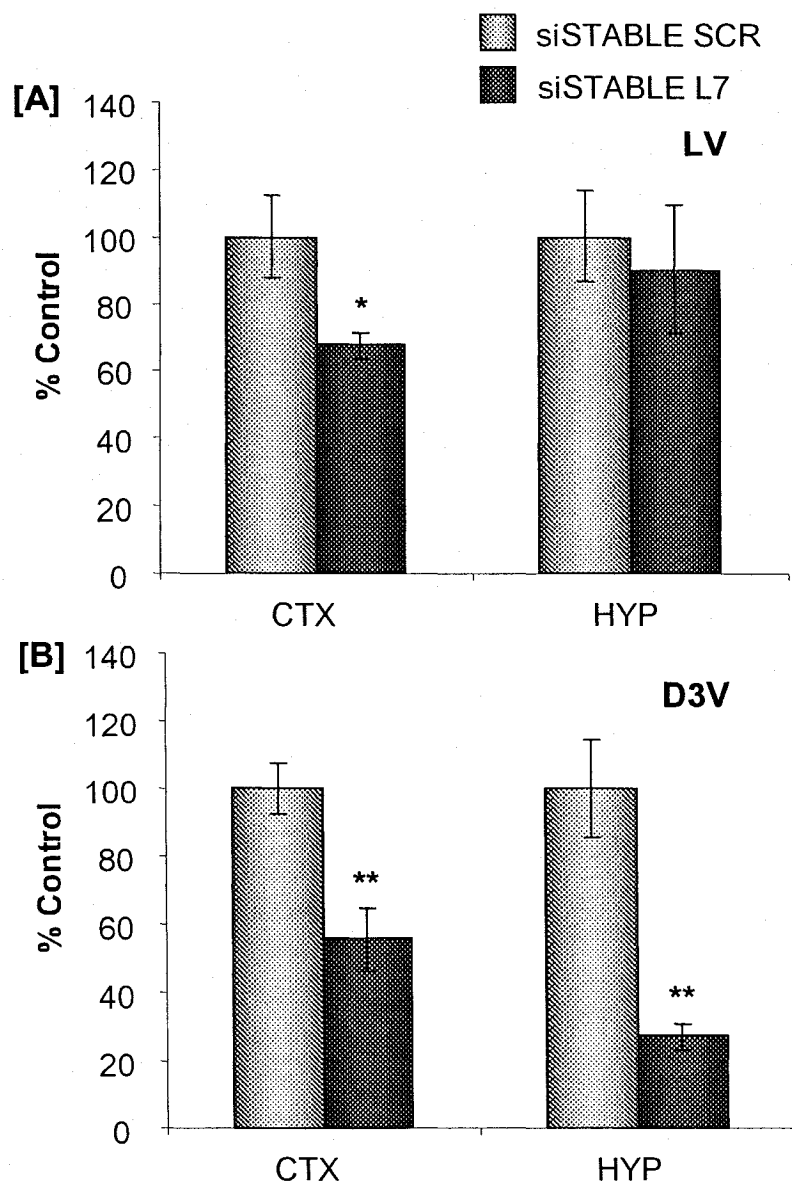
Figure 27: Effects of siSTABLE L7 on leptin expression in the rat brain: inhibition of feeding-induced expression in fasted rats. Leptin gene expression was analysed by realtime RT-PCR 24h after the icv injection of siSTABLE L7 (5.75µg/rat), or the control molecule, into the brain of fasted rats. [A] Injecting siSTABLE into the lateral ventricle significantly decreased leptin mRNA in the cortex (-30%), but not in the hypothalamus. [B] In contrast leptin gene expression was significantly attenuated in the cortex (-40%) and hypothalamus (-75%) following injection into the D3V. Data are expressed as a percentage of the control ± SEM (n=4-5 for each group). (*p<0.05, **p<0.01)

Effects Of Leptin Knockdown On *rstn* Expression In CTX And HYP

Following the LV injection of siSTABLE L7 cortical *rstn* gene expression was also reduced by 32%, although this was not statistically significant. However when siSTABLE L7 was injected into the D3V a 50% reduction in *rstn* mRNA was detected ($p < 0.05$) relative to the controls. In contrast no change in hypothalamic *rstn* mRNA was detected in either experiment (Fig 28B).

Effects Of Silencing Leptin On *socs-3* Expression In CTX And HYP

Injecting siSTABLE L7 into the lateral ventricle was associated with a small, non-significant, increase in cortical *socs-3* gene expression (Fig 29A), whereas no change was detected in the hypothalamus. Conversely the D3V injection of siSTABLE L7 had no effect on cortical *socs-3*, but did induce a non-significant increase (~25%) in hypothalamic *socs-3* expression, relative to control values (Fig 29B).

Leptin Knockdown Does Not Impact *fiaf* Expression

As seen in Figure 30, *fiaf* gene expression was analyzed in the cortex and hypothalamus following the injection of siSTABLE L7 into the LV. Since no changes in *fiaf* gene expression were detected in either tissue (CTX or HYP), *fiaf* was not analysed in subsequent experiments.

VIIIb. Chronic siSTABLE L7 Infusion (14days) Into Lateral Ventricle

The Effects Of siSTABLE Infusion On Weight Gain And Food Intake

Daily body weights were recorded from 2 separate experiments over a period of 14 days beginning on the day of mini pump implantation (Day 0). No significant changes in weight gain were detected between the leptin knockdown (siSTABLE L7; n=12) or control groups (siSTABLE SCR; n=12) relative to body weights recorded on the day of pump implantation (Fig 31). Similarly when overnight food intake was monitored on the final day there were no measurable changes between groups (data not shown).
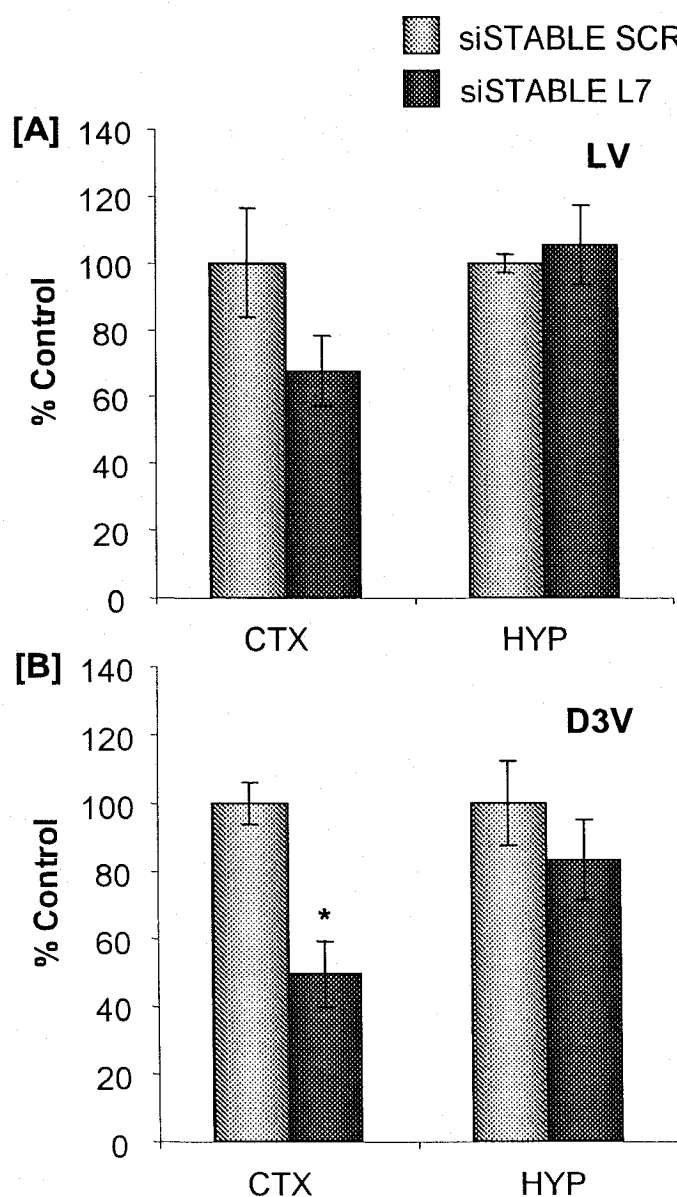
Figure 28: Effects of siSTABLE L7 on *rstn* expression in the rat brain: inhibition of feeding-induced expression in fasted rats. *Rstn* gene expression was analysed by realtime RT-PCR 24h after the icv injection of siSTABLE L7 (5.75µg/rat), or the control molecule. [A] Leptin knockdown was associated with a non-significant reduction in cortical *rstn* when rats were injected into the lateral ventricle. [B] *Rstn* was significantly reduced (-50%) in the cortex, but not in the hypothalamus, of rats injected into the D3V. Data are expressed as a percentage of the control ± SEM (n=4-5 for each group). (*p<0.05)
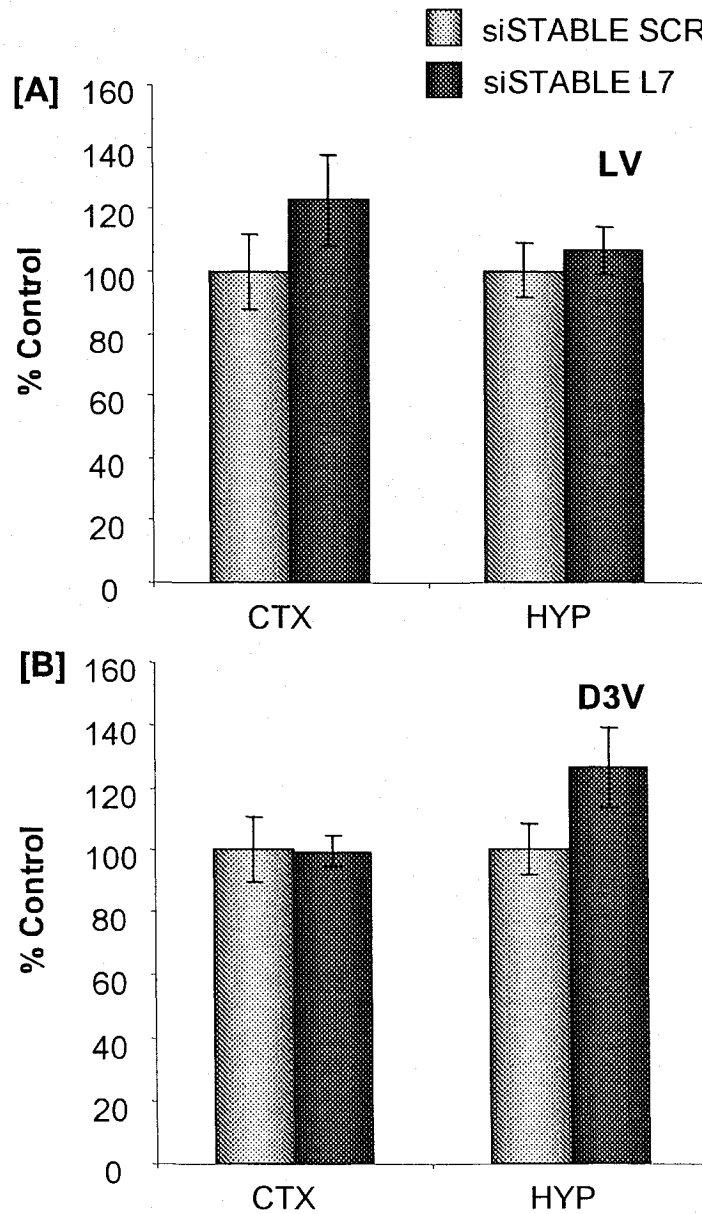
Figure 29: Effects of siSTABLE L7 on *socs-3* expression in the rat brain: inhibition of feeding-induced expression in fasted rats. No significant changes in *socs-3* gene expression were detected in either the cortex (CTX) or hypothalamus (HYP) by realtime RT-PCR after the injection of siSTABLE L7 into the [A] lateral ventricle or [B] dorsal third ventricle. Data are expressed as a percentage of the control ± SEM (n=4-5 for each group).
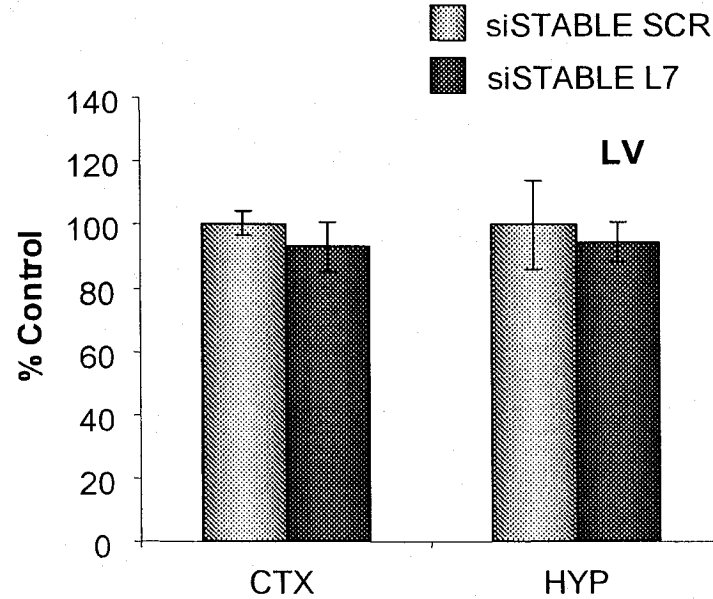
Figure 30: Effects of siSTABLE L7 on *fiaf* expression in the rat brain: inhibition of feeding-induced expression in fasted rats.
Realtime RT-PCR analysis failed to detect any significant changes in *fiaf* expression following the injection of siSTABLE L7 into the lateral ventricle, thus its expression was not analysed in subsequent experiments. Data are expressed as a percentage of the control ± SEM (n=4-5 for each group).
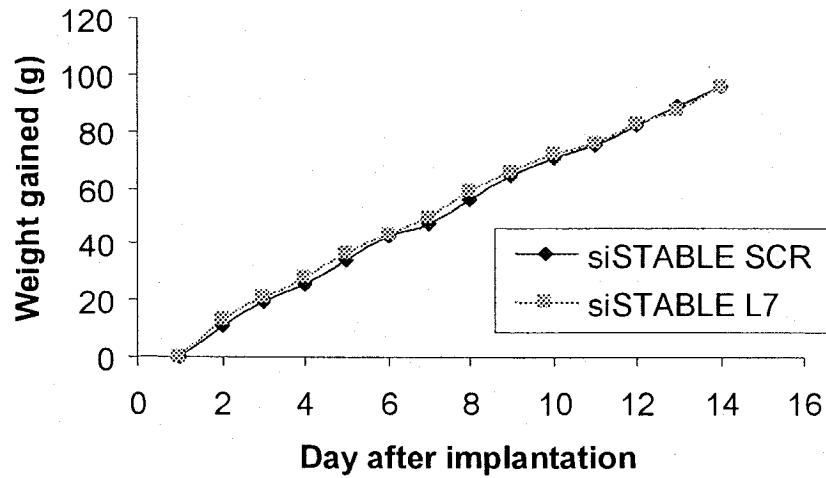
Figure 31: Effects of infusing siSTABLE L7 into the lateral ventricle on weight gain

Individual bodyweights were collected from rats daily between 10:00h and 14:00h starting on the day of mini pump implantation. Weight gain was calculated by subtracting the rats starting weight from their daily weight over the 14 day experiment. Average weight gain was calculated for both groups (n=8) and is plotted above. No significant variations in body mass were detected at anytime point.

Ipsilateral Vs. Contralateral Leptin Knockdown

In the initial experiment cortical leptin gene expression was assessed in both hemispheres. This sought to verify that siSTABLE L7 could reduce leptin mRNA in more distal sites 14 days after minipump implantation, despite the fact that siSTABLE L7 was infused into the right lateral ventricle. As seen in Figure 32 leptin expression was reduced by 52% and 44% in the ipsilateral and contralateral brain regions, respectively, as measured by realtime RT-PCR. However leptin expression was highly variable and failed to reach significance.

The Effects Of Longterm Leptin Knockdown On Adipokine Gene Expression

The continuous infusion of siSTABLE L7 induced modest decreases in hypothalamic (-30%) and cortical (-24%) leptin gene expression, but these reductions failed to attain significance (Fig 33A). In contrast leptin gene expression was unaffected in adipose tissue or in the pituitary suggesting that silencing was localized to the CNS alone. Similar to our acute leptin knockdown data, when cortical leptin was silenced using siSTABLE L7, *rstn* gene expression was also reduced by 35% ($p < 0.05$) in the cortex. In contrast leptin knockdown induced a 132% ($p < 0.05$) increase in hypothalamic and a 200% ($p < 0.0001$) increase in pituitary *rstn* gene expression (Fig 33B). In contrast *rstn* gene expression was unaffected in adipose tissue. Although no significant changes in *socs-3* were detected in any tissue analyzed, cortical *socs-3* appeared to be lower in the leptin knockdown group (Fig 33C).

The Effects Of An ICV Leptin Injection On Rstn And Socs-3 Gene Expression

Shortly (30min) following the icv injection of leptin into the LV a modest reduction in hypothalamic *rstn* gene expression was detected (-25%; p=N.S.), whereas a significant reduction in cortical *rstn* was detected (-40%; $p < 0.05$) (Fig 34A). However as expected the injection of leptin induced significant increases in hypothalamic (130%, $p < 0.001$) and cortical (70%; $p < 0.001$) *socs-3* gene expression (Fig 34B).
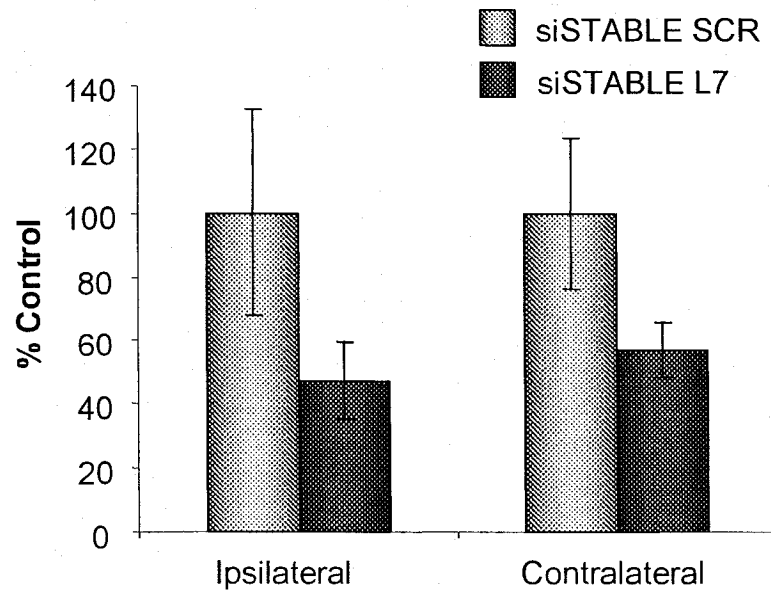
Figure 32: The effects of unilateral siSTABLE L7 infusion on bilateral leptin gene expression
Leptin gene expression was analysed by realtime RT-PCR in the ipsilateral and contralateral cortical hemispheres and revealed that the unilateral infusion of siSTABLE L7 was capable of inducing bilateral gene silencing, although these data failed to attain significance. Data are expressed as a percentage of the control ± SEM (n=4 for each group).
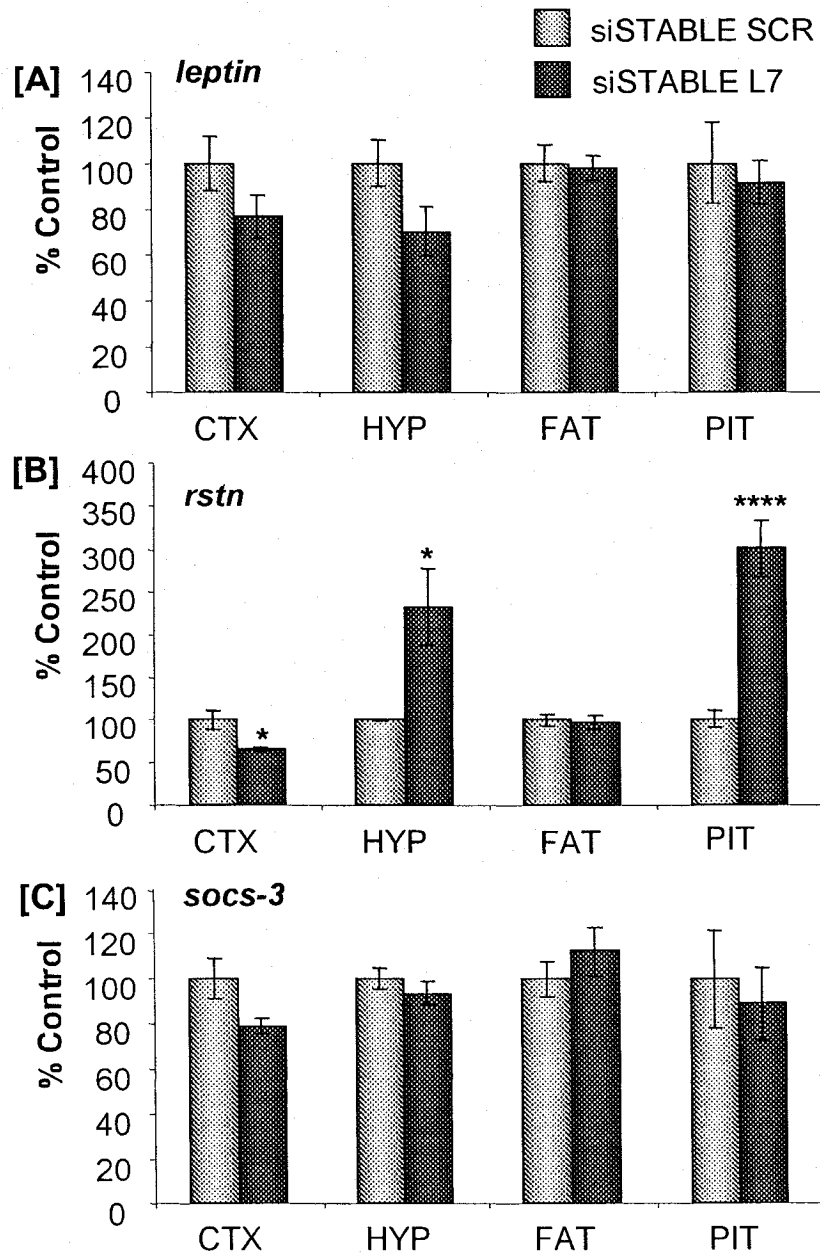
Figure 33: The effects of siSTABLE L7 infusion on adipokine gene expression. Gene expression was analysed in 4 tissues following the 14 day infusion of siSTABLE L7 into the lateral ventricle (4μg/day) by realtime RT-PCR. [A] Leptin was only modestly, but non-significantly, decreased in the cortex and hypothalamus, and was unchanged in the pituitary or adipose tissue. [B] Intriguingly *rstn* gene expression was significantly suppressed in the cortex (-35%), and was increased in the HYP (132%) and PIT (+200%) but unchanged in fat. [C] No significant changes in *socs-3* gene expression were detected in any of the tissues analysed. Data were pooled from duplicate experiments and are expressed as a percentage of the control ± SEM (n=7-8). (*p<0.05, ****p<0.0001)
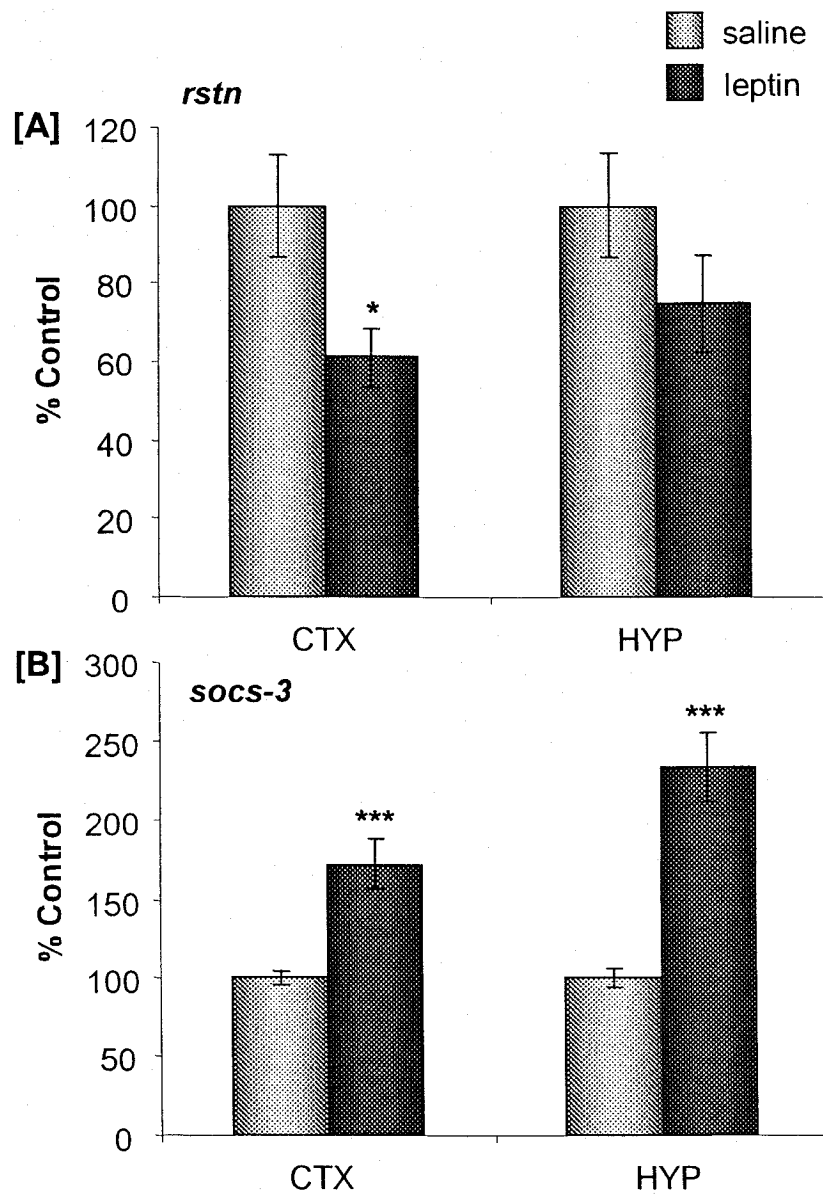
Figure 34: The effects of ICV leptin on *rstn* and *socs-3* expression
Rstn and *socs-3* gene expression were analysed in the cortex and hypothalamus 30min following the icv injection of recombinant leptin (7.5μg/rat) into the lateral ventricle using realtime RT-PCR. [A] *Rstn* gene expression was significantly suppressed in the cortex (-40%) and tended to be slightly lower in the hypothalamus (-25%). [B] As expected leptin induced the expression of *socs-3* in the cortex (+72%) and hypothalamus (+133%). (*p<0.05, ***p<0.001)

IX. Discussion

These studies clearly demonstrated that a single injection of siSTABLE L7 into the lateral ventricle, or into the dorsal third ventricle, of fasted rats was capable of significantly attenuating the nutritionally-induced increases in leptin gene expression in the rat brain relative to animals injected with the non-specific control molecule. However the site of injection greatly influenced the degree of silencing. For example when siSTABLE L7 was injected into the lateral ventricle only cortical leptin gene expression was significantly reduced (-32%, $p<0.05$). In contrast, injecting siSTABLE L7 into the dorsal third ventricle resulted in a significant attenuation in both cortical (-45%, $p<0.01$) and hypothalamic (-72%, $p<0.01$) leptin gene expression. Moreover the icv injection of siSTABLE L7 induced a modest, but significant, increase in overnight weight gain regardless of the site of injection, relative to animals injected with the non-specific control molecule. In addition the injection of siSTABLE L7 also decreased the expression of cortical resistin (*rstn*), but had no effect on hypothalamic *rstn*, or on *socs-3*. Recombinant leptin was also injected into the lateral ventricle to verify whether it had the opposite effects on *rstn* expression relative to the changes detected after leptin knockdown. As expected, and as reported previously (Morrison et al. 2005), significant increases in *socs-3* were detected in both the cortex and hypothalamus following the icv injection of recombinant leptin into the lateral ventricle. However the central icv injection of leptin also induced modest decreases in cortical (-40%; $p<0.05$) and hypothalamic *rstn* (-25%; $p=N.S.$) gene expression 30 min after delivery. In contrast to these acute studies, the continuous infusion of 'naked' siSTABLE L7 into the lateral ventricle induced a non-significant reduction in both cortical (-24%) and hypothalamic (-30%) leptin gene expression, and no effects on bodyweight were detected over the 14 day period. Like in the acute silencing studies, the infusion of siSTABLE L7 induced a significant reduction in cortical resistin mRNA (-35%, $p<0.05$). In contrast the chronic leptin knockdown

resulted in significant increase in hypothalamic (+132%, p<0.05) and pituitary (+200%; p<0.0001) *rstn* gene expression. However no significant changes in socs-3 were detected in any of the 4 tissues analysed.

A major concern was whether the effective target identified in vitro, siSTABLE L7, would be capable of mediating gene knockdown in vivo. Although modest reductions in cortical (-24%) and hypothalamic (-30%) leptin gene expression were achieved when siSTABLE L7 was continuously infused into the lateral ventricle, these reductions were of a much smaller magnitude than the decreases detected in C6 cells (Fig 14; -50%, p<0.01). However the lower RNAi silencing efficiency achieved in vivo is not unexpected. Hoyer et al. (2006) identified an effective target that was capable of reducing EGFP expression by nearly 90% in vitro, but in vivo the reductions achieved in EGFP mRNA expression varied, with a maximal inhibition occurring in the hippocampus (-50%) two weeks after the start of siRNA infusion (Hoyer et al. 2006). Similarly it was found that a siRNA capable of reducing the dopamine D1 receptor by more than 75% in vitro was ineffective in vivo, but this failure might be attributed to the relatively short period of application (3 days vs. 14 days) (Isacson et al. 2003). Similarly a siRNA targeting brain-derived neurotrophic factor (BDNF) was also capable of reducing its expression by 75% in vitro, and no reductions were detected 3h after it was injected in vivo. Although siRNA molecules appear less effective at silencing target gene expression in vivo, this might reflect the inefficient methods of delivery used in vivo.

In contrast to the minipump experiments, the injection of siSTABLE L7 into the D3V of fasted rats was highly efficient at blocking the nutrition-mediated induction of hypothalamic leptin gene expression (-70%; p<0.01) 24h after rats were refed. However no changes in hypothalamic leptin gene expression were detected when siSTABLE L7 was injected into the lateral ventricle of fasted rats. This suggests that the site of delivery has a profound effect on the effectiveness of this acute gene silencing approach. In

contrast, a similar degree of leptin silencing was achieved in the cortex using both sites of delivery (35 to 45% reduction), suggesting that siRNA need to be injected as close as possible to brain-regions of interest in order to achieve maximal reductions in target gene expression (refer to Figure 23 and 24). Although siRNA have been reported to cross between tightly associated neurons (Zhao et al. 2006), this method of siRNA transportation is likely slow since the widespread knockdown reported in the mouse CNS was only detected after 2 weeks, but not 1 week, of continuous siRNA infusion (Thakker et al. 2004). Therefore the greater reductions in leptin gene expression achieved when siSTABLE L7 was injected into the D3V, relative to when it was delivered into the LV, could be due to the closer association between the D3V and the brain regions being analysed. Thus siRNA molecules were likely able to readily access these target tissues.

RNAi appears to be an effective approach to block the induction of brain leptin in vivo. The injection of siSTABLE L7 was highly effective at blocking the refeeding-mediated increases in brain leptin gene expression in fasted rats (fig 27), which were of similar magnitude to the blockade achieved using siSTABLE L7 in C6 cells (Fig 15). These results are similar to others who have achieved a nearly 100% blockade of target gene induction in the CNS when siRNA were delivered either via an intrathecal, or intracerebral, route (Baker-Herman et al. 2004; Satriotomo et al. 2006). However in these acute RNAi studies a transfection reagent was employed (Oligofectamine™) to facilitate the intracellular delivery of siRNA, which might have further enhanced the degree of silencing, partially accounting for the greater reductions in leptin gene expression achieved by the single injection of siSTABLE L7 vs. the chronic infusion (Fig 27 vs. Fig33A). Similarly the continuous infusion of siRNA molecules may have saturated RNAi pathways and induced a 'repelling' (Hong et al. 2005), or feedback mechanism, thus reducing the silencing properties of siSTABLE L7. Taken together these findings suggest that RNAi is an effective approach to block the induction of brain

adipokine gene expression. Furthermore this might be a useful technique to block the age-dependent increases in brain adipokine expression in order to study their role in development, or in the pathology of inflammatory or brain injury-induced anorexia or brain repair (see Chapters 5 and 6).

Although the use of siRNA to silence gene expression in the CNS is a relatively new approach to study gene function, there have been no clear investigations verifying the ideal dose of siRNA required to induce gene silencing. This issue is further confounded by the wide variations in doses that have been reported in the literature. For example in neonatal rats as little as 7ng of siRNA was capable of reducing gene expression in the developing CNS (Hassani et al. 2005), whereas others have used upwards of 400µg/day to achieve reductions in target gene expression in adult mice (Thakker et al. 2004) and rats (Dorn et al. 2004). However in the former study a lipid-mediated reagent was used (JetSI^{TM}) to facilitate delivery in smaller animals, whereas the latter studies involved the infusion of 'naked' siRNA into adult rodents, which might explain some of the variance in dosing. The silencing efficiency of target molecules has also been reported to vary significantly (recall Fig 14), and could also be impacting the dosage required to achieve significant reductions in target gene expression. There have also been reports of using siRNA doses that fall between these extremes (5µg/day (Tan et al. 2005; Salahpour et al. 2007) or 11.5µg (Baker-Herman et al. 2004; Satriotomo et al. 2006)), but these studies also employed lipid-mediated carriers to facilitate intracellular delivery. The question has already been asked whether there are adverse consequences of flooding endogenous RNAi pathways (Stevenson 2004). In mice it appears that overloading the RNAi machinery is lethal (Grimm et al. 2006), and feedback mechanisms help attenuate the silencing effects of siRNA when high doses were employed (Hong et al. 2005). Thus a higher dose of siRNA does not necessarily equate to greater gene silencing, and limiting the amount of siRNA used should also

reduce the potential for activating non-specific or 'off-target' RNAi pathways. A relatively modest dose was used in the present studies; for acute ICV work only 5.75µg siRNA/rat was injected, and 4ug/rat per day was continuously infused using the osmotic mini pumps. It should also be noted that unlike the above studies a stability-enhanced siRNA, that is resistant to degradation, was being employed. Thus siSTABLE L7 concentrations should remain elevated longer than unmodified siRNA molecules and might permit the use of lower concentrations. Although a significant reduction in leptin gene expression was achieved using these doses in the acute studies, a higher dose may have resulted in a more profound knockdown during chronic siRNA infusion and is worth examining in future studies.

Although very modest reductions in brain leptin gene expression were achieved in the minipump experiments, significant changes in *rstn* gene expression were detected in the various tissues analysed (Fig 33B). In the cortex leptin knockdown was associated with a reduction in *rstn*, whereas *rstn* mRNA was increased in the HYP and PIT. A major concern is whether the effects on *rstn* gene expression are part of a non-specific RNAi response, or whether brain-derived leptin truly regulates *rstn* gene expression in these other tissues by elusive mechanisms. The tissue-dependent changes in *rstn* suggest that these effects are part of a specific response since if non-specific RNAi pathways were responsible then similar changes in *rstn* expression would have been detected in both brain tissues being analysed, and no effect would have occurred in the pituitary. Moreover the opposing effects on *rstn* gene expression seen in the cortex and hypothalamus are not unexpected. Recently it was shown that treating neonatal rat pups with leptin differentially activated leptin signaling pathways in various brain regions (Walker et al. 2007). More specifically leptin activated the JAK-STAT pathway in the HYP, but failed to activate STAT-3 and instead induced a MAPK response in the hippocampus (for signaling pathways refer to Figure 6, Chapter 1) (Walker et al. 2007). It

seems plausible that leptin-knockdown also modifies these leptin signaling cascades in a regionally-dependent manner, and this could lead to the tissue-specific changes in *rstn* mRNA that were detected in the rat brain. Similar reductions in cortical *rstn* were also detected in the acute knockdown experiments, although no changes in hypothalamic *rstn* were detected, further suggesting an interrelationship exists between leptin and *rstn* expression in the rat CNS. However the acute icv injection of leptin also induced modest reductions in cortical and hypothalamic *rstn* gene expression. Although the effects on cortical *rstn* were somewhat unexpected, this might be the result of the short time between injection and tissue collection (30min) relative to the knockdown experiments (24h and 14days). In contrast leptin appeared to inhibit hypothalamic *rstn*, and these reductions may have achieved significance if leptin had been infused continuously over a period of several hours or days to match the time frame used in the leptin knockdown experiments. In view of the fact that siRNA do not appear to escape from the CNS, since no leptin silencing was detected in either PIT or FAT, these experiments suggests that brain-derived leptin is modulating *rstn* gene expression in the pituitary. The effects of the hypothalamus and leptin on pituitary *rstn* are not totally without precedent. It was previously reported that pituitary *rstn* mRNA was significantly lower in leptin-deficient ob/ob mice relative to their lean littermates (Morash et al. 2004). Similarly hypothalamic lesions, induced by neonatal MSG treatment, also lead to a robust attenuation in pituitary *rstn* gene expression (Morash et al. 2002). In total these data suggest that brain leptin regulates the expression of other adipokines in the rodent CNS, but might also impact the expression of adipokines in other tissues (i.e. PIT) via an adipokine feedforward mechanism.

In these experiments only a modest change in body mass was detected during the acute knockdown studies, whereas the continuous infusion of siSTABLE L7 had no effect. The failure of leptin knockdown to mediate changes in bodyweight in the latter

study could be due to the limited silencing achieved by the infusion of 'naked' siSTABLE L7 into the rat brain. However these results are not surprising since leptin heterozygotic mice, which are deficient in a single leptin allele, also have normal body weights despite their modest reductions in circulating leptin (6.5ng/mL OB/ob vs . 9.6ng/mL OB/OB) (Chung et al. 1998). However leptin haploinsufficiency does result in an increase in the proportion of body fat, which might be part of a compensatory mechanism to restore leptin levels (Chung et al. 1998). In humans leptin heterozygosity is also associated with normal bodyweight, fasting blood glucose and insulin levels are normal, and plasma leptin levels appear unchanged relative to humans with two functional leptin alleles (Strobel et al. 1998). Similarly leptin receptor-heterozygotic mice (DB/db) also have an increase in the percentage of body fat, but do not appear to be any heavier than their wild type littermates (Chung et al. 1998). Moreover this phenotypic effect of leptin receptor deficiency has also been documented in rats (Heo et al. 2002) and humans (Farooqi et al. 2007). Similarly when leptin receptor expression was silenced in the rat midbrain, using viral-RNAi, no changes in body mass were detected and only slight increases in food intake appeared about 2 weeks after knockdown was initiated (Hommel et al. 2006). Therefore in these leptin RNAi studies the magnitude of silencing may have been too subtle, or the length of knockdown may have been too acute, in order to produce any obvious phenotypical changes. However in future studies it might be worth trying to quantify body composition and plasma leptin concentrations, after leptin knockdown as described by others (Harris and Martin 1984; Richard and Trayhurn 1985). Prolonging leptin silencing, by infusing siSTABLE L7 over greater periods, might also generate a more overt phenotype. Similarly we do not yet know what the consequences of putting our knockdown rats on a high fat, or a 'westernized', diet which might also induce greater variations in bodyweight.

In addition to regulating appetite and body weight, leptin has also been implicated in the development of the central nervous system. Recall leptin deficient mice (ob/ob) have smaller brains than their wild type littermates (Steppan and Swick 1999), and this was partially restored with leptin treatment (Ahima et al. 1999). Leptin also appears to regulate the expression of various synaptic proteins in rats and mice (Ahima et al. 1999; Walker et al. 2004a; Walker et al. 2007). Similarly treating the human neuroblastoma cell line, SH-SY5Y, with leptin protected cells from apoptosis (Russo et al. 2004), and my studies in C6 glioblastoma revealed that silencing leptin induced a 2-fold increase in cell death. Thus knocking down leptin expression, with siSTABLE L7, may induce long term changes in brain structure by impacting cell survival or synaptogenesis. In addition the timing of leptin knockdown could also influence its effectiveness on weight gain, especially if leptin gene expression was suppressed during critical neonatal periods associated with neurogenesis and the development of hypothalamic feeding circuits (Bouret et al. 2004a; Bouret and Simerly 2004; Bouret and Simerly 2006).

In conclusion siSTABLE L7 appears to be an effective RNAi target that is capable of silencing leptin gene expression in the rat brain, especially in the cortex and hypothalamus. However the magnitude of silencing achieved appears dependent on the site of injection and the mode of siRNA delivery (i.e. 'naked' vs. complexed with Oligofectamine[TM]). Although no overt phenotypical changes were detected in these studies, this was not totally unexpected given the subtle changes that have been reported in leptin, and leptin receptor, heterozygotes (Chung et al. 1998; Strobel et al. 1998). However it should be noted that total leptin deficiency is very rare in human populations (Considine et al. 1995), and that subtle allelic variations are more likely to predispose individuals to the development of obesity. Thus our model of brain leptin knockdown could be a more accurate representation of what might be seen clinically,

relative to current animal models of obesity that are totally leptin deficient (ob/ob), or completely devoid of the functional leptin receptor (i.e. db/db mice or fatty (fa/fa) Zucker rats). However brain-derived leptin appears to regulate the expression of another adipokine, resistin, suggesting that adipokine crosstalk exists in the hypothalamus. Intriguingly the icv injection of resistin has been reported to induce effects similar to leptin in that it increased hypothalamic cFOS expression and reduced appetite (Tovar et al. 2005). The icv injection of resistin has been reported to induce hepatic gluconeogenesis (Ahima et al. 2006; Singhal et al. 2006). Similarly brain leptin appears to modulate the expression of pituitary *rstn* suggesting the possible existence of a hypothalamic adipokine feedforward mechanism. **Thus brain-derived leptin could be part of a complex brain adipokine system that is involved in the subtle regulation of appetite and metabolism. Furthermore this system might provide the hypothalamus with the fine tuning it requires to achieve the high degree of efficiency (99.97%; (Hervey 1969)) with which it regulates bodyweight, but this needs to be investigated further. In summary this model of reducing brain leptin has opened the doors to many other investigations on brain adipokines and their potential effects on appetite and brain development.**

Chapter 3: *Other Adipokines Are Expressed In The CNS*

I. Introduction

Many, if not all, adipokines appear to modulate the output from hypothalamic

metabolic pathways that are implicated in energy homeostasis (Ahima et al. 2006).

However as discussed in Chapter 1, it remains unclear whether peripherally expressed

adipokines are capable of crossing the highly impenetrable blood brain barrier (BBB).

Thus our hypothesis that the brain would express leptin was extended to include all

adipokine genes since they also appear to exert metabolic effects via brain-dependent

pathways(s). For example the expression of two adiponectin receptors (Adipo R1 and

Adipo R2) has also been detected in the mouse brain (Yamauchi et al. 2003), and the

icv injection of adiponectin decreased bodyweight by modifying appetite and energy

expenditure, while stimulating *c-fos* expression (Qi et al. 2004). In contrast the central

administration of adiponectin had no effect on bodyweight or *c-fos* in obese agouti mice

suggesting that adiponectin exerts its central metabolic effects via an AgRP-dependent

mechanism (Masaki et al. 2003). However peripherally administered adiponectin does

not appear to cross the BBB, although it did inhibit the release of interleukin-6 from

mouse brain endothelial cells (Pan et al. 2006; Spranger et al. 2006). Recently

adiponectin was reported to be abundantly expressed in the chicken brain and pituitary

(Maddineni et al. 2005), which we have confirmed by RT-PCR in the mouse

hypothalamus and pituitary (Brown et al. unpublished observations). Thus like leptin,

centrally expressed adiponectin might be modifying appetite via a local

autocrine/paracrine mechanism.

The studies described below focused on the role of brain-derived resistin (*rstn*)

and FIAF (*fiaf*), which are also transcribed in the mouse brain and pituitary gland

(Morash et al. 2002; Morash et al. 2004; Wiesner et al. 2004; Wilkinson et al. 2005).

More recently the expression of *rstn* and *fiaf* was confirmed in a novel hypothalamic neuronal clonal cell line by RT-PCR (see below). In addition these immortalized N-1 hypothalamic neurons express a variety of other neuropeptides and receptors that are typical of mouse hypothalamic neurons, suggesting their suitability as an in vitro model for these initial studies on brain *rstn* and *fiaf* (Belsham et al. 2004). As done for leptin, we employed an RNAi-based approach to specifically silence *rstn* and *fiaf* expression in the N-1 cell line model, and in differentiated 3T3-L1 adipocytes, to verify whether local autocrine/paracrine adipokine signaling could be impacting signaling pathways or cellular energy metabolism.

Resistin And Insulin Resistance

Resistin was initially implicated as an insulin desensitizing hormone since the injection of the purified recombinant hormone impaired glucose tolerance in mice. Conversely the immunoneutralization of resistin improved blood glucose concentrations and increased insulin sensitivity (Steppan et al. 2001). Subsequent studies supported a role for resistin in the regulation of hepatic gluconeogenesis since its transgenic overexpression elevated plasma glucose levels (Rangwala et al. 2004). Conversely resistin knockout mice have reduced fasting blood glucose concentrations, and this was associated with the decreased expression of hepatic gluconeogenic enzymes (Banerjee et al. 2004). However the role of resistin in human biology remains less apparent. Unlike laboratory rodents, where *rstn* is predominantly expressed in adipose tissue, *rstn* mRNA levels were highest in bone marrow and circulating mononuclear cells (Savage et al. 2001; Ort et al. 2005). Moreover *rstn* expression was not detected in human adipose tissue by some (Ort et al. 2005), although very low levels have been reported by others (Nagaev and Smith 2001; Savage et al. 2001; Janke et al. 2002). There is also no clear consensus on whether resistin production is increased in adipose tissue of obese or diabetic humans (Nagaev and Smith 2001; Savage et al. 2001; Janke et al. 2002), and

plasma resistin levels do not appear to differ between healthy individuals and those with type 1 or type 2 diabetes (Fehmann and Heyn 2002). Thus the role of resistin in human glucose metabolism remains a contentious issue (Adeghate 2004; McTernan et al. 2006). In contrast resistin appears to possess potent inflammatory properties both in rodents and humans suggesting it might link obesity with inflammation (Gomez-Ambrosi and Fruhbeck 2001; Kaser et al. 2003; Silswal et al. 2005; McTernan et al. 2006).

Resistin Is Widely Expressed In The Body

Although resistin was initially thought to be exclusively expressed in adipose tissue (Steppan et al. 2005), there is little doubt now that it is transcribed in multiple non-adipose sites. For example *rstn* mRNA has been detected in mouse hepatic cells (Tsukamoto 2005), as well as several rat tissues including the stomach, small intestine, colon, adrenal, muscle and liver to name a few (Nogueiras et al. 2003). Our laboratory provided the first evidence that *rstn* was also expressed in the mouse brain and pituitary (Morash et al. 2002; Morash et al. 2004), and this has since been confirmed by others in the rat (Tovar et al. 2005). More recently the N-1 neuronal cell line was also shown to expresses *rstn* (see below). Similarly *rstn* gene expression has been detected throughout the human body (Ort et al. 2005). Moreover its expression in humans appears to be highest in non-adipose tissues including circulating mononuclear cells (Savage et al. 2001), macrophages (Jung et al. 2006) and in bone marrow (Ort et al. 2005). This ubiquitous pattern of *rstn* expression has complicated the original hypothesis that resistin could be the link between obesity and insulin resistance, an issue that remains to be resolved (Adeghate 2004; Steppan and Lazar 2004; McTernan et al. 2006). However this pattern of expression is similar to what has been found for leptin, suggesting that resistin is likely another pleiotropic adipokine.

Resistin And Brain Function

Although no resistin receptors have been cloned, the first resistin binding peptide was recently identified, and the brain appears to respond to exogenous resistin (Liu et al. 2006). Brunetti et al (2004) reported that human resistin (0.1-10nM) inhibits the depolarization-induced, but not the basal, secretion of catecholamines from rat hypothalamic synaptosomes (Brunetti et al. 2004). However this report failed to outline how much hypothalamic tissue was used to prepare the synaptosomes, and it remains possible that nerve endings from the median eminence, a circumventricular organ that would normally be accessible to circulating resistin, were included. Tovar et al. (2005) provided further evidence that resistin influences central energy metabolism. The microinjection of mouse resistin fragments (10μg per rat, aa 26-49 or aa 23-42) into the lateral ventricle transiently reduced food intake in both fasted and satiated adult male rats. Although this effect was reproducible over several days, repeated injections of resistin fragments failed to reduce bodyweight. In addition, the central injection of resistin increased expression of *c-fos* in the arcuate nucleus (ARC) of fasted, but not fed, rats (Tovar et al. 2005). Similarly it has been reported that the icv injection of resistin induces hepatic gluconeogenesis and insulin resistance in mice (Muse et al. 2005; Ahima et al. 2006; Singhal et al. 2006). Therefore resistin appears capable of regulating appetite and glucose homeostasis via brain-dependent pathways. However resistin circulates as a large hexamer and, like adiponectin (Pan et al. 2006; Spranger et al. 2006), seems unlikely to cross the BBB. Therefore it was hypothesized that *rstn* would also be transcribed by the brain and pituitary gland, which proved to be correct (Morash et al. 2002; Morash et al. 2004).

Detection And Regulation Of Resistin Expression In Brain

Rstn mRNA was readily detected by RT-PCR in the brains of adult C57BL6 and CD-1 mice using 2 alternative sets of primers that generate amplicons of 278 and 330 bp

(Morash et al. 2002). Products of the expected size were readily detectable after 30 PCR amplification cycles in fat, hypothalamus and cortex using both sets of primers in the two mouse strains (Morash et al. 2002). Moreover *rstn* gene expression was compared in saline perfused and non-perfused tissues to exclude the possibility that the detected PCR products were being amplified from contaminating blood cells, since *rstn* expression has been detected in human monocytes (Savage et al. 2001). Since no differences in *rstn* expression were detected between the two types of tissue preparations this excluded the possibility that blood cells were a non-specific source of *rstn* and further suggests that the mouse brain and pituitary are loci of *rstn* expression. Similar RT-PCR studies in the rat also failed to detect *rstn* expression in blood cells (Nogueiras et al. 2003), and further suggests that they are not a source of resistin in rodents. Additionally *rstn* expression was significantly higher in microdissected basal hypothalamus (MBH; ~3-fold; p<0.005) relative to the remainder of the hypothalamus as detected using semi-quantitative RT-PCR, and this was consistent with the immunolocalization of resistin protein (see below) (Wilkinson et al. 2005). Subsequently the enrichment of *rstn* mRNA in MBH of the rat brain was confirmed by in situ hybridization, but *rstn* expression was also separately detected in arcuate and ventromedial nuclei of the hypothalamus, the hippocampus and cerebral cortex (Tovar et al. 2005). Similarly low levels of *rstn* mRNA were detected from whole fetal, but not adult, human brain (Ort et al. 2005). However measuring *rstn* expression in discrete human brain regions might produce more convincing results that the human brain is also a locus of *rstn* expression.

In mice hypothalamic resistin gene expression was unaffected by age (postnatal day (PD) 3 to PD 40), or nutritional status (Morash et al. 2002; Morash et al. 2004). Although studies in the rat also showed that *rstn* mRNA was reduced by fasting in adipose tissue, it was unchanged in a variety of other tissues including stomach and

muscle (Nogueiras et al. 2003). In marked contrast to the detected changes in fat and pituitary, hypothalamic *rstn* mRNA was not different between *ob/ob* and non-obese mice (Morash et al. 2004). However these studies do not preclude gene expression changes that might be occurring in discrete hypothalamic nuclei and this might be clarified by using *in situ* hybridization. Alternatively immunohistochemistry was used to detect a complex network of resistin positive fibres that extend rostrally from the ARC to the preoptic area. However in keeping with RT-PCR data, hypothalamic resistin-immunoreactivity (ir) was unaffected by fasting (48h) or by high fat diet, but periventricular staining was greatly increased in the lactating mouse. Marked reductions in resistin positive fibres were also detected in the ob/ob mouse brain and in neonatal mice made underweight for their age by raising them in large litters (Wilkinson et al. 2005). Intriguingly this pattern of reduced resistin-ir in *ob/ob* mice is similar to the deficits in ARC neuronal projections described by Bouret and coworkers (see section III Chapter 1)(Bouret et al. 2004a; Bouret and Simerly 2004). However these projections were restored by neonatal leptin treatment (Bouret et al. 2004b), thus it would be interesting to see if the same were true for resistin-ir. Double-label immunofluorescence also demonstrated that these resistin positive fibres co-localized with α-MSH-ir, suggesting that *rstn* is expressed in POMC neurons (Wilkinson et al. 2005). Moreover this provides a further example of an adipokine co-localizing with a hypothalamic neuropeptide, as was the case for leptin-ir with oxytocin and vasopressin in the rat paraventricular and supraoptic nuclei (Morash et al. 2002). The functional consequence of such co-production of peptides is currently unresolved, and this is especially true for *rstn*, since, as noted, a resistin receptor remains to be identified. Nevertheless, these data suggest that resistin localizes within the hypothalamic circuitry that is regulating appetite and energy homeostasis (Elmquist and Flier 2004). An important question is whether resistin is endogenously produced by POMC neurons, or alternatively, is resistin taken up by

these cells from peripheral circulation? Although resistin appears to pool and accumulate in synovial fluid (Bokarewa et al. 2005), it is difficult to understand how POMC neurons could specifically concentrate resistin to the exclusion of other ARC cells. Regardless of whether resistin is biosynthesized or merely accumulated by POMC neurons, there is accumulating data suggesting that the hypothalamus is a resistin target tissue (see above). To summarize, resistin mRNA is present in the brain where it appears to exert neurochemical effects on hypothalamic synaptosomes (nerve endings) and impacts appetite and gluconeogenesis via centrally-dependent pathways in rats and mice (Brunetti et al. 2004; Muse et al. 2005; Tovar et al. 2005; Singhal et al. 2006). Thus studies were undertaken in the novel N-1 hypothalamic neuronal cell line to investigate the potential effects of endogenous *rstn* on cellular signaling and energy metabolism

Fasting-Induced Adipose Factor (FIAF)

Adipose tissue appears to be the primary source of FIAF, a secreted protein also known as angiopoietin-like protein 4 (ANGPTL4) and PPARγ angiopoietin related protein (PGAR) (Kim et al. 1999; Kersten et al. 2000; Yoon et al. 2000). Although FIAF has been shown to inhibit lipoprotein lipase activity, it has also been implicated in glucose metabolism and angiogenesis (Le Jan et al. 2003; Xu et al. 2005; Mandard et al. 2006). Similarly FIAF appears to protect mice against the development of diet-induced obesity (DIO) in germ free mice, and *fiaf* knockout mice have significantly larger fat pads than controls (Backhed et al. 2004; Backhed et al. 2007). Moreover *fiaf* overexpression has been reported to improve glucose tolerance and appears to modify the expression of mitochondrial respiratory chain proteins (Xu et al. 2005; Wang et al. 2007). Thus it should come as no surprise that insulin sensitizing PPARγ ligands, such as pioglitazone, increase the expression of *fiaf* (Yoon et al. 2000). Since the hypothalamus is also regarded as an adiposity and glucose-sensing system (Mobbs et al. 2005), I hypothesized that it too would be influenced, at least in part, by local *fiaf* expression.

Like leptin and resistin, *fiaf* gene expression has been detected in several different mouse tissues including fat, liver, lung, kidney, spleen (Kersten et al. 2000) and placenta (Yoon et al. 2000). However *fiaf* mRNA was also readily detected in the mouse brain and pituitary by RT-PCR (Wiesner et al. 2004), rat brain and pituitary (see Chapter 2, section VI), and in the N-1 neuronal cell line (N-1; see below). Similarly *fiaf* expression was detected in human glioblastoma and oligodendrocyte tissue (Le Jan et al. 2003), and in a human glioblastoma cell line (Lal et al. 2001). Like *rstn*, hypothalamic *fiaf* expression was unaffected by fasting in CD-1 male mice, whereas significant increases (approx. 2.5-fold) in *fiaf* were detected in both visceral fat, as expected, and pituitary gland (Wiesner et al. 2004). Again the use of in situ hybridization might be necessary to detect fasting-induced changes in *fiaf* mRNA within discrete hypothalamic nuclei in the mouse brain. As with *rstn*, preliminary studies were conducted in the N-1 cell line model to gain insight into the function of endogenously expressed *fiaf*.

N-1 Cells, A Novel Hypothalamic Cell Line Model

The complex nature of the hypothalamus may have precluded the above studies from detecting changes in hypothalamic *rstn* and *fiaf* gene expression in vivo, especially with regards to fasting-induced changes in centrally-derived adipokines. This led to the speculation that changes were occurring in discrete cellular populations, which would be undetectable by performing standard RT-PCR analysis on the entire mediobasal hypothalamus (Morash et al. 2004). Although many studies on adipokine gene expression have taken advantage of the differentiated 3T3-L1 adipocyte cell line model, until recently no suitable model appeared to exist for the study of hypothalamic-derived adipokines. Several brain cell lines have been available for many years, such as PC12 or Neuro2A neuroblastomas, but they seemed inappropriate for the study of hypothalamic gene expression since they are not fully differentiated neurons. This led to the recent development and characterization of a cohort of immortalized hypothalamic

neuronal cell lines that express a variety of hypothalamic neuropeptides and receptors that are implicated in central energy homeostasis including NPY, POMC, and ghrelin (Belsham et al. 2004). Moreover these neurons appear to respond to hormonal stimuli in a manner similar to that observed in the intact hypothalamus. For example leptin reduced the expression of hypothalamic neurotensin (NT) in vivo (Sahu 1998), a finding confirmed using these cell lines (Cui et al. 2005). I hypothesized that *rstn* and *fiaf* would also be expressed to varying degrees in this array of hypothalamic cell lines, which proved to be correct (unpublished observations- D.D. Belsham-personal communication). The N-1 cell line was employed in the present studies, which co-expresses both *rstn* and *fiaf*. Their neuronal nature was confirmed by staining for the specific neuronal marker NeuN (see Figure 35) (Brown et al. 2006a), and the expression of the glial cell marker, glial fibrillary acidic protein (GFAP), was undetectable by RT-PCR (Belsham et al. 2004). Additionally the use of an in vitro model will establish whether adipokines can have a direct effect on N-1 hypothalamic cells since the icv injection of resistin might stimulate other types of brain cells, such as brain endothelium, to release anorexigenic or inflammatory neuropeptides, which could indirectly modify gene expression or energy homeostasis.

Resistin-Dependent Regulation Of Socs-3

Suppressor of cytokine signaling-3 (SOCS-3) is an intracellular inhibitor of leptin and insulin signaling (Bjorbaek et al. 1999; Ueki et al. 2004). As such it has been implicated in the development of leptin resistance and the pathology of diet-induced obesity (DIO)(Munzberg et al. 2004). Following leptin receptor stimulation STAT-3 mediates the expression of *socs-3* which then binds tyrosine 985 of the intracellular domain of the long form of the leptin receptor, or associates with JAK-2, in order to block further leptin receptor signaling (recall Figure 7 in Chapter 1) (Bjorbaek et al. 1999; Bjorbaek et al. 2000). Thus *socs-3* appears to be part of an autoinhibitory

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[A]

[B]   *fiaf*   *rstn*
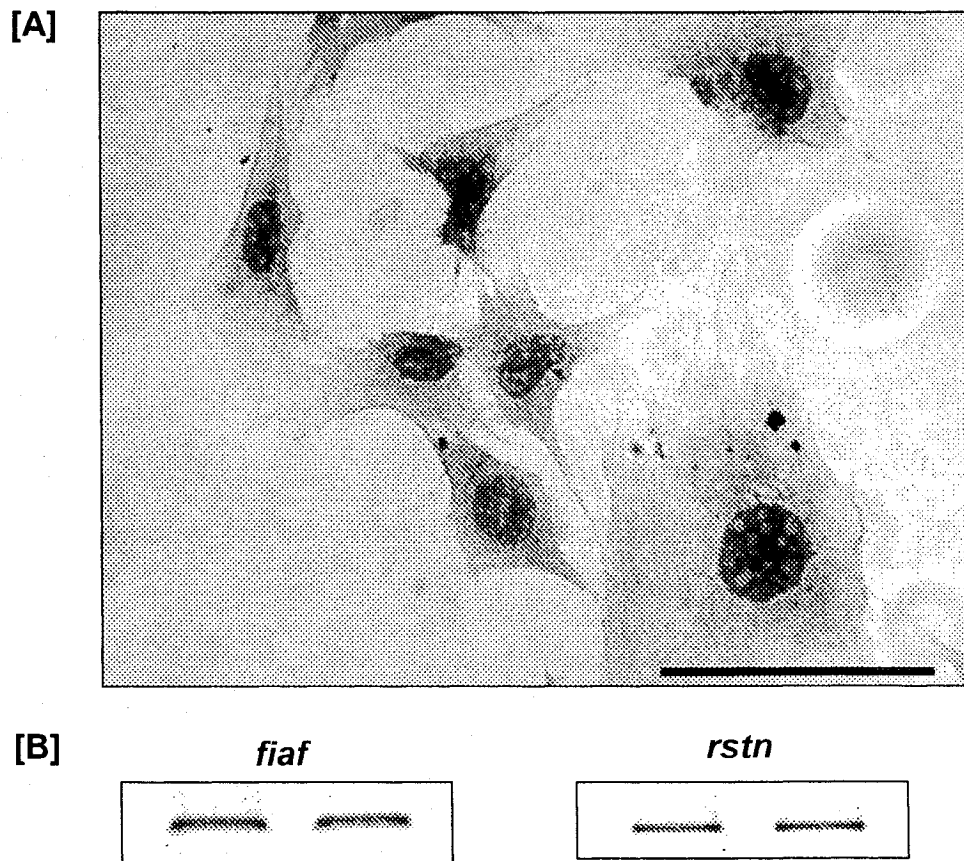
Figure 35: Characteristics of the N-1 cell line

[A] N-1 cells are immunopositive for the neuron-specific nuclear marker NeuN further confirming their neuronal nature. In brief N-1 cells were seeded on glass coverslips 24h prior to being fixed with 4% PFA. Cells were blocked with horse serum and incubated with a primary antibody to NeuN (1:100; Chemicon; Temecula, CA), followed by biotinylated horse ant-mouse (1:500; vector laboratories; Burlington, ON). Staining was visualized with diaminobenzidine (DAB), and the scale bar denotes 100µM. Staining was abolished by omission of the primary antiserum (not shown). [B] *Fiaf* and *rstn* gene expression were also readily detected in unstimulated N-1 neurons by realtime RT-PCR. Reactions were separated on 2% agarose gels stained with ethidium bromide to confirm that products of the expected size were amplified.

feedback mechanism of leptin signaling. Similarly chronic leptin receptor activation, which occurs in hyperleptinemic states such as obesity, is hypothesized to induce sustained elevations in *socs-3* and appears to be atleast partially responsible for obesity-associated leptin resistance (Munzberg and Myers 2005). This was supported by the observation that *socs-3* mRNA was increased by 50% in the arcuate nucleus of animals made obese by feeding them a high fat diet (Munzberg et al. 2004). Although *socs-3* knockout is embryonic lethal, *socs-3* haploinsufficiency protected mice against the development of DIO by increasing leptin sensitivity (Howard et al. 2004). Recently resistin was shown to induce *socs-3* expression in differentiated 3T3-L1 adipocytes, and this was speculated to be part of the insulin desensitizing mechanism that occurs in vivo (Steppan et al. 2005). Although the overexpression of *socs-3* in adipose tissue of mice induced local insulin resistance, it failed to induce systemic insulin resistance (Shi et al. 2006). Similarly the hepatocyte-specific deletion of *socs-3* was not protective against the development of peripheral insulin resistance, and resulted in a phenotype that is consistent with a chronic inflammatory state (Torisu et al. 2007). Thus the elevation of peripheral *socs-3* expression alone seems incapable of inducing systemic insulin resistance, and suggests that resistin acts on alternative pathways to reduce insulin sensitivity. In contrast the brain-specific, or the even more precise POMC neuronal-specific, deletion of *socs-3* is highly protective against the development of DIO and is associated with improved glucose tolerance (Mori et al. 2004; Kievit et al. 2006). Thus resistin might also be modulating *socs-3* expression in the CNS in order to modify glucose metabolism by impairing leptin and insulin sensitivity. This was tested by treating N-1 neurons with recombinant resistin, or by modifying endogenous *rstn* mRNA by inhibiting its expression using RNAi or using a plasmid vector to induce its overexpression (see below).

5' Adenosine Monophosphate (AMP)-Activated Protein Kinase (AMPK) And Hypothalamic Energy Sensing

AMPK was first isolated and characterized from the rat liver (Carling et al. 1994), but has since been detected in numerous other tissues including the brain and fat (Verhoeven et al. 1995). It is a heterotrimeric protein complex composed of a single catalytic (α), and 2 regulatory (β and γ), subunits. Moreover different isoforms exist for the various subunits which can be combined to generate several heterotrimeric variants (Hardie et al. 2003). Small reductions in cellular energy stores, as detected by incremental increases in intracellular adenosine 5'-monophosphate (AMP) concentrations, are capable of activating AMPK by inducing the phosphorylation of threonine 172, which is located in the activation loop of the catalytic α subunit (Hardie et al. 2003). Several studies have induced the activation of AMPK by using 5-aminoimidazole-4-carboxamide riboside (AICAR), a nucleoside analogue that is converted to 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranotide (ZMP), which acts as a stable AMP mimetic (Hardie et al. 2003). However AMPK can also be activated by other upstream kinases, including the calcium activated calcium/calmodulin-dependent protein kinase β (CaMKKβ) (Ramamurthy and Ronnett 2006). Although in vitro AMPK enzymatic assays are available, many studies have used Western blot analysis to determine the amount of phosphorylated AMPKα (pAMPKα) relative to total AMPKα. Thus the resulting ratio (i.e. pAMPKα/ total AMPKα) serves as an index of AMPK activity, since an increase in the ratio pAMPKα/total AMPKα is indicative of elevated AMPK activity.

AMPK has also been implicated in the regulation of whole-body energy homeostasis. For example the activation of peripheral AMPK, using the thienopyridone family of ligands, induced fatty acid oxidation in Sprague-Dawley rats and lowered plasma glucose concentrations and reduced weight gain in ob/ob mice (Cool et al.

2006). Leptin has also been shown to activate AMPK in peripheral tissues where it appears to inhibit lipogenesis and stimulates fatty acid oxidation, although its effects on AMPK activation are tissue-specific (Kahn et al. 2005; Kola et al. 2006). Therefore it can be inferred that the activation of peripheral AMPK is protective against weight gain. Resistin also induces metabolic changes via an AMPK-dependent mechanism. In contrast to leptin, the induction of hyperresistinemia inhibited AMPKα phosphorylation in various peripheral tissues (skeletal muscle, -60%; liver, -60%; and adipose tissue, -40%) that were collected from insulin clamped rats (Sato et al. 2005a). Treating isolated rat hepatocytes overnight with resistin (1μg/ml) also modestly reduced the ratio of pAMPKα/total AMPKα (Muse et al. 2004). Conversely hepatic AMPK activity appears to be elevated by about 2-fold in resistin knockout mice (Banerjee et al. 2004). AMPK does not appear to mediate any of the metabolic improvements that have been reported in *fiaf* knockout (KO) mice (Backhed et al. 2007). Thus leptin and resistin, but not FIAF, appear to exert part of their metabolic effects via AMPK-dependent pathways.

The close association between central energy sensing pathways, and those that control appetite, lead to the hypothesis that inducing hypothalamic AMPK activity would modify appetite and food intake in rodents. As predicted the icv injection of anorexigenic hormones, such as leptin and insulin, modified hypothalamic AMPK activity (Minokoshi et al. 2004). However unlike in peripheral tissues, leptin inhibited AMPK activation in the hypothalamus (Kahn et al. 2005; Kola et al. 2006). Similarly when an adenovirus expressing a dominant negative form of AMPK was injected into the mouse hypothalamus this lead to a reduction in appetite, whereas the injection of a virus encoding a constitutively active form of AMPK increased food intake (Minokoshi et al. 2004). Thus stimulating central AMPK activity appears to increase appetite and induced feeding. Hypothalamic AMPK is also implicated in the modulation of peripheral blood glucose. For example the pharmacological activation of AMPK, by the icv injection of

AICAR, induced a greater than 3-fold increase in hepatic gluconeogenesis in hypoglycemic Sprague Dawley rats (McCrimmon et al. 2004; McCrimmon et al. 2006). Likewise the intraperitoneal injection of streptozotocin (STZ) induced diabetes and hyperphagia in rats, and this was associated with increased levels of phosphorylated AMPK in the hypothalamus (Namkoong et al. 2005). However it is worth noting that regional, or even possibly subcellular, variations in central AMPK activity might induce differential metabolic responses (Ramamurthy and Ronnett 2006). Regardless hypothalamic AMPK appears to modulate appetite and gluconeogenesis, suggesting its involvement in the pathology of diabetes and obesity. Although resistin appears to modulate AMPK activation in peripheral tissues, its effects on central AMPK have not yet been investigated.

Elucidating The Role Of Resistin And FIAF In The N-1 And 3T3-L1 Cell Lines

In summary, there is substantial evidence that adipokines are not produced exclusively by adipocytes, but are ubiquitously expressed throughout the body in numerous tissues and cell types, including the brain. I hypothesized that autocrine/paracrine adipokine systems might also be functioning in brain and adipose tissue, and these locally-derived adipokines could be modifying cellular energy metabolism or local signaling pathways implicated in the development of insulin resistance and obesity. This hypothesis was tested by analyzing the effects of resistin and FIAF on the expression of socs3, a target gene implicated in the etiology of diabetes and obesity (Munzberg and Myers 2005). Further studies were conducted on the effects of resistin and AMPK phosphorylation, an intracellular energy sensing enzyme (Kahn et al. 2005). These studies were conducted in 3T3-L1 adipocytes, a well-described in vitro model for the study of adipokine regulation, and in the novel N-1 hypothalamic neuronal cell line (Belsham et al. 2004). Both cell lines were (a) treated with recombinant resistin or (b) had endogenous adipokine gene expression silenced using RNAi. Additionally N-1

neurons were transfected with *rstn* and *fiaf* overexpression vectors in order to elevate adipokine gene expression to levels that are normally detected in adipocytes. The changes in gene expression (*rstn, fiaf* and *socs-3*) were analysed using realtime RT-PCR, whereas Western blot analysis was performed to determine the ratio of phosphorylated AMPKα/total AMPKα.

The following studies were conducted to establish whether brain-derived *rstn* could be impacting the expression or regulation of critical targets involved in the control of hypothalamic energy homeostasis. This was accomplished by verifying whether *rstn* modifies *fiaf* or *socs-3* mRNA levels in N-1 neurons, or influences the activity of AMPK. Parallel studies were conducted in 3T3-L1 adipocytes to establish whether these effects could be cell-type specific.

II. Materials And Methods

N-1 Neuron Cell Culture

Immortalized mouse N-1 hypothalamic neurons were maintained in DMEM culture medium containing 10% fetal bovine serum (FBS) at 37°C in 5 % CO_2 / 95% air (Belsham et al. 2004). Cells were split prior to attaining confluence and were used between passage numbers 20 and 30 to limit the possibility that cell dedifferentiation was occurring. For all experiments cells were plated at 100,000 cells/well in Nunc 6 well plates.

For resistin studies N-1 cells were grown in serum free DMEM for 16-18h prior to being treated with murine recombinant resistin (30min; 25, 100, 200 or 400 ng/ml, PeproTech; Rocky Hill, NJ) and RNA was isolated 30min later as previously described (see Chapter 2 section III). For RNAi studies cells were transfected with 100nM of the STEALTH molecules using optimized conditions for Lipofectamine 2000 (see below; 2.25µl:1µg STEALTH), and propagated for a further 24h until RNA isolation. In overexpression studies N-1 neurons were serum-starved overnight prior to being

transfected for 24h with the *rstn* or *fiaf* expression vectors using 3.2 µg plasmid/well and the optimized conditions for Lipofectamine 2000 (See below; Invitrogen).

3T3-L1 Adipocyte Differentiation And Cell Culture

3T3-L1 fibroblasts (ATCC; Manassas, VA) were plated in 6 well Nunc plates, or 250ml flasks, and maintained in DMEM containing 10% FBS at 37°C in 5% CO_2/ 95% air. Two days after cells were confluent medium was replaced with DMEM containing 10% FBS, 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 1µM Dexamethasone, and 10µg/ml insulin. Cells were maintained in this differentiation medium for 72h and then replaced with DMEM containing 10% FBS and 5µg/ml bovine insulin. Soon after lipid droplets began to appear in cells and these differentiated adipocytes were grown for a further 8-10 days prior to treatment. This protocol was previously used to obtain cultures where more than 90% of cells expressed an adipocyte phenotype (Shojima et al. 2002; Song et al. 2002).

For resistin studies cells were differentiated in 6 well plates as described above, and maintained for a further 8-10days. Just prior to treatment medium was replaced with 1.5ml of serum free DMEM and cells were exposed to recombinant resistin for 30min (25 and 200ng/ml; Peprotech) before RNA was isolated. For RNAi studies differentiated adipocytes were trypsinized from the 250mL flasks and replated at 100,000 cells/well in 12 well Nunc plates and maintained for a further 24h in DMEM/10%FBS/5µg/ml insulin (Sigma; Oakville, ON), as previously described (Haruta et al. 1995; Usui et al. 2004). Cells were transfected 24h later with 100nM of the STEALTH molecules using optimized conditions for Lipofectamine 2000 (See below; 1.5µl:1µg STEALTH), and total RNA was isolated 24h later.

siRNA Transfection Optimization

Both N-1 cells (30,000 cells/well) and differentiated 3T3-L1 adipocytes (50,000cells/well) were seeded on glass cover slips in 12 well Nunc plates. Just prior to

transfection the cell medium was replaced in each well with 500μL of serum-free DMEM (Invitrogen). A fixed amount of a FAM-labeled siRNA (50pmol/well; Invitrogen) was diluted in 20μL of OptiMEM media (Invitrogen). Various amounts of transfection reagent (1, 2, 3 and 4μL) were diluted to a final volume of 25μL with OptiMEM. After a 5 min incubation at room temperature the FAM-labeled siRNA (20μL) and transfection complexes (25μL) were transferred into a single tube and mixed by swirling with the end of a pipette tip. The solutions were incubated for a further 25min prior to adding onto cells (45μL/well in a 12-well plate). Cells were transfected for a period of 4h, and then washed with Dulbecco's phosphate buffered saline (D-PBS) and fixed for 5min with 4% PFA. Cells were subsequently stained for 5 min using a 500nM DAPI solution prepared in D-PBS (Molecular Probes). Cover slips were mounted onto slides using the Slowfade® Antifade kit (Molecular Probes). Slides were photographed using a Retiga 1300 (QImaging; ON, Canada) digital camera attached to a Leitz Laborlux S microscope. Transfection efficiency was confirmed by visualizing the uptake of the fluorescently labeled siRNA (data not shown).

Plasmid Transfection Optimization

Cell lines were plated on cover slips in 12 well plates as above, and medium was replaced with 500μL of serum-free DMEM just prior to transfection. For plasmid transfection optimization an enhanced green fluorescent protein (EGFP) expressing plasmid was used since it was a similar size to the expression vectors that were used in subsequent experiments. The EGFP expressing plasmid was kindly provided by Dr. Paul Linsdell (Department of Physiology and Biophysics) and purified as outlined previously (See Chapter 2 section III). A fixed amount of plasmid (1.6μg/ well) was diluted to 40μL in OptiMEM. Varying amounts of lipofectamine 2000 (1, 2, 3 and 4μL) were diluted in OptiMEM to a final volume of 45μL, and incubated at room temperature for 5 min. The plasmid (40μL) and transfection complexes (45μL) were placed into a single tube and

mixed by swirling with a pipette tip and incubated for a further 25min. Cells were transfected for 4h, then medium was aspirated and replaced with DMEM containing 10%FBS. After a further 20h cells were washed with D-PBS and fixed for 5min using 4% PFA. Cover slips were mounted onto slides using the Slowfade® Antifade kit (Molecular Probes). Transfection efficiency was confirmed visually by the number of cells that expressed EGFP.

STEALTH siRNA Design And Transfection

Chemically modified STEALTH RNAi targets for mouse *rstn* and *fiaf* were designed using the STEALTH (STH) RNAi design tool (www.invitrogen.com). Two targets were designed for *rstn* (STH R4 and STH R12) and *fiaf* (STH F1 and STH F2), along with a non-specific control. These chemically-modified siRNA targets were obtained from Invitrogen (Burlington) (See Table 3 for siRNA sequences). Transfection conditions were optimized for the cell lines using the BLOCK-IT transfection kit (Invitrogen) according to the manufacturer's instructions and are similar to experiments in C6 cells (Chapter 2 Section III), or as outlined above. Interferon beta (IFNβ) gene expression was also analysed to rule out potential non-specific effects that could be occurring.

Rstn And Fiaf Expression Vectors

A full length *rstn* complementary DNA (cDNA) fragment was amplified by PCR using previously published primers (Sato et al. 2005a). The PCR product was purified using the QIAquick gel extraction kit according to the manufacturer's protocol (Qiagen). Both the pcDNA 3.1 (-) plasmid vector (Invitrogen) and purified amplicon were digested overnight at 37°C with Eco RI and Bam HI (Invitrogen) and then cleaned up using the QIAquick gel extraction kit (Qiagen). The full length resistin cDNA was then ligated into the linearized plasmid overnight at room temperature using T4 DNA ligase (Invitrogen). The *fiaf* expression vector was generously provided by Dr. A. Xu (Department of

Medicine, University of Hong Kong, China)(Xu et al. 2005). Both the *fiaf* and *rstn* expressing plasmids were transformed and propagated in E. Coli (DH5α; Invitrogen) and purified using the Purelink™ HiPure Plasmid Midiprep kit (Invitrogen; for details refer to Chapter 2, section III). After purification both plasmids were also subjected to realtime RT-PCR analysis for either *rstn* or *fiaf* to further confirm the presence of the desired inserts.

Realtime RT-PCR

Total RNA (1.5μg) was reverse transcribed (RT) and PCR amplified using the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's protocol using previously published primers and Taqman™ probes for *rstn*, cyclophilin (Brown et al. 2005b), *socs-3* (Steppan et al. 2005) and *fiaf* (Wiesner et al. 2006)(please see Chapter 2, Section VII for realtime primer/probe design and Table 2 for primer sequences). In brief, RNA was diluted to 12μL and then heat denatured for 5min at 70°C. Samples were returned to ice prior to the addition of 15μL of the 2x reverse transcription master mix and 3μL of the SuperScript™ III RT enzyme master mix (Invitrogen). The RT reaction consisted of a 10min incubation at 25°C, 45min incubation at 42°C, followed by a 5min 85°C termination step, and the resulting cDNA (30μL) was stored at -20°C. For PCR amplifications samples were amplified in duplicate and only 1 gene was analysed per reaction. Individual PCR reactions consisted of a 2x Platinum® quantitative PCR SuperMix-UDG, 7pmol of the sense and antisense primers, 1pmol of the appropriate dual-labeled probe, 3μL of cDNA, to a final volume of 33μL in sterile water. Reactions were heated to 10min at 95°C, followed by 50 amplification cycles of 95 °C for 20s and 60°C for 60s using a BioRad thermal cycler and an iQ realtime PCR detection system. A standard curve, that was prepared using a serial dilution of a reference sample, was included in each realtime run to correct for possible variations in product amplification. Relative copy numbers were obtained from standard curve values,

and were normalized to the values obtained for our house keeping gene, cyclophilin. Data are expressed as a percentage of the control +/-SEM. It should be noted that no significant variations in the expression of the housekeeping gene were observed between groups when evaluated using either the threshold cycle (C_T), or using the relative levels of expression, and this is consistent with previous reports (Bond et al. 2002). IFNβ gene expression was analysed in triplicate using the Platinum SYBR Green qPCR kit (Invitrogen) using previously published primers and conditions (Stewart et al. 2005).

Oil Red O Staining

Cellular lipids were stained using Oil Red O as outlined by others (Qiao et al. 2006), and used as an measure of 3T3-L1 adipocyte differentiation. In brief differentiated 3T3-L1 cells seeded in 12 well plates were transfected 24h later with 100nM of either of the various RNAi targets against *rstn* (STH R4 and STH R12), *fiaf* (STH F and STH F2), or the non-specific STH CTL, as described above. After culturing cells for a further 5 days in DMEM/10%FBS/5µg/mL insulin, cells were fixed for 1h in 4% PFA, and stained for 2h with 1ml of a stock solution of Oil Red O that was prepared by diluting 0.1mg/ml of Oil Red O dye (Sigma) in isopropanol. Cells were washed, and air dried for 1h, and then isopropanol (300µL/well) was added for 30min to dissolve incorporated stain. Absorbances (490nm) were measured on a microplate reader (BioRad), and corrected for by taking background measurements on isopropanol alone. Data are expressed as a percentage of the values obtained for cells transfected with STH CTL ± SEM.

The Effects Of Resistin On AMPK Activation In N-1 Neurons And 3T3-L1 Adipocytes

For AMPK studies N-1 neurons or differentiated 3T3-L1 adipocytes were cultured in 6 well plates as already outlined. Prior to resistin studies, cells were serum starved overnight, and then treated for 45 min with either 100 or 1000ng/ml of recombinant

resistin. In resistin overexpression studies cells were transfected for 5h with the resistin expressing plasmid, as outlined above, and then medium was replaced with DMEM/10%FBS and cells were cultured overnight prior to isolating cellular lysates. In contrast endogenous resistin expression was silenced using STH R4 by using the optimized conditions outlined above, and cellular lysates were isolated 24h later.

Western Blot Analysis Of AMPKα

Following treatment(s), cells were washed twice and then cells were harvested by scraping using D-PBS and transferred into Eppendorf tubes. After centrifugation, cellular pellets were lysed in Radioimmunoprecipitation RIPA buffer (containing 1mM phenylmethylsulphonylfluoride, 10μg/mL antipain, 10μg/mL leupeptin, 10μg/mL pepstatin, 5mM EDTA and 5mM EGTA) and sample concentrations were determined using the DC protein assay (Bio-Rad; Mississauga, ON). Samples (40μg) were separated on a 10% SDS-PAGE gel and transferred overnight onto HYBOND-C nitrocellulose membrane (Amersham Bioscience; Baie d'Urfé, QC). Membranes were washed with Tween-20 tris buffered saline (TTBS) prior to blocking in TTBS containing 10% milk powder. Blots were probed overnight with an anti-phosphorylated (Thr 172)-AMPKα (pAMPK) antibody (1:1000; Cell Signaling; Danvers, MA), washed 4 times with TTBS and then exposed to a donkey anti-rabbit horseradish peroxidase (HRP) conjugated antibody (1:5000; Amersham Bioscience). Bands for pAMPK were detected by chemiluminescence using SuperSignal™ West Femto Chemiluminescent substrate (Pierce; Rockford, IL). Membranes were washed and then probed with an antibody recognizing total AMPKα (1:1000; Cell Signaling). After 4 further washes in TTBS blots were exposed to donkey anti-rabbit HRP (1:5000; Amersham Bioscience). Again bands for total AMPKα were detected by chemiluminescence using SuperSignal™ West Femto (Pierce). Blots were then scanned into Photoshop 7.0 on a Macintosh G5 computer. Band intensity was quantified using NIH Image (v1.63), and relative ratios of

pAMPKα/total AMPKα were calculated. Data are expressed as a percentage of control ±

SEM.

Statistics

Data were analyzed using ANOVA using the Newman-Keuls post hoc test, or

using the Student's *t-test* where appropriate using graphpad PRISM v4.0a. Significance

was set at $p<0.05$.

III. Results

The Effects Of Resistin Treatment

Recombinant resistin dose-dependently inhibited *socs-3* expression in N-1

neurons, with a maximal reduction of 29% resulting after treatment with both the

200ng/ml ($p<0.01$) and 400ng/ml ($p<0.05$) doses (Fig 36A). In contrast resistin treatment

had no significant effect on *fiaf* mRNA in N-1 cells (Fig 36B). Treatment of 3T3-L1

adipocytes with recombinant resistin failed to modify *socs-3* expression (Fig 37A),

however *fiaf* mRNA was significantly inhibited with both the low (-31%; $p<0.01$) and high

dose (-40%; $p<0.01$) of resistin (Fig 37B). Note that recombinant resistin had no effect

on endogenous *rstn* gene expression in either cell line (data not shown).

Effects Of Rstn And Fiaf Overexpression In N-1 Cells

Transfecting N-1 cells with a *rstn* expressing plasmid (pRESISTIN) increased

endogenous *rstn* gene expression by 36000-fold, ($p<0.0001$), mRNA levels that are

comparable to those found in 3T3-L1 adipocytes (Fig 38A). However, relative to cells

transfected with an empty vector (pEMPTY), *fiaf* and *socs-3* gene expression were

reduced by 40% ($p<0.001$) and 25% ($p<0.05$) respectively (Fig 38B and 38C).

Conversely overexpressing *fiaf* (pFIAF) yielded a 230-fold increase ($p<0.0001$) in *fiaf*

mRNA, again to levels that are more comparable to those found in adipose tissue (Fig

39B). In contrast *rstn* and *socs-3* gene expression were unchanged (Fig 39A and 39C).
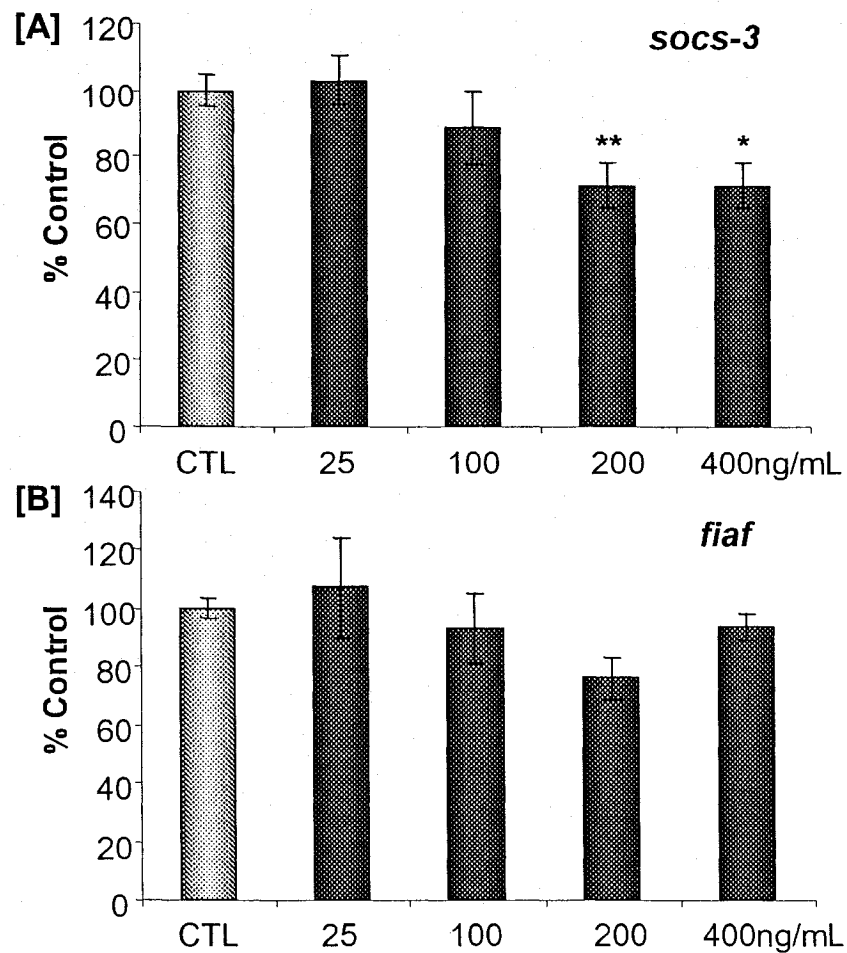
Figure 36: The effects of resistin on *socs-3* and *fiaf* gene expression in N-1 neurons

Serum starved N-1 cells were treated with various doses of recombinant resistin for 30 min prior to RNA isolation. *Socs-3* and *fiaf* gene expression were analysed using realtime RT-PCR. [A] Resistin dose-dependently inhibited *socs-3* gene expression in N-1 cells with maximal inhibition occurring with the 200ng/mL dose. [B] *Fiaf* mRNA was unaffected by resistin treatment. Data were pooled from multiple experiments and are expressed as percentage of the control ± SEM (n=4-8 for each dose). (*$p<0.05$, **$p<0.001$)
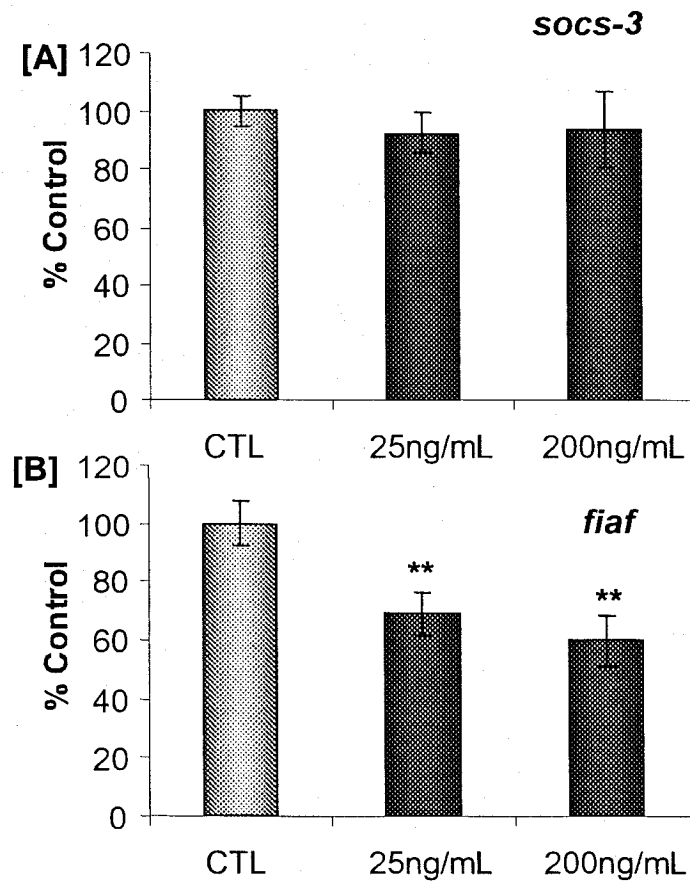
Figure 37: The effects of resistin on socs-3 and fiaf expression in 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were treated with a low and high dose of recombinant resistin for 30 min and socs-3 and fiaf gene expression were analysed using realtime RT-PCR. [A] Unexpectedly resistin treatment had no effect on socs-3 mRNA in 3T3-L1 cells. [B] In contrast fiaf gene expression was significantly inhibited by resistin treatment. Data were obtained from triplicate experiments and are expressed as a percentage of the control ± SEM (n=7-10 for each dose). (**$p<0.01$)
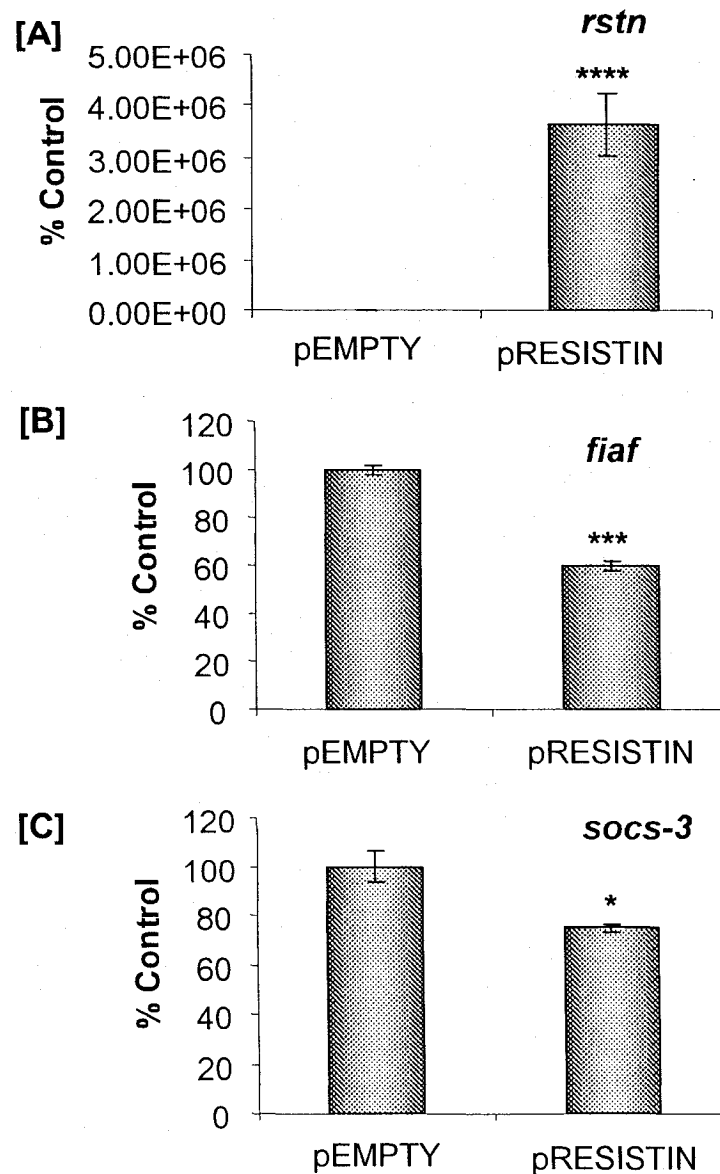
Figure 38: The effects of resistin overexpression on *fiaf* and *socs-3* in N-1 neurons. Gene expression was analysed after transfecting N-1 cells for 6h with a *rstn* overexpression plasmid, or an empty vector, using realtime RT-PCR. [A] As expected *rstn* gene expression was significantly higher in cells transfected with the expression vector. [B] Overexpressing *rstn* led to a significant reduction in *fiaf* mRNA (-40%). [C] *Socs-3* gene expression was also suppressed (-25%) by the overexpression of *rstn*. Data were pooled from duplicate experiments and are expressed as a percentage of the control ± SEM (n=6). (*p<0.05, ***p<0.001, ****p<0.0001)
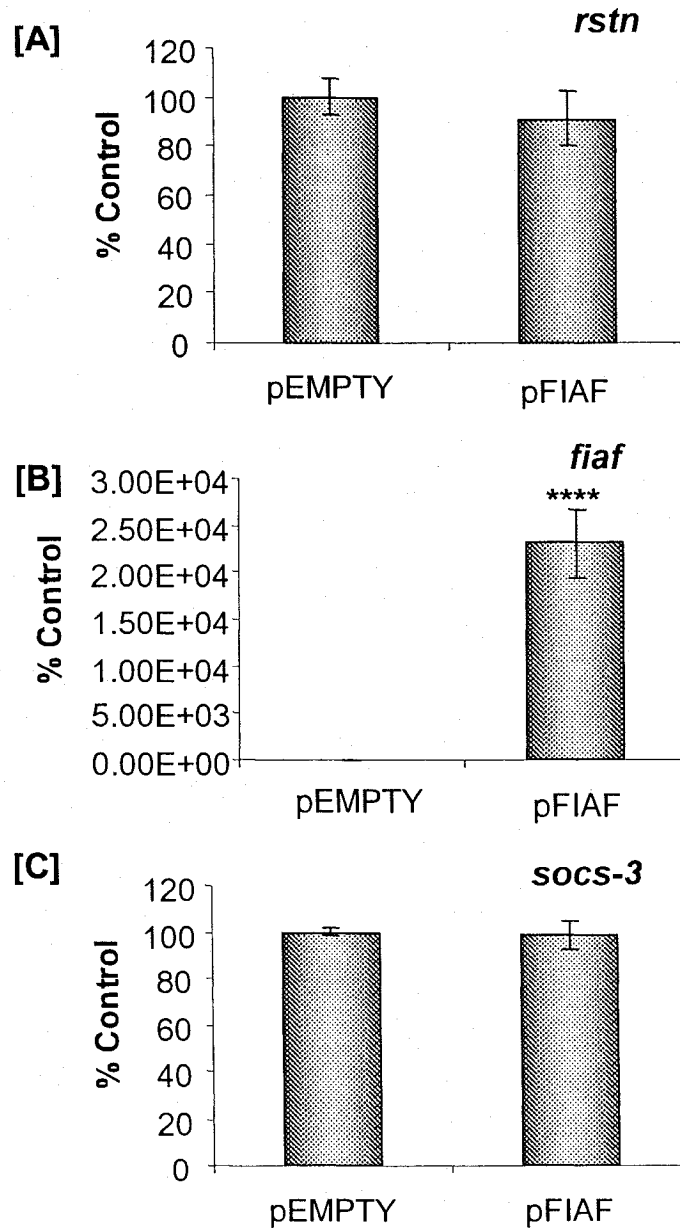
Figure 39: The effects of fiaf overexpression on rstn and socs-3 expression in N-1 neurons. Following transfection with a fiaf expression vector, or an empty vector, gene expression was analysed in N-1 neurons by realtime RT-PCR. [A] Rstn gene expression was unchanged by fiaf overexpression. [B] As expected fiaf mRNA was significantly increased in cells transfected with the fiaf vector. [C] Fiaf overexpression also failed to modify socs-3 gene expression. Data were pooled from duplicate experiments and are expressed as a percentage of the control ± SEM (n=8). (****p<0.0001)

Resistin RNAi Knockdown In N-1 Cells

N-1 cells were transfected with a 100nM of either STH R4 or STH R12, the resistin-specific RNAi targets. Although STH R4 reduced *rstn* mRNA by more than 50% (p<0.001), STH R12 only lowered *rstn* by 25% (p<0.01) (Fig 40A). Similarly STH R4 *increased fiaf* by 30% (p<0.01), whereas STH R12 inhibited *fiaf* by 70% (p<0.001) (Fig 40B). Likewise STH R4 elevated *socs-3* by 21% (p<0.01), but STH R12 resulted in a 35% (p<0.001) reduction (Fig 40C). Note that neither STH R4, nor STH R12, had any effect on interferon beta (IFNβ), a gene implicated in the non-specific interferon response (Fig 40D).

RNAi-Mediated Silencing Of Fiaf In N-1 Cells

As above, N-1 cells were transfected with either of the *fiaf*-specific siRNA targets (100nM; STH F or STH F2) As seen in Fig 41B, both targets reduced *fiaf* mRNA by more than 50% (p<0.001). In contrast *rstn* and *socs-3* mRNA tended to be slightly higher, but this was not significant (Fig 41A and 41B). Similarly neither of the *fiaf*-specific targets had any effect on IFNβ (Fig 41D).

Effects Of Resistin Knockdown In Serum Starved N-1 Cells

As demonstrated in Chapter 2 (section IVa) the effects of gene silencing can be prolonged by reducing cell proliferation using reduced serum conditions. As expected, when cells were transfected with the most effective siRNA target against resistin (STH R4; 100nM) and cultured in serum-free medium, an even greater reduction in *rstn* mRNA resulted (60%; p<0.01) (Fig 42A vs. Fig 40A). Similarly the detected increases in *fiaf* (+46%; p<0.01) and *socs-3* (+65%; p<0.01) gene expression were greater than what was detected in cells grown under normal serum conditions (recall Fig 42B vs. 40B and Fig 42C vs. Fig 40C). In contrast, tranfecting cells with STH F2 (100nM), then culturing cells in serum free DMEM as above, failed to further modify *rstn* or *socs-3* expression (data not shown).
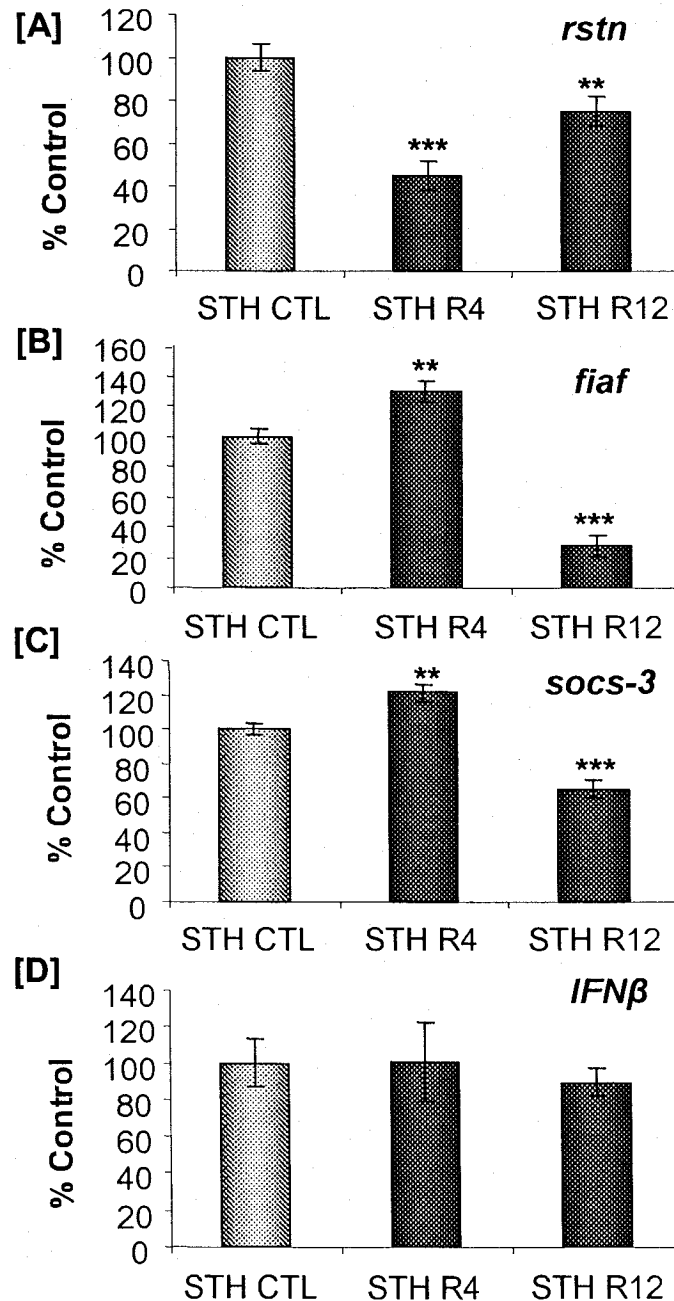
Figure 40: *Rstn* knockdown in N-1 cells. N-1 cells were transfected with 100nM of either the non-specific STH CTL molecule or two RNAi targets against *rstn* (STH R4 and STH R12). Gene expression was analysed 24h later using realtime RT-PCR. [A] As expected *rstn* gene expression was significantly decreased by 55% using STH R4 and by 25% using STH R12. [B] In contrast STH R4 induced a significant increase in *fiaf* (+30%), whereas STH R12 inhibited *fiaf* (-70%). [C] Similarly STH R4 elevated *socs-3* expression (+20%), but STH R12 had the opposite effect (-35%). [D] Neither target had a significant effect on *IFNβ* gene expression. Data were pooled from triplicate experiments and are expressed as a percentage of the control ± SEM (n=12). (**p<0.01, ***p<0.001)
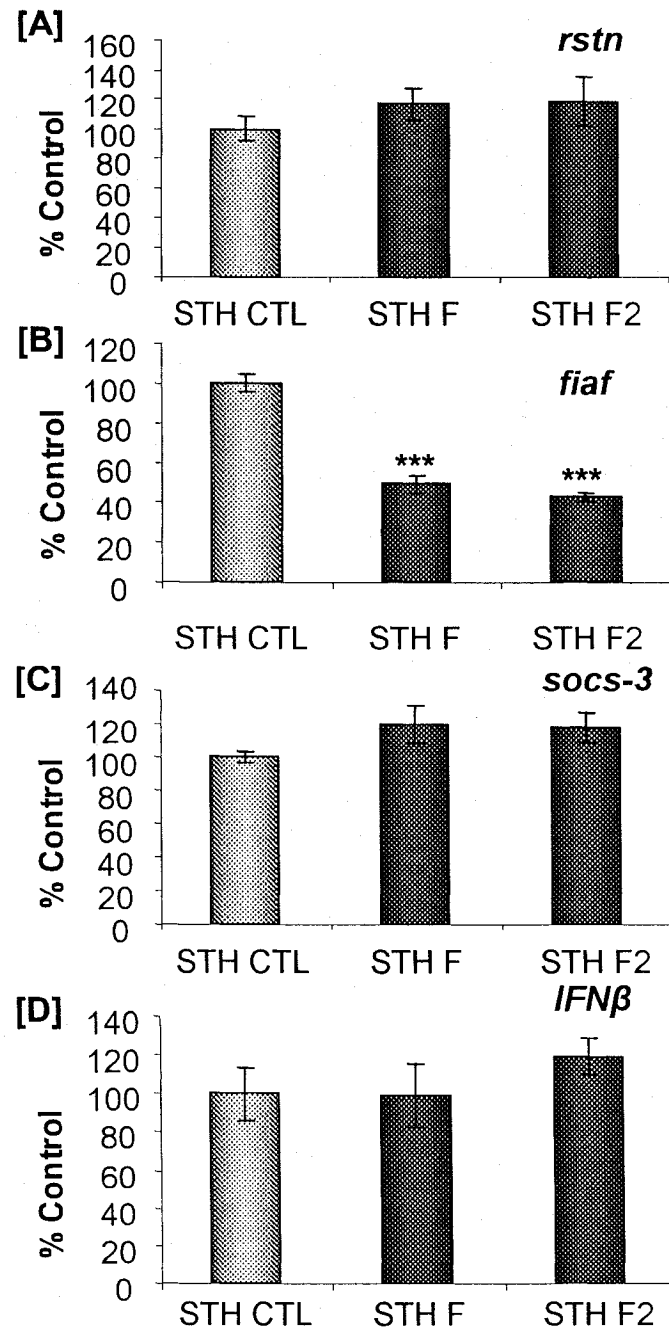
Figure 41: *Fiaf* knockdown in N-1 cells. Gene expression was analysed in N-1 cells 24h following transfection with two fiaf-specific siRNAs (STH F or STH F2; 100nM), or the non-specific control (STH CTL), using realtime RT-PCR. [A] No significant increases in *rstn* gene expression were detected with either of the *fiaf*-specific targets. [B] Both STH F and STH F2 significantly reduced (-50%) *fiaf* gene expression in N-1 neurons. [C] *Socs-3* mRNA was unaffected by *fiaf* silencing. [D] *IFNβ* gene expression was not significantly modified by either fiaf-specific siRNA. Data were pooled from triplicate experiments and are expressed as a percentage of the control ± SEM (n=12). (***p<0.001)
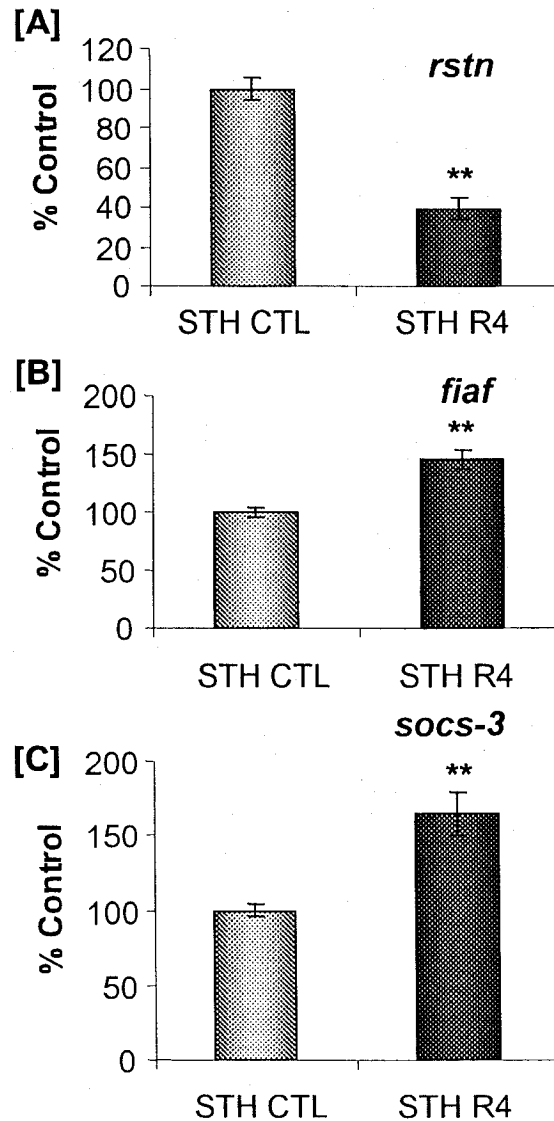
Figure 42: The effects of *rstn* **knockdown in serum starved N-1 cells.** N-1 cells were transfected with STH R4 (100nM), or STH CTL, and gene expression was analysed 24h after culturing in serum-free DMEM. [A] *Rstn* gene expression was significantly inhibited in N-1 cells (-60%). [B] As expected *fiaf* gene expression was significantly increased by STHR4 (+45%), and this increase was larger than the detected changes under normal culture conditions (recall figure 40). [C] Similarly *socs-3* gene expression was increased by 65% following *rstn* silencing. Data were pooled from triplicate experiments and are expressed as a percentage of the control ± SEM (n=12). (**p<0.01)

### siRNA-Mediated Knockdown Of Rstn In 3T3-L1 Cells

As with N-1 cells, differentiated 3T3-L1 adipocytes were transfected with 100nM of either of the rstn-specific siRNAs. As seen in Fig 43A STH R4 reduced rstn gene expression by 52% ($p<0.001$), and STH R12 by 74% ($p<0.001$). In contrast to N-1 cells, STH R4 induced a small reduction in fiaf (-20%; p=n.s.) and socs-3 (-20%; $p<0.05$) (Fig 43 B and 43C). However STH R12 did not have any significant effect. Similarly neither STH R4, nor STH R12, modified IFNβ expression (Fig 43D).

Silencing Fiaf In 3T3-L1 Adipocytes

Cells transfected with STH F or STH F2 (100nM; 24h) resulted in a 42% ($p<0.001$) and 28 % ($p<0.001$) reduction in fiaf expression, respectively (Fig 44B). Although neither of the fiaf-specific siRNA had any effect on rstn mRNA (Fig 44A), STH F induced a 30% reduction in socs-3 ($p<0.001$) (Fig 44C). Similar to N-1 cells, neither target modified IFN β gene expression.

The Effects Of Rstn And Fiaf Knockdown On Adipogenesis

Just following differentiation, but before major lipid accumulation had occurred, 3T3-L1 adipocytes were transfected with both of the rstn-specific (STH R4 and STH R12; 100nM) or fiaf-specific (STH F and STH F2; 100nM) RNAi targets. Cells were cultured for a further 5 days before staining cells with Oil Red O, As seen in Fig 45A both of the resistin-specific siRNA molecules resulted in a significant reduction in lipid accumulation (20%, $p<0.001$). Conversely fiaf knockdown, with either RNAi target, had no effect on lipid accumulation (Fig 45B).

The Effects Of Resistin On AMPK Activation In N-1 Neurons

As done for socs-3, N-1 cells were treated with two doses of recombinant resistin (45min; 100 or 1000ng/ml) which induced a 30% ($p<0.05$) and 20% (p= N.S.) increase in the ratio of pAMPKα/total AMPKα (Fig 46A). Similarly overexpressing resistin induced a 47% ($p<0.001$) increase in phosphorylated AMPKα in N-1 neurons (Fig 46B). In contrast
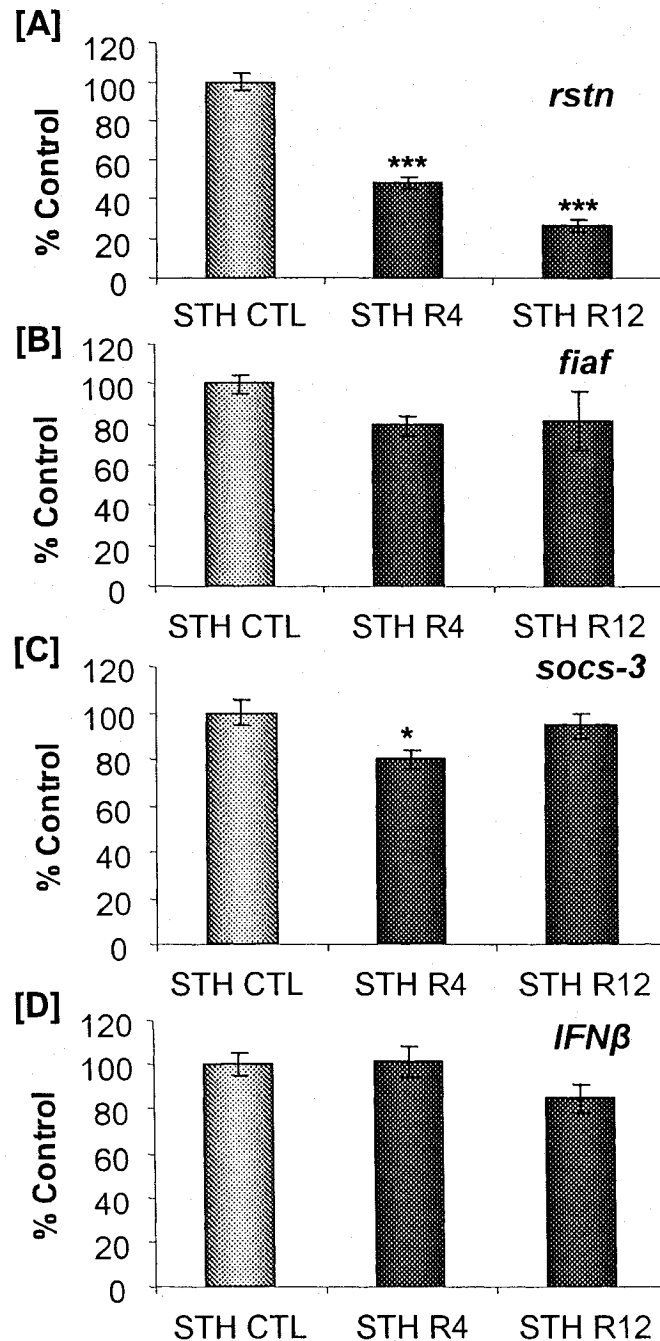
Figure 43: *Rstn* knockdown in 3T3-L1 adipocytes

Gene expression was analysed in differentiated 3T3-L1 cells 24h following transfection with 100nM of the two *rstn* siRNAs (STH R4 and STH R12), or the non-specific STH CTL molecule, using realtime RT-PCR. [A] *Rstn* gene expression was significantly reduced by 50% using STH R4, and by 70% using STH R12. [B] No changes in *fiaf* expression were detected. [C] STH R4 significantly decreased *socs-3* expression (-20%), but STH R12 had no effect. [D] Neither target had a significant effect on *IFNβ* gene expression. Data were pooled from triplicate experiments and are expressed as a percentage of the control ± SEM (n=12). (*p<0.05, ***p<0.001)
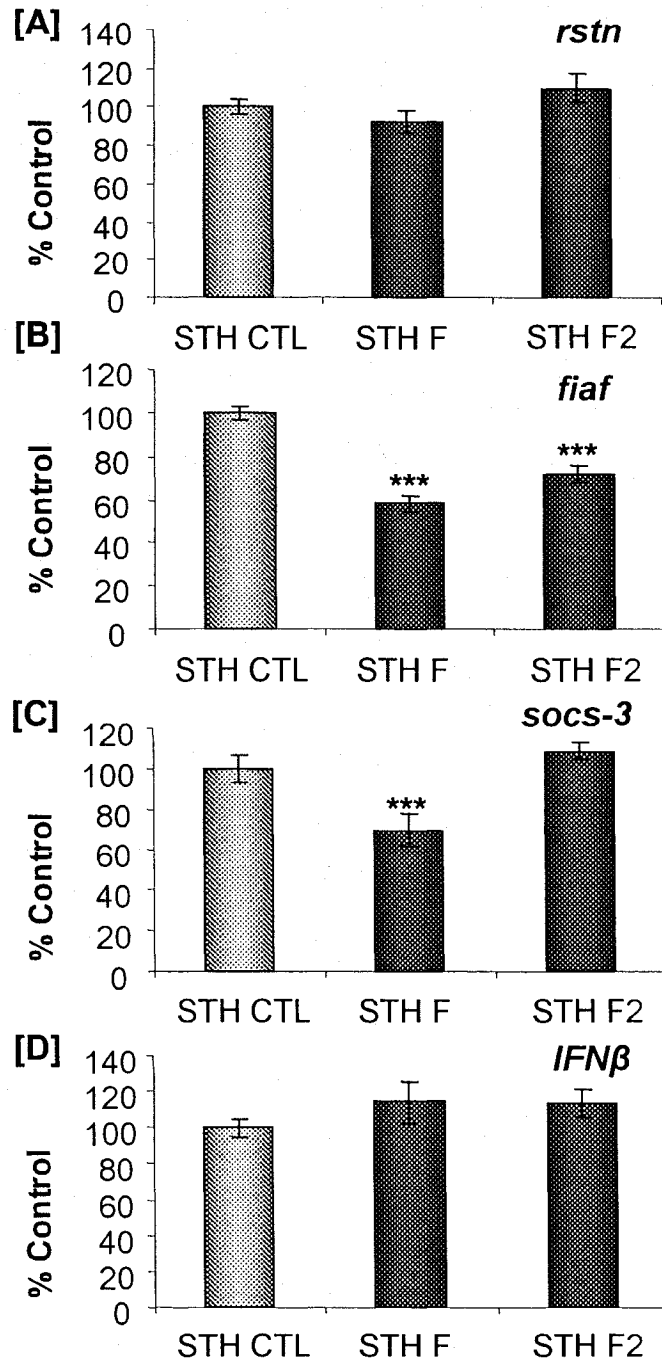
Figure 44: *Fiaf* **knockdown in 3T3-L1 cells.** 3T3-L1 adipocytes were transfected with siRNAs targeting *fiaf* (STH F or STH F2; 100nM), or the control (STH CTL), and gene expression was analysed 24h later using realtime RT-PCR. [A] Neither of the *fiaf*-specific targets induced significant changes in *rstn* gene expression. [B] Significant reductions in *fiaf* were induced by STH F (-40%) and STH F2 (-30%). [C] Although STH F2 had no effect, *socs-3* gene expression was inhibited by STH F (-30%) [D] *IFNβ* gene expression was unchanged. Data were pooled from triplicate experiments and are expressed as a percentage of the control ± SEM (n=12). (***p<0.001)
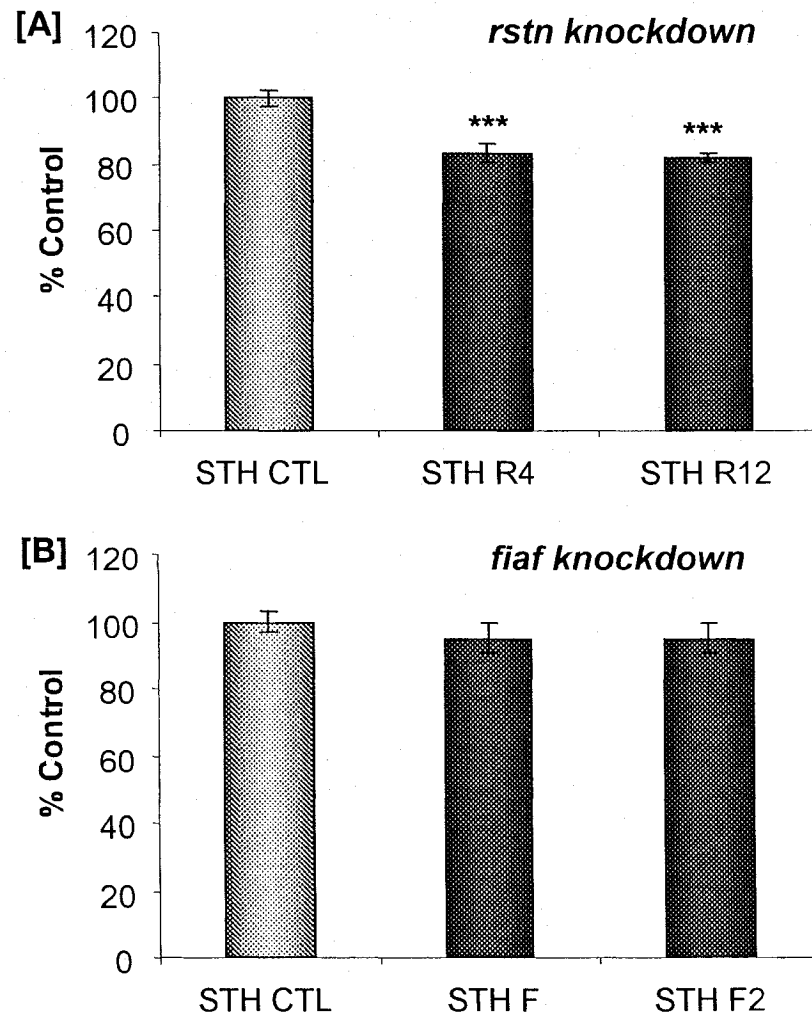
Figure 45: The effects of adipokine knockdown on 3T3-L1 adipocyte differentiation. Just following differentiation 3T3-L1 cells were transfected with either of the siRNAs against *rstn* or *fiaf* (100nM) and cultured for a further 5 days before staining cells with the lipid stain, Oil red O. [A] Resistin knockdown significantly reduced adipocyte differentiation (-20%). [B] In contrast *fiaf* knockdown did not have any effect on lipid accumulation. Data were pooled from duplicate experiments and are expressed as a percentage of the control ± SEM (n=8). (***p<0.001)
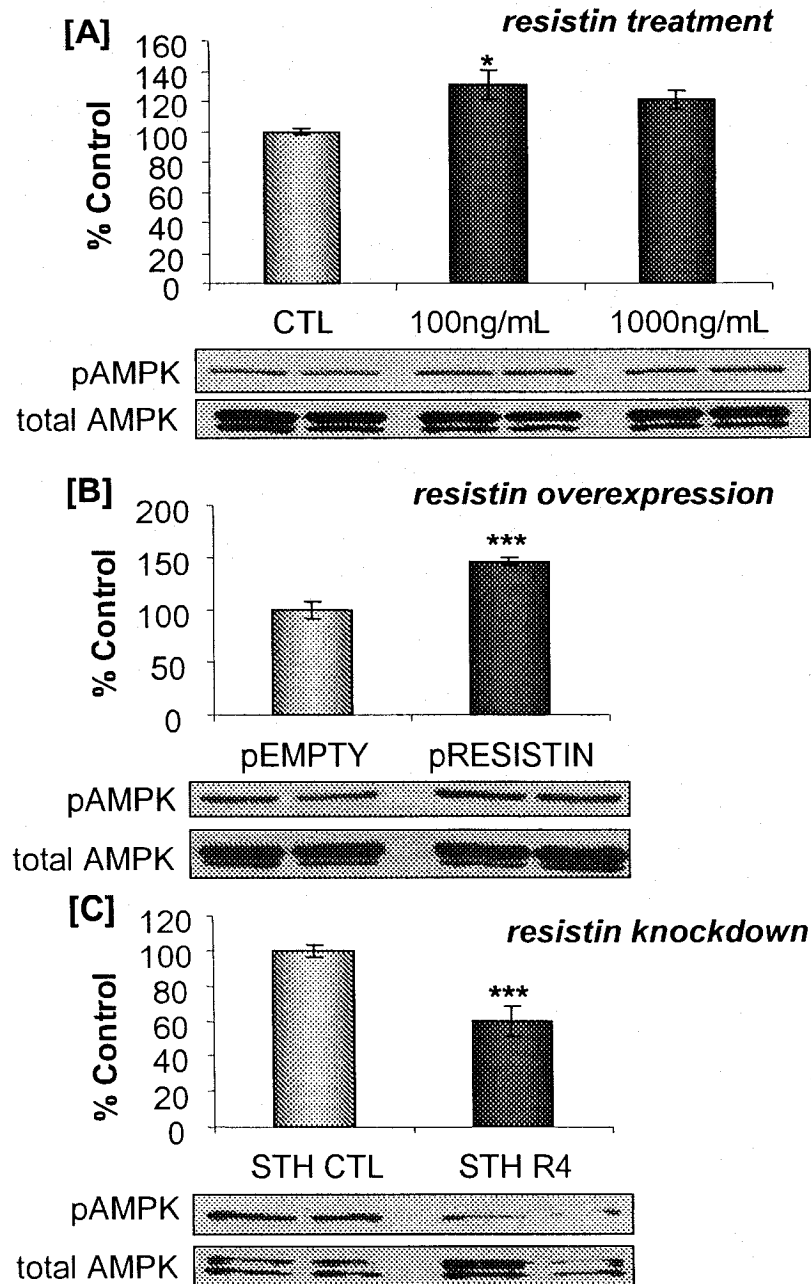
**Figure 46: The effects of resistin on AMPK activation in N-1 neurons.** The effects of resistin on AMPK activation in N-1 cells was assessed by detecting the relative ratio of active (pAMPK) to total AMPK using Western blot analysis. [A] Resistin treatment (45min) significantly increased the amount of active AMPK (+30%), although it was not significant with the higher dose. [B] When cells were transfected for 24h with a resistin overexpression vector pAMPK levels were significantly increased (+45%). [C] Conversely *rstn* knockdown attenuated pAMPK activation (-40%). Representative western blots are shown for pAMPKα and total AMPKα. Data are expressed as a percentage of the control ± SEM and were obtained from duplicate or triplicate experiments (n=4-8). (*p<0.05, ***p<0.001)
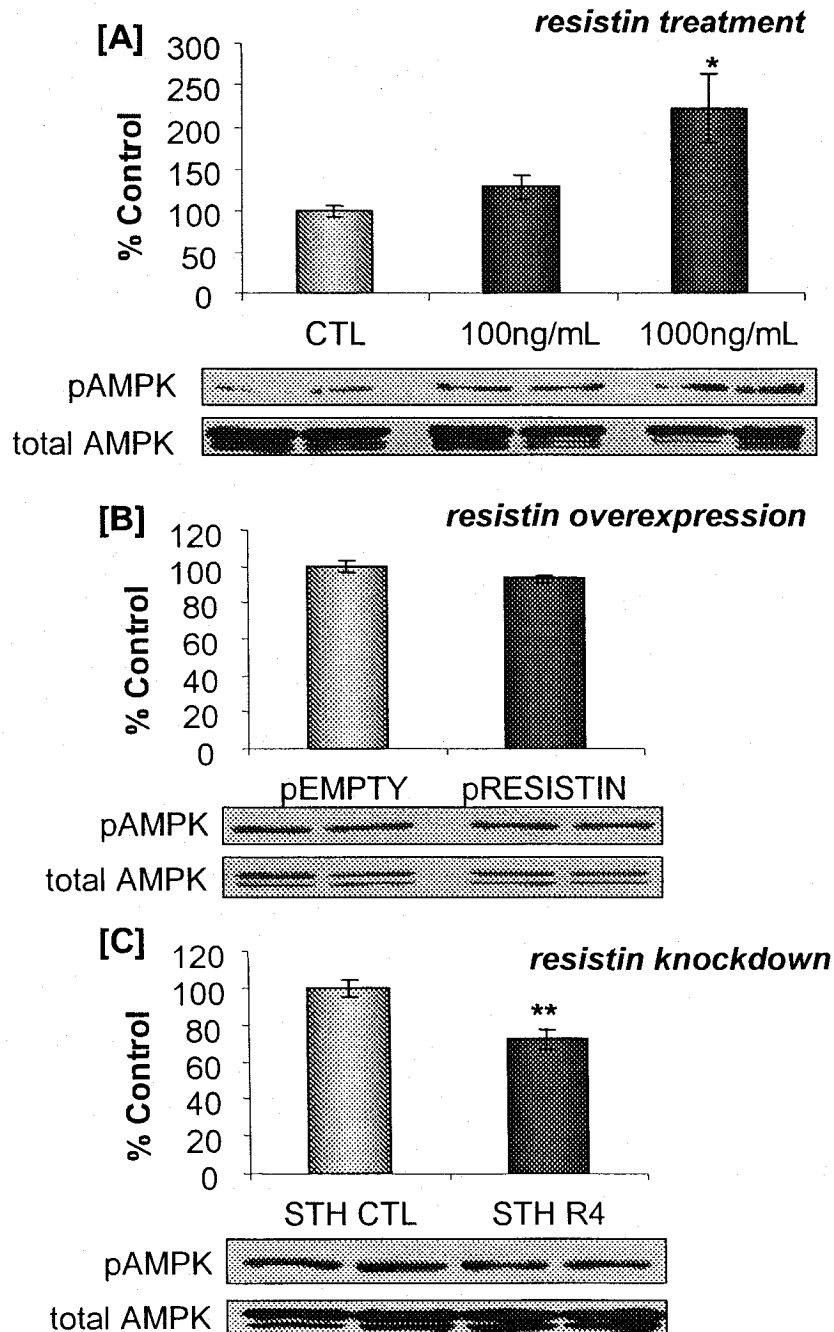
Figure 47: The effects of resistin on AMPK activation in 3T3-L1 adipocytes. AMPK activation was also assessed in 3T3-L1 adipocytes using Western blot analysis. [A] Treating cells with resistin (45min) dose-dependently activated AMPK (30% or123%). [B] No significant increase in pAMPK was detected after 24h when 3T3-L1 cells were transfected with a resistin expression vector [C] *Rstn* knockdown significantly decreased pAMPK activation (-30%). Representative western blots are shown for pAMPKα and total AMPKα. Data were obtained from duplicate or triplicate experiments and are expressed as a percentage of the control ± SEM (n=5-9). (*p<0.05, **p<0.01)

when cells were transfected with STH R4, the *rstn*-specific siRNA, a 40% (p<0.001) reduction in pAMPKα was detected (Fig 46C).

The Effects Of Resistin On AMPK Activation In 3T3-L1 Adipocytes

When 3T3-L1 adipocytes were treated with resistin the lower dose (100ng/ml) induced a 28% increase in pAMPK, whereas the high dose (1000ng/ml) resulted in a 123% (p<0.05) increase in the relative ratio of pAMPK/total AMPK (Fig 47A). In contrast overexpressing resistin failed to modify the amount of pAMPKα (Fig 47B). However transfecting differentiated 3T3-L1 cells with STH R4 induced a 28% (p<0.01) reduction in pAMPKα (Fig 47C).

IV. Discussion

These data suggest that resistin modifies cellular signaling (i.e. *socs-3*) and energy sensing (i.e. AMPK) pathways in N-1 hypothalamic neurons and in differentiated 3T3-L1 adipocytes, although these effects are cell-type specific. For example treating N-1 neurons with recombinant resistin, or alternatively overexpressing *rstn*, lead to significant reductions in *fiaf* and *socs-3* gene expression. Conversely when endogenous *rstn* gene expression was specifically silenced, using RNAi, significant increases in *fiaf* and *socs-3* mRNA were detected in N-1 cells. Exogenous resistin treatment, or the overexpression of *rstn,* was also associated with increased AMPK activity in these neurons, whereas resistin knockdown significantly lowered the ratio of pAMPKα/total AMPK. However modifying *fiaf* expression, either by overexpression or by silencing it using RNAi, had no significant effect on *rstn* or *socs-3* in N-1 cells, thus eliminating the possibility of a reciprocal relationship with resistin. Although resistin treatment also decreased *fiaf* gene expression in 3T3-L1 adipocytes, unexpectedly no significant changes in *socs-3* were detected. The silencing of *rstn* in 3T3-L1 cells, by RNAi, also attenuated *fiaf* and *socs-3* expression and it appeared to inhibit 3T3-L1 adipocyte differentiation. Treating 3T3-L1 cells with recombinant resistin also resulted in an

increase in phosphorylated AMPKα, although *rstn* overexpression appeared to have no effect. Conversely silencing resistin appeared to lower the ratio of pAMPKα/total AMPKα in 3T3-L1 adipocytes, although the changes were smaller than those detected in N-1 neurons. Taken together these in vitro experiments indicate that resistin, from both endogenous and exogenous sources, modulates the expression of *fiaf* and *socs-3*, as well as influences AMPK activity, in N-1 neurons and 3T3-L1 adipocytes in a cell line-dependent manner. Furthermore these data implicate endogenous resistin in the regulation of local energy homeostasis and signaling events involved in the control of appetite and peripheral energy homeostasis.

Substantial evidence has been generated which clearly demonstrates that, like adipose tissue, the brain also expresses several adipokines including *rstn* and *fiaf* (Wilkinson et al. 2007). As mentioned above, many studies on adipokine gene expression have taken advantage of the 3T3-L1 adipocyte cell line model, but until recently there appeared to be no suitable mouse cell lines available to study the regulation of centrally-derived adipokines. Two other common brain cell line models that were previously available, GT1-7 cells or N1E-115, also appeared poorly suited for studying hypothalamic energy homeostasis. For example N1E-115 neurons were isolated from a spontaneous tumor in the spinal cord, whereas GT1-7 cells do not co-express critical genes implicated in energy homeostasis, such as neuropeptide Y (NPY) or agouti-related peptide (AgRP) (Cai et al. 2007). In contrast the array of hypothalamic neuronal cell lines that were developed and characterized by Belsham et al. (2004) appear to be more suitable models for obesity related studies since their gene profiles are representative of the intact hypothalamus (Belsham et al. 2004). For example many of these cell lines co-express a variety of neuropeptides implicated in adipokine cell signaling and energy homeostasis, including NPY and AgRP or proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART) (Belsham et al.

2004; Cui et al. 2005; Titolo et al. 2006). Moreover these hypothalamic-derived neurons respond to external stimuli in a manner that is consistent with changes seen in the intact hypothalamus. For example glucose inhibited AMPK activity in vivo (Minokoshi et al. 2004), and this was recently confirmed in the N43-5 cell line (Cai et al. 2007). Another example is the demonstration that estradiol acutely inhibits NPY expression, data that are consistent with the reported anorexigenic effects of estradiol in vivo (Titolo et al. 2006). However the effects of estradiol were cell-line dependent, and they had unique gene expression profiles, suggesting that these cell lines were derived from two distinct hypothalamic regions (Titolo et al. 2006). Thus like the intact hypothalamus, where regional variations in gene expression are not uncommon, different cell lines might generate unique responses to hormones. In the present studies the N-1 cell line was employed since it co-expresses *rstn* and *fiaf,* like the intact hypothalamus (recall Figure 35), in addition to expressing the long form of the leptin receptor mRNA (Belsham et al. 2004). The use of this in vitro model permitted the conclusion that the effects induced by resistin in N-1 cells are occurring directly. As noted already (see section I), the reductions in appetite (Tovar et al. 2005), or increases in hepatic gluconeogenesis (Muse et al. 2005; Singhal et al. 2006), that have been reported following the icv injection of resistin do not exclude the possibility that resistin might stimulate other types of brain cells to release anorexigenic or inflammatory neuropeptides that subsequently modify hypothalamic energy homeostasis. Thus these data revealed that resistin directly inhibits *fiaf* and *socs-3* expression and activates AMPK in N-1 cells. However an important next goal will be to confirm resistin has a similar effect on gene expression in the intact hypothalamus in vivo.

Although no receptors for resistin have been cloned or characterized, evidence suggests that they are present in the brain. As noted above, the icv injection of resistin was reported to induce hepatic gluconeogenesis in mice (Muse et al. 2005; Singhal et al.

2006), and icv injection of resistin peptide fragments acutely reduced food intake in both

fasted and satiated rats (Tovar et al. 2005). However repeated daily icv injections of

resistin over the period of a week failed to reduce bodyweight, and this is consistent with

the normal bodyweights reported for hyperresistinemic mice (Rangwala et al. 2004), as

well as mice lacking resistin (*rstn -/-*; (Banerjee et al. 2004). Brunetti et al. (2004) also

reported that resistin inhibited the depolarization-induced secretion of norepinephrine

and dopamine from rat hypothalamic synaptosomes (Brunetti et al. 2004). The effective

concentration of resistin was in the nanomolar range, clearly much lower than the icv

dose (10µg/rat) used by Tovar et al (2005) to induce *c-fos* expression. This difference

might reflect the greater bioactivity of recombinant resistin, used by Brunetti et al (2004),

compared to the resistin fragments used by Tovar et al. (2005). Our evidence, that

recombinant resistin significantly reduced the expression of *socs-3* in hypothalamic

neurons, provides tentative support that the hypothalamus is also a resistin target tissue.

The inhibitory concentrations of resistin used in these studies (25-400ng/ml) are within

the range of effective doses reported by others for *in vitro* investigations. For example,

resistin (50nM; approx. 600ng/ml) inhibited insulin-stimulated glucose uptake in rat

skeletal muscle cells (Palanivel et al. 2006), and reduced dopamine release from

synaptosomes (125ng/ml; (Brunetti et al. 2004). Recombinant human resistin also

increased preadipocyte proliferation *in vitro* (375ng/ml; (Ort et al. 2005)). However these

concentrations exceed the reported plasma resistin levels for both humans (10ng/ml;

(Lee et al. 2003)) and mice (approx. 5ng/mL; (Lee et al. 2005)). Moreover the effective

levels of resistin that regulate *socs-3* expression in our experiments are approximately

10-fold higher than those reported to elevate *socs-3* mRNA in 3T3-L1 adipocytes

(Steppan et al. 2005). However, in my hands this lower dose of resistin (25ng/ml) had no

effect on *socs-3* mRNA in serum-starved N-1 cells, nor in 3T3-L1 adipocytes. These

differing responses may have arisen due to the different sources of recombinant resistin

that were used (i.e. in house purification vs. commercial source (Peprotech)). In total the data suggest that the N-1 hypothalamic cell line is also a resistin target, and is in further support of the hypothesis that the hypothalamus is a resistin sensitive tissue.

The specificity of RNAi has recently been questioned, with some off-target responses having been reported including the induction of interferon-stimulated genes (ISG), such as interferon β (IFNβ) (Jackson and Linsley 2004). Improvements in siRNA design, and the introduction of chemical modifications to prolong the silencing effect, appear to reduce the risk of activating these non-specific RNAi responses (Jackson et al. 2006; Snove and Rossi 2006). For example our STEALTH™ siRNA molecules had no effect on IFNβ mRNA levels in either cell line following the siRNA-mediated silencing of *rstn* and *fiaf,* suggesting that they did not induce non-specific RNAi pathways. However other off-target RNAi pathways have also been identified that appear independent of the ISG-mediated responses. As noted in Chapter 2, high doses of siRNA induced the expression of intracellular endonuclease leading to a more rapid attenuation of gene silencing (Hong et al. 2005). Similarly the hepatic expression of small hairpin RNAs (shRNA) appeared to saturate RNAi pathways and proved fatal in mice (Grimm et al. 2006). Although measuring IFNβ expression limits the possibility that off-target pathways are activated, it alone does not totally exclude the possible activation of other non-specific pathways. Additionally two effective siRNA targets against *rstn* were identified in 3T3-L1 cells (STH R4 and STH R12), but only one of these appeared to work well in N-1 cells. It seems likely that the ineffective molecule, STH R12, was inducing non-specific effects in N-1 cells since it only moderately lowered *rstn* expression, and the detected effects on *fiaf* and *socs-3* were the opposite to what was observed in cells transfected with the other resistin siRNA, STH R4. Perhaps STH R12 induced the expression of endogenous endonucleases in N-1 cells, which in turn may have induced a global silencing response since the expression of 3 out of the 4 genes analysed had been

significantly attenuated (see Figure 40). However the effects of STH R4 appear to be specific since treating N-1 neurons with resistin, or overexpressing *rstn,* induced the expected inhibitory effects on *fiaf* and *socs-3* expression while increasing the phosphorylation of AMPK, responses that were the exact opposite of those generated by STH R4.

Could the observed changes in *socs-3* expression be of physiological (or pathological) significance? Following *rstn* knockdown, *socs-3* mRNA was significantly increased by 20% (p<0.01) under normal culture conditions, or by 65% (p<0.01) in serum starved N-1 neurons. Also, as expected, resistin treatment reduced *socs-3* mRNA in N-1 cells. The differential increase in *socs-3* could be due to a slightly greater reduction in *rstn* expression that was achieved following serum starvation in N-1 neurons, but may also have resulted by the removal of serum borne components that could also be impacting *socs-3* expression. Additionally the reported increases in *socs-3* that were observed in 3T3-L1 adipocytes are the opposite of what was detected in N-1 cells, suggesting that resistin's actions are cell-type, or possibly tissue-specific. *Socs-3* was previously implicated as a potent feedback inhibitor of leptin and insulin signaling, and is thought to be involved in the development of obesity-related leptin resistance (Howard et al. 2004; Munzberg et al. 2004). Thus excessive weight gain is associated with increased hypothalamic *socs-3* expression (Munzberg et al. 2004), whereas *socs-3* haploinsufficiency (i.e. a 50% mRNA reduction) is protective against the development of diet-induced obesity (DIO) (Howard et al. 2004). These effects on bodyweight are likely dependent on central pathways since the neuron-specific (Mori et al. 2004), or POMC-specific (Kievit et al. 2006), deletion of *socs-3* is protective against DIO and lead to improvements in glucose tolerance. In contrast the liver-specific deletion of *socs-3* induced systemic insulin resistance and led to significant increases in bodyweight (Torisu et al. 2007). In 3T3-L1 adipocytes resistin was reported to induce *socs-3*

expression, and this was hypothesized to be part of the mechanism involved in the development of peripheral insulin resistance (Steppan et al. 2005). Although our *rstn* knockdown experiments did produce the expected decrease in *socs-3* mRNA, treatment of 3T3-L1 cells with resistin failed to increase *socs-3* expression. As noted above, this discrepancy may have arisen due to variations in conditions used to induce adipocyte differentiation of 3T3-L1 cells as was the source of the recombinant resistin. Moreover the overexpression of *socs-3* in adipose tissue induced modest decreases in bodyweight and failed to induce systemic insulin resistance (Shi et al. 2006). This suggested that resistin must be targeting other tissues, such as the brain, in order to modify peripheral insulin sensitivity.

The magnitude of the *socs-3* gene expression changes detected in N-1 neurons, either following either *rstn* knockdown or after resistin treatment, are similar to those that appears to profoundly modify leptin and insulin sensitivity in vivo (Munzberg et al. 2004). Thus resistin might be expected to induce significant changes in body mass in vivo. However resistin deficiency alone is incapable of inducing significant changes in bodyweight (Banerjee et al. 2004), nor does the transgenic overexpression of *rstn* (Rangwala et al. 2004), or the repeated icv injections of resistin peptide fragments (Tovar et al. 2005). Perhaps the lack of change in body mass could be the result of compensational changes that maintain metabolic stability. For example mice that are deficient in both leptin and resistin have significantly greater bodyweights than those that are lacking a single adipokine, but paradoxically their blood glucose levels are significantly lower than those measured in ob/ob mice (Qi et al. 2006). Thus resistin-deficiency might be impacting hypothalamic energy metabolism and bodyweight regulation, but other compensational pathways or mechanisms are likely preventing it from manifesting into an overt phenotype. Thus neuronal resistin appears to influence

cytokine signaling in N-1 cells and this could have implications for the regulation of central energy homeostasis in vivo.

The activation of AMPK was assessed to further verify whether resistin could be influencing cellular energy metabolism. The results of these studies suggest that resistin activates AMPK both in N-1 neurons and 3T3-L1 adipocytes since acute resistin treatment increased the ratio of phosphorylated AMPKα/total AMPKα. Similar increases in pAMPKα were detected following the overexpression of *rstn* in N-1, but not in 3T3-L1 cells. This lack of effect in 3T3-L1 cells could be due to the relatively modest increases in resistin that occur following *rstn* overexpression since this cell line already expresses fairly high levels of *rstn*. In contrast *rstn* expression is relatively weak in N-1 cells, thus using a plasmid-based system to increase *rstn* expression to levels that are normally present in adipose tissue had a significant impact on AMPK activation. Additionally the RNAi-mediated reduction in *rstn* induced the expected decreases in pAMPKα in both cell lines. In adipose tissue AMPK appears to modulate lipid metabolism by altering both transcriptional and enzymatic activity (Long and Zierath 2006; Steinberg et al. 2006). For example the overexpression of a non-secreted form of resistin impaired fat re-esterification in rat adipose tissue (Pravenec et al. 2006). In contrast resistin knockdown reduced lipid accumulation in 3T3-L1 cells, as seen by the reduced Oil red O staining, and confirms what had already been reported in these cells (Gong et al. 2004).Thus adipocyte-derived resistin might regulate intracellular lipid metabolism via an AMPK-dependent mechanism. In contrast hypothalamic AMPK appears to modulate appetite and glucose homeostasis (McCrimmon et al. 2004; Minokoshi et al. 2004; Kahn et al. 2005). Although the diabetogenic effects of resistin are still being elucidated, these data are consistent with the hypothesis that resistin exerts at least part of its effects via a hypothalamic-dependent mechanism, which may possibly involve an AMPK-associated component. For example the icv injection of resistin was reported to induce hepatic

gluconeogenesis (Muse et al. 2005; Singhal et al. 2006) much like the reported effects of activating AMPK in the VMH (McCrimmon et al. 2004; McCrimmon et al. 2006). However it should be noted that in the latter study the authors assumed AMPK was activated following the injection of 5-aminoimidazole-4-carboxamide riboside (AICAR), but AMPK activation was never assessed directly. AICAR might also be modifying the activity of other energy sensing enzymes that could also be inducing hepatic gluconeogenesis since this compound does interfere with multiple metabolic pathways (Hardie et al. 2003). These clearly show that resistin stimulated AMPK activation in N-1 cells, and suggests that it might be part of the mechanism responsible for inducing the detected increases in peripheral glucose metabolism that occurred following the icv injection of resistin (Muse et al. 2005; Singhal et al. 2006). Conversely when resistin expression was silenced using RNAi a decrease in phosphorylated AMPK was detected, which would be expected to lower glucose production in vivo. A comparable model is the resistin-knockout mice, which are reported to have significantly lower fasting plasma glucose levels relative to wildtype littermates (Banerjee et al. 2004). This raises the questions of a) whether resistin is actually regulating glucose metabolism in vivo via a central AMPK-dependent pathway, and b) could this regulation be dependent on an endogenous hypothalamic resistin autocrine/paracrine system?

The significance of *fiaf* expression in N-1 neurons is unknown. FIAF is a member of a group of angiopoietin-like proteins (ANGPTL) implicated in lipid metabolism and the metabolic syndrome (Kersten 2005). As its name implies, FIAF (fasting-induced adipose factor; also known as ANGPTL4 and PGAR) is rapidly induced by fasting (Kersten et al. 2000). Total body fat in heterozygotic (+/-) and *fiaf* knockout (-/-) mice was reported to be 37% and 55% higher than controls (Backhed et al. 2004), and overexpression of *fiaf* improved glucose tolerance (Xu et al. 2005), suggesting its involvement in energy metabolism in vivo. Our laboratory reported that the brain and pituitary also express high

levels of *fiaf* mRNA (Wiesner et al. 2004), and *fiaf* is dramatically upregulated in anoxic/ischemic brain (See Chapter 6;(Wiesner et al. 2006)). In the experiments described here, resistin clearly inhibited *fiaf* expression in N-1 neurons and in 3T3-L1 adipocytes. However *rstn* knockdown also resulted in an unexpected 20% reduction in *fiaf* in 3T3-L1 cells. But *fiaf* is induced during adipogenesis (Yoon et al. 2000), which was also inhibited by 20% following *rstn* knockdown, therefore resistin knockdown appears to inhibit *fiaf* expression indirectly by blocking adipogenesis. However further studies are still necessary to probe the importance of *fiaf* in hypothalamic function.

It has previously been hypothesized that adipokines might be part of a complex autocrine/paracrine adipose circuit (Mohamed-Ali et al. 1998; Kim and Moustaid-Moussa 2000). For example adiponectin exerts autocrine regulation of adipocyte proliferation and differentiation as well as augments the insulin responsiveness of the glucose transport system in these cells (Fu et al. 2005). As reported here, silencing resistin inhibited 3T3-L1 adipocyte differentiation via an autocrine mechanism, as assessed by Oil Red O, which is consistent with a previous report (Gong et al. 2004). The results of the experiments in N-1 neuronal cells revealed that such a circuit may also exist for neuronal-derived adipokines. As noted, receptors for resistin and FIAF have yet to be identified, however treating N-1 cells with resistin, or modifying endogenous *rstn* using RNAi or overexpression vectors, clearly show that resistin modifies the expression of *socs-3* and *fiaf* as well as impacts AMPK activity in these cells. It also remains uncertain whether resistin can cross the blood brain barrier since it appears to circulate as a large hexamer (Patel et al. 2004), and like adiponectin, this complex seems unlikely to penetrate the blood brain barrier (Spranger et al. 2006). Thus the putative regulatory function of brain-derived resistin, and other adipokines, might occur independently of the feedback effects from peripherally-derived adipokines since these resistin-producing neurons are likely inaccessible to the peripherally-derived peptides. On the other hand,

even if resistin is transported into the brain, I speculate that endogenous brain resistin might 'tune' the afferent peripheral resistin signals, which might be a general rule for brain-derived adipokines (Wilkinson et al. 2007). The apparent 'crosstalk' that occurred between resistin and FIAF in N-1 cells mirrors the changes detected between leptin and resistin in the intact rat brain (Chapter 2, section VIIIb). This interrelationship between adipokines might induce minor adjustments, as an endogenous metabolic tuning mechanism, in order to modify the perception or intensity of peripheral adiposity signals. Therefore a central adipokine system might help the hypothalamus to generate appropriate responses to fluctuations in peripheral energy stores, but this now needs to be confirmed by in vivo.

In summary resistin exerts autocrine/paracrine control over cellular energy metabolism (i.e AMPK) and signaling pathways (i.e. *socs-3*) that have been implicated in the development of obesity and diabetes. Thus modifications in central adipokine expression could have implications for the regulation of local energy metabolism and signaling in vivo. For example the hypothalamic-specific reduction in resistin would be expected to increase *socs-3* expression, leading to increases in appetite or bodyweight. Similarly silencing central *rstn* expression might also reduce hypothalamic AMPK activity, leading to decreased hepatic gluconeogenesis. These effects are consistent with the reported changes measured in resistin and leptin-deficient mice (Qi et al. 2006), and as expected, the exact opposite of what has been reported following the icv injection of resistin (Muse et al. 2005; Tovar et al. 2005; Singhal et al. 2006). Thus, like 3T3-L1 adipocytes, the N-1 cell line is an important and useful model for studying the regulation of brain-derived adipokines and the pathways implicated in central energy homeostasis. Furthermore these studies suggest that the in vivo

knockdown of hypothalamic resistin might impact central energy homeostasis, and should now be tested in vivo.

Chapter 4: Role Of CEBPα and PPARγ In The Regulation Of Brain-Derived Adipokines

I. Introduction

We have provided substantial evidence that many adipokine genes are expressed, not only in adipose tissue, but in the brain and pituitary as well (Wilkinson et al. 2007). In addition they appear to be developmentally and nutritionally regulated in these non-adipose tissues (Morash et al. 2002; Morash et al. 2004; Wiesner et al. 2004). However the low level of adipokine gene expression in these tissues raised questions as to whether they served any physiological purpose. Our RNA interference (RNAi) studies in C6 glioblastoma cells and in N-1 hypothalamic cells clearly suggest that centrally expressed adipokines modify cell survival, impact the expression of genes implicated in cellular signaling (e.g. *socs-3*) or control the activity of enzymes involved in central energy homeostasis (i.e. AMPK) (See Chapters 2 and 3). As such, these novel central targets might be having a profound effect on brain function, or even perhaps whole body energy homeostasis. Although numerous transcription factors have been implicated in the regulation of adipokines, the two predominant families are the CCAAT enhancer binding proteins (CEBPs) and peroxisome proliferator-activated receptors (PPARs). However their effects on adipokine gene expression appear cell type or tissue-specific, and they are often dependent on the presence of other co-activators that have also been implicated in the initiation of gene transcription. It seems likely that these factors may also modulate the expression of brain-derived adipokines, although their effects will not necessarily be the same as those detected in adipocytes. **Understanding the molecular mechanisms that regulate adipokine expression in non-adipose tissues could provide further insight into their purpose and function.**

The Role Of CEBPα And PPARγ In Adipogenesis

The differentiation of fibroblast cells into adipocytes involves the coordinated

activation of multiple biochemical pathways linked to the induction of various adipogenic

transcription factors, including CEBPs and PPARs, over a period of several days (See

Figure 48). Although other cell lines can be induced to differentiate into adipocytes

including F442A, 3T3-L2 or ob1771, a sub strain of the Swiss mouse 3T3 cell line, 3T3-

L1, has been studied in greatest detail (Green and Meuth 1974; Cowherd et al. 1999).

Four general stages of adipogenesis were identified in this model cell line including 1)

the preconfluent and proliferative stage, 2) growth arrest 3) hormonal-mediated clonal

expansion followed up by 4) growth arrest and terminal differentiation (Cowherd et al.

1999). However the timing of these events is critical and certain key adipogenic genes

are only activated during specific stages of differentiation (see Figure 48). Various

compounds were shown to inhibit adipogenesis including 5-bromo-2-deoxyuridine or

retinoic acid (RA) (Green and Meuth 1974; Schwarz et al. 1997). Although RA mediates

its effects by interfering with the expression of PPARγ, it is only capable of inhibiting

adipogenesis when applied to preconfluent fibroblasts (Schwarz et al. 1997; Shao and

Lazar 1997). Despite several protocols having been used to induce adipocyte

differentiation, one of the most effective methods includes coordinating the timing and

treatment of cells with a differentiation cocktail that contains dexamethasone (DEX),

isobutylmethylxanthine (IBMX), and insulin. Unlike the very early protocols that only

attained a 10-50% adipocyte conversion (Green and Meuth 1974), more than 90% of

cells treated with this cocktail have been reported to differentiate into adipocytes

(Shojima et al. 2002; Song et al. 2002). Cellular lipid accumulation is stimulated by

insulin and by raising intracellular cAMP concentrations through the use of

phosphodiesterase (PDE) inhibitors such as IBMX (Green and Kehinde 1974; Green and

Kehinde 1975; Cowherd et al. 1999; Otto and Lane 2005). Similarly DEX increases the
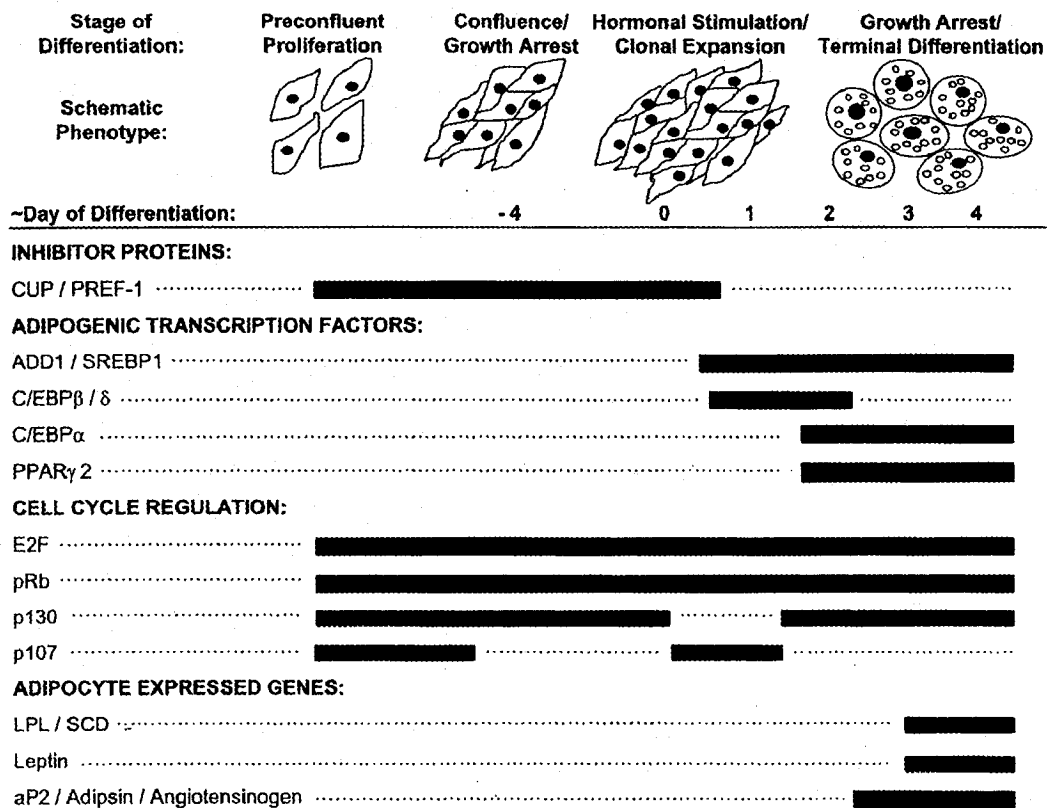
Figure 48: The stages of adipogenesis in 3T3-L1 adipocytes

The differentiation of 3T3-L1 fibroblasts into adipocytes has been divided into 4 general stages that are associated with unique cell type morphologies. In addition certain 'adipogenic' transcription factors are only expressed during specific stages of differentiation (e.g. CEBPβ/δ), or are induced in the final stages and involved in maintaining an adipocyte phenotype (e.g. PPARγ). Various compounds can facilitate the differentiation process by either stimulating the expression of certain transcription factors (i.e. DEX and CEBPβ) or facilitating lipid accumulation (i.e cAMP, IBMX or insulin). (Figure adapted from Cowherd et al.1999)

expression of CEBPβ, which stimulates the expression of adipogenic transcription factors including CEBPα and PPARγ that induce an array of adipocyte-related genes. CEBPα and PPARγ also stimulate the expression of several adipocyte-related genes (MacDougald and Lane 1995) including leptin (He et al. 1995), resistin (Hartman et al. 2002), adiponectin (Gustafson et al. 2003) and FIAF (Yoshida et al. 2004). Many of the current anti-diabetic drugs, such as thiazolidinediones (TZDs), target these transcription factors leading to changes in adipokine gene expression, which is thought to mediate at least part of their effects on insulin sensitization.

Both the CEBP and PPAR families of transcription factors bind to DNA as either homo- or heterodimers. CEBP is a member of the basic-region leucine zipper (bZIP) class of DNA-binding proteins. Although multiple isoforms of CEBP have been identified, CEBPα, CEBPβ, and CEBPδ appear to be the most important in adipogenesis (Cowherd et al. 1999; Otto and Lane 2005). These transcription factors contain a transactivation domain, DNA binding basic region, and a leucine rich dimerization domain that permits the formation of homo- or heterodimers with other CEBP proteins prior to binding DNA (see Figure 49, adapted from (Goldsby et al. 2000)) (Otto and Lane 2005). Similarly various members of the PPAR family were detected in adipose tissue including PPARα, although the PPARγ isoform is regarded as the master regulator of adipogenesis (Otto and Lane 2005). PPARs also contain several domains that are involved in either DNA binding, transactivation, ligand binding or dimerization. However unlike CEBPs, in order for PPARs to bind DNA they must heterodimerize with retinoid X receptors (RXR) (Aranda and Pascual 2001). However both families of transcription factors form dimers in order to bind PPAR- or CEBP-specific response elements that are normally located in the proximal promoter regions of numerous genes leading to modifications in the transcription and expression of several genes, including adipokines.
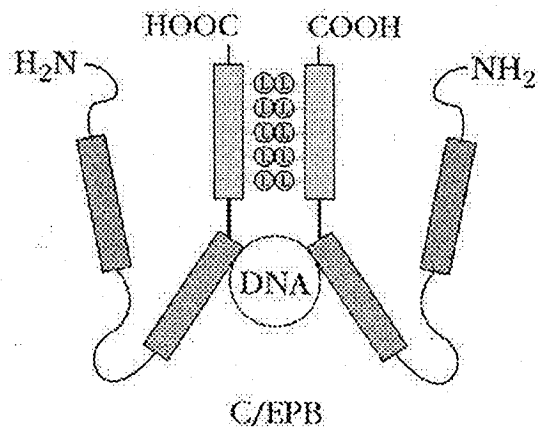
Figure 49: CEBPα mediated transcription
The CEBP family of transcription factors forms either homo- or heterodimers that are dependent on their leucine rich domains. Subsequently dimers are able to associate and bind specific DNA promoter regions in order to modify gene transcription. (Figure adapted from Goldsby et al. 2000)

Adipokine Genes, CEBPs And PPARs

The induction of adipokine gene expression, including *rstn* (Haugen et al. 2001; Kim et al. 2001) and *fiaf* (Yoon et al. 2000), usually occurs during adipogenesis. As already noted, these adipokines have been implicated in the regulation of glucose and lipid homeostasis (Kersten 2005; McTernan et al. 2006), and like most adipokines, their physiological actions appear to be at least partially dependent on central hypothalamic pathways (Ahima et al. 2006). The expression and regulation of both of these adipokines is at least partially-dependent on CEBPα and PPARγ. For example resistin promoter analysis in mice and humans revealed a proximal CEBP transcriptional start site (Hartman et al. 2002; Seo et al. 2003), and the overexpression CEBPα was capable of inducing resistin expression in 3T3-L1 adipocytes, L6 myocytes and human kidney 293T cells. Intriguingly the overexpression of CEBPα failed to induce *rstn* in NIH3T3 fibroblasts nor in mouse myeloid progenitor 32D cells (Song et al. 2002; Zhou et al. 2006). This lack of response might be explained by the presence of endogenous CEBP inhibitory mechanisms that are not present in all cell types (Song et al. 2002). Although RXR and PPARγ ligands were also capable of inhibiting *rstn* expression, no obvious PPARγ response elements (PPRE) were detected in the proximal resistin promoter (Hartman et al. 2002), although a potential PPRE was identified 10Kbp upstream of the resistin gene in human macrophages (Patel et al. 2003). A more plausible mechanism was recently put forth that suggests PPARγ interacts with specificity protein 1 (Sp1) in order to bind a proximal region of the mouse resistin promoter in 3T3-L1 cells which is responsible for mediating transcriptional repression (Chung et al. 2006). Mutational analysis of this site suggested that it was required for the TZD-mediated repression of *rstn* expression (Chung et al. 2006). The mouse *fiaf* promoter was also found to contain several PPREs, in addition to an activator protein 1 (AP-1), AP-2, CEBP and Sp1 transcriptional start sites (Yoshida et al. 2004). Although treating rat hepatoma cells with

a PPARγ agonist increased *fiaf* expression, both PPARα and PPARβ agonists were much more effective (Ge et al. 2005). However I am unaware of any specific investigations looking at the role of CEBPα in the regulation of *fiaf*. Thus CEBP and PPAR appear to be key factors involved in the regulation of adipokine gene expression, although their effects are cell-type specific.

CEBPα And PPARγ Are Also Present In The CNS

Although CEBP is predominantly expressed in hepatocytes and fat cells, its expression has also been detected in numerous other mouse tissues (Williams et al. 1991). For example CEBPα mRNA was detected in the mouse and human brain (Williams et al. 1991; Blalock et al. 2004), and its expression was increased by lipopolysaccharide (LPS) in whole mouse brain extracts (Saito et al. 1999). Similarly hypoxia/ischemia (H/I) induced CEBPα expression in numerous brain regions as soon as 6h after the hypoxic insult, which persisted for at least 1 week (Walton et al. 1998). Although this injury induced the greatest increases in CEBPα expression in the hippocampus, its expression was elevated throughout the ipsilateral mouse brain as detected by in situ hybridization, and confirmed by immunohistochemistry (Walton et al. 1998). Similarly CEBPα mRNA was detected in the human hippocampus using microarray analysis, and it appeared to be modestly higher (~25%) in brain tissue collected from patients diagnosed with Alzheimer's disease (Blalock et al. 2004). Several adipogenic transcription factors were also detected by Western blot analysis in mouse cortical astrocytes that were cultured in vitro, including CEBPα, CEBPβ, and CEBPδ (Cristiano et al. 2005). In preliminary studies we used realtime RT-PCR to confirm that the N-1 hypothalamic cell line also expresses CEBPα (Brown et al. unpublished). Since the brain expresses CEBPα this suggested that it might be mediating the expression of brain-derived adipokines, but it could also be involved in central inflammation and brain repair since its expression is increased following brain damage.

Like CEBP, the expression of peroxisome proliferator-activated receptors (PPARα, PPARβ/δ and PPARγ) and their heterodimeric binding partners, retinoid X receptors (RXRα, RXRβ and RXRγ) has also been detected in the CNS. Moreover PPARs expressed in the brain are implicated in cellular differentiation and maturation, central inflammation and neurodegeneration. For example PPARs were readily detected in mouse cortical neurons and rat cortical astrocytes and their regulation appeared to be dependent on the stage of brain cell differentiation (Cimini et al. 2005; Cristiano et al. 2005). However its expression is cell-type dependent since unlike astrocytes, where PPARγ was increased during differentiation, its expression was suppressed in cortical neurons (Cimini et al. 2005; Cristiano et al. 2005). Similarly PPARγ expression appears to be highest in the developing embryonic mouse brain, and is substantially lower in the mature adult brain, as detected by Western blot analysis (Wada et al. 2006), further suggesting its involvement in the development and maturation of the CNS. Northern blot analysis was used to detect PPARγ mRNA in the adult human brain (Elbrecht et al. 1996), and an immunohistochemical investigation revealed that PPARγ was enriched in discrete regions of the adult rat CNS (Moreno et al. 2004). PPARγ expression was also detectable in the hypothalamus of adult male mice by RT-PCR (Wiesner et al. 2004), and the adult rat hypothalamus was weakly immunopositive for PPARγ and its heterodimeric binding partner RXRγ (Moreno et al. 2004). Additionally PPARγ expression was rapidly induced in the adult rat brain by middle cerebral artery occlusion (MCAO), and it remained significantly elevated for up to 14 days following injury, as detected by RT-PCR (Victor et al. 2006). Thus PPARγ also appears to be involved in neuronal development, maturation, and brain damage, but it might also be regulating the expression of centrally-derived adipokines.

Valproic Acid And CEBPα

Valproic acid (VPA), an anticonvulsant, is used clinically as a mood stabilizer and antiepileptic drug. However an unwanted side effect of VPA treatment includes weight gain which has been partially attributed to an increased resistance to anorectic hormones, such as leptin and insulin, although the exact mechanisms influencing body mass remain largely unresolved (Pylvanen et al. 2002; Greco et al. 2005; El-Khatib et al. 2007). The documented increases in body mass are particularly concerning for VPA therapy since it not only reduces patient compliance, but also elevates the risk of developing diabetes and cardiovascular disease (Pylvanen et al. 2002; El-Khatib et al. 2007). Recently it was hypothesized that VPA therapy may induce a state of leptin resistance, much like obesity, leading to the increases in appetite and body mass (El-Khatib et al. 2007), although the responsible mechanisms remain to be uncovered.

At the molecular level VPA treatment has been shown to inhibit histone deacetylase (HDAC) activity (Gottlicher et al. 2001; Lagace and Nachtigal 2004). However VPA also blocks the expression and transcriptional activity of CEBPα in differentiated 3T3-L1 cells, leading to time- and dose-dependent decreases in leptin and adiponectin gene expression (Lagace et al. 2004; Qiao et al. 2006). Treating 3T3-L1 cells with a more potent and specific HDAC inhibitor, trichostatin A (TSA), failed to reduce leptin mRNA (Lagace et al. 2004). In contrast TSA treatment also decreased adiponectin mRNA suggesting that VPA might inhibit its expression by multiple mechanism(s) (Qiao et al. 2006). While both studies attributed the changes in adipokine expression to CEBPα, since VPA was thought to interfere with its transcriptional activity, only one detected actual decreases in CEBPα expression (Qiao et al. 2006). Similarly treating undifferentiated 3T3-L1 cells with VPA *induced* CEBPα-mediated transcription, suggesting that the inhibitory effects of VPA are dependent on other adipocyte-related genes (Qiao et al. 2006). Thus the effects of VPA on CEBPα transcriptional activity are

likely tissue-specific. Repeatedly injecting mice with VPA also significantly reduced the

concentration of circulating adiponectin, and both adiponectin and CEBPα mRNA were

significantly lower in adipose tissue of animals treated with VPA, relative to the controls

(Qiao et al. 2006). Having established that the N-1 cell line also expresses CEBPα, I

hypothesized that VPA would also regulate the expression of *rstn, fiaf* and *socs-3*.

All-Trans Retinoic Acid Inhibits CEBPα And PPARγ

Retinoic acid (RA) is implicated in the regulation of CEBPα- and PPARγ-

mediated transcriptional activity via a retinoic acid receptor (RAR)-dependent

mechanism. Once RA binds RAR it competes with PPARγ for its heterodimeric partner,

RXR. In other words RAR sequesters RXR, thus indirectly inhibiting PPARγ activity.

Similarly RAR inhibits the expression and transcriptional activity of CEBPα in 3T3-L1

adipocytes (Schwarz et al. 1997; Shao and Lazar 1997). Since RA exerts an inhibitory

effects on these adipogenic transcription factors it was hypothesized that it would also

modify the expression of *rstn* (Felipe et al. 2004). As expected 9-*cis* retinoic acid

suppressed *rstn* mRNA levels, however all-*trans* retinoic acid (ATRA) was much more

effective, and these reductions in *rstn* were attributed to the inhibitory effects of RA on

CEBPα (Felipe et al. 2004). Similarly ATRA was shown to inhibit leptin expression and

secretion in 3T3-L1 adipocytes, and in cultures of human adipose tissue, in a time- and

dose-dependent manner (Hollung et al. 2004). However ATRA had no effect on leptin

mRNA in human primary osteoblasts or in BeWo cells, a placenta trophoblast cell line,

although it did inhibit leptin secretion from the latter (Hollung et al. 2004). Thus it was

concluded that ATRA modifies adipokine gene expression in a time-, dose-, tissue- and

species-specific manner (Hollung et al. 2004). ATRA also dose-dependently reduced the

total amount of body fat, and significantly decreased the bodyweights, of NMRI male

mice without modifying daily caloric intake (Felipe et al. 2004). Like in vitro, ATRA was

also capable of inhibiting leptin gene expression and secretion in vivo (Felipe et al.

2005). However these decreases in leptin would be expected to induce weight gain and suggested that ATRA induced weight loss by other anorexigenic mechanisms. For example ATRA inhibited the expression of neuropeptide Y (NPY), an orexigenic neuropeptide, in the human SH-SY5Y neuroblastoma cell line (Magni et al. 2000). Additionally it was unclear whether RAR directly modulated NPY, or if ATRA indirectly modified the expression of other genes that subsequently inhibited NPY expression (Magni et al. 2000). ATRA has been shown to inhibit JAK-STAT signaling in rat primary astrocytes, and in rat C6 glioblastoma cells, by rapidly (1h) inducing the expression of socs-3 (Choi et al. 2005). Although these effects were thought to be part of an anti-inflammatory response, this signaling cascade also mediates the anorectic effects of leptin in the hypothalamus (recall Figure 6) (Bjorbaek et al. 1997). Therefore ATRA may induce weight loss by impacting central cytokine signaling pathways, or by modifying the expression of other hypothalamic neuropeptides, such as brain-derived adipokines.

Rosiglitazone And The Brain

Typically PPARγ ligands are used clinically as anti-diabetic drugs since they induce insulin sensitization through multiple mechanisms (Vasudevan and Balasubramanyam 2004). Various classes of PPARγ ligands have been identified, however the TZD family of drugs including rosiglitazone, ciglitazone, pioglitazone and troglitazone, appear highly specific for PPARγ. Moreover many adipokines appear to be regulated by PPARγ including rstn and fiaf (Yoon et al. 2000; Steppan et al. 2001). In the rodent CNS TZDs possess anti-inflammatory and neuroprotective properties (Sundararajan et al. 2006). For example rosiglitazone was thought to offer neuroprotection by inducing the expression of socs-2 and socs-3 and pioglitazone increased neuronal glucose metabolism (Dello Russo et al. 2003; Park et al. 2003). However these effects were suggested to be PPARγ-independent since they occurred rapidly (20min-2h) before changes in gene expression could occur (Dello Russo et al.

2003), and were not further enhanced by the exogenous overexpression of PPARγ (Park et al. 2003). However it should be pointed out the overexpression of PPARγ alone would not have increased its transcriptional activity unless there was not an adequate intracellular supply of its heterodimeric binding partner, RXR, already present. Similarly when rosiglitazone was injected 1h prior to the onset of ischemia in adult C57BL/6 mice it reduced brain infarct volume when assessed 48h later (Luo et al. 2006). Using electrophoretic mobility shift assays (EMSA), these authors went on to show that PPARγ ligands increased the ability of brain-derived PPARγ to bind DNA. It seems likely that in addition to modifying local glucose metabolism and cytokine signaling in the CNS, TZDs might also alter the expression of brain-derived adipokines.

Adipogenic Transcription Factors And Adipokine Regulation In The CNS

CEBPs and PPARs are key transcription factors involved in adipogenesis and in the regulation of adipokine gene expression, including *rstn* and *fiaf*. However these transcription factors are also expressed in the CNS where they have been implicated in brain cell differentiation, mediate local inflammatory responses and appear to offer neuroprotection. Several compounds modulate the activity of these transcription factors including VPA, ATRA and rosiglitazone, and they have been shown to modify adipokine gene expression in adipocytes both in vitro and in vivo. However these drugs also modify appetite and metabolism by elusive mechanisms that might be independent of their effects on peripheral adipokine expression. **Since VPA and ATRA influence the transcriptional activity of CEBPα I hypothesized that they would also regulate *rstn, fiaf* and *socs-3* expression in N-1 neurons and in the hypothalamus. Cells were also treated with trichostatin A (TSA), a more potent and specific HDAC inhibitor, or rosiglitazone, a highly specific PPARγ ligand, to verify whether these alternate pathways could be responsible for the modifying central adipokine gene expression. To further confirm the changes in gene expression were CEBPα-**

dependent, the expression of CEBPα was specifically silenced in N-1 cells using RNAi. Conversely N-1 neurons were transfected with a CEBPα overexpressing plasmid to see if these effects on gene expression could be reversed. Parallel experiments were also conducted in differentiated 3T3-L1 adipocytes to verify whether the measured gene expression changes were cell line-dependent. In vivo investigations were also performed to verify whether these compounds were capable of modifying adipokine gene expression in vivo.

II. Materials And Methods

N-1 Cell Culture

Immortalized N-1 hypothalamic cells were maintained in DMEM containing 10% FBS at 37°C in 5 % CO_2 / 95% air (Belsham et al. 2004). Cells were plated at 100,000 cells/well in Nunc 6 well plates 24h prior to treatment. For pharmacological studies cells were treated with either 1 mM 2-propylpentanoic acid (Valproic acid; Sigma, Oakville ON), 5nM trichostatin A (TSA, Sigma), 1μM of all-*trans* retinoic acid (ATRA; Sigma) or 1μM of rosiglitazone (ROSI; a generous gift from Dr. George Robertson, Department of Psychiatry, Dalhousie University). Depending on the drug, total RNA was isolated either 24h or 48h following treatment using the RNEasy mini kit following the DNase protocol (Qiagen; Mississauga Ont.) as described in Chapter 2 section III.

3T3-L1 Differentiation And Cell Culture

3T3-L1 fibroblasts (ATCC; Manassas, VA) were plated in 6-well Nunc plates and maintained in DMEM containing 10% FBS at 37°C in 5 % CO_2 / 95% air. Two days after cells were confluent medium was replaced with DMEM containing 10% FBS, 0.5mM 3-isobutyl-1-methylxanthine (IBMX), 1μM Dexamethasone, and 10μg/ml Insulin. Cells were maintained in this differentiation medium for 72h and then returned to DMEM containing 10% FBS and 5μg/ml Bovine insulin. Soon after lipid droplets began to appear in cells and these differentiated adipocytes were cultured for a further 8-10 day

prior to treatment. As noted in Chapter 3, this protocol was used to obtain cultures where more than 90% of cells expressed the adipocyte phenotype (Shojima et al. 2002; Song et al. 2002). As above cells were treated with either 1mM VPA, 5nM TSA, 1μM ATRA or 1μM ROSI. Depending on the compound, total RNA was isolated either 24h or 48h following treatment using the RNEasy mini kit following the DNase protocol (Qiagen; Mississauga Ont.).

RNAi-Mediated Knockdown Of CEBPα In N-1 Cells

Endogenous CEBPα expression was specifically silenced in N-1 cells using an RNAi approach similar to that already described in Chapter 2 and 3. In brief, a CEBPα target was designed using the STEALTH RNAi design tool (www.invitrogen.com) and obtained from Invitrogen (STH CEBPα), as well as a non-specific control molecule (STH CTL) (For sequences refer to Table 3). Cells were transfected with 100nM of a STEALTH small interfering RNA (STH; Invitrogen) molecule targeting CEBPα, or a non-specific control sequence, using lipofectamine 2000 (Invitrogen) using previously optimized conditions as outlined in Chapter 3. Total RNA was isolated 24h or 48h later using the RNEasy mini kit following the DNAse protocol (Qiagen). In addition interferon β (IFNβ) gene expression was also analysed using realtime RT-PCR to assess the possibility that these RNAi targets activated non-specific RNAi pathways (Jackson and Linsley 2004).

CEBPα Overexpression In N-1 Neurons

A CEBPα expression vector was obtained from Dr. R. N. Day (Departments of Medicine and Cell Biology, University of Virginia Health Sciences Centre, Charlottesville, Virginia) (Schaufele et al. 2003). Plasmids were transformed and propagated in E. Coli (DH5α; Invitrogen, Burlington ON), and purified using the Purelink™ HiPure Plasmid Midiprep kit (Invitrogen) as outlined in Chapter 2. In overexpression studies N-1 cells were transfected with 3.2 μg plasmid/well of either an empty vector, or one containing

the CEBPα cDNA sequence, using previously optimized conditions for lipofectamine 2000 (Invitrogen; please refer to Chapter 3). Total RNA was isolated 24h following treatment using the RNEasy mini kit following the DNase protocol (Qiagen).

Mice

Male mice (CD-1; postnatal day 30-32 and weighing 28-32g) were obtained from Charles River Breeding farm (St. Constant, QC) and were acclimatized for 1 week prior to treatments. Animals were maintained in small groups and housed in plastic cages with free access to Purina Rat Chow and drinking water, and exposed to a 12h light:12h dark photoperiod. For VPA experiments the drug was dissolved in saline and mice received either a single subcutaneous injection of VPA (Sigma; 100mg/Kg; 0.1mL), or vehicle. In ATRA experiments mice were injected with various doses of drug (ATRA, SIGMA, St. Louis; 20, 50 or 100mg/Kg; 0.1mL s.c.) or vehicle. Rosiglitazone (ROSI) was obtained as tablets of Avandia™ (GlaxoSmithKline) and they were carefully crushed into a fine powder that was suspended in a minimal volume of DMSO to form a slurry. Mice received a single injection of vehicle or ROSI (0.1mL; 100mg/Kg, s.c.). In all experiments mice were weighed just prior to injection, and again before sacrifice. Animals were killed by decapitation 24h post-injection and tissues (mediobasal hypothalamus (HYP) and visceral adipose tissue (FAT)) were collected as described in Chapter 2, and snap frozen on dry ice and stored at -70°C until RNA isolation. The experimental protocol was reviewed and approved by the Dalhousie University Committee on Laboratory Animals. RNA was isolated from FAT using TRIzol® (Invitrogen; Burlington ON), and from HYP using the RNEasy mini kit following the DNase protocol (Qiagen; Mississauga ON) as described in section VII of Chapter 2.

Realtime RT-PCR

Total RNA (1.5μg) was reverse transcribed (RT) and PCR amplified using the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Invitrogen) according to the

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manufacturer's protocol using previously published primers and Taqman™ probes for *rstn*, cyclophilin (Brown et al. 2005b), *socs-3* (Steppan et al. 2005) and *fiaf* (Wiesner et al. 2006) as already described in Chapter 3. In brief, RNA was diluted to 12μL and then heat denatured for 5min at 70°C. Samples were returned to ice prior to the addition of 15μL of the 2x reverse transcription master mix and 3μL of the SuperScript™ III RT enzyme master mix (Invitrogen). The RT reaction consisted of 10min incubation at 25°C, 45min incubation at 42°C, followed by a 5min 85°C termination step, and the resulting complementary DNA (cDNA; 30μL) was stored at -20°C. For PCR amplifications samples were amplified in duplicate and only 1 gene was analysed per reaction. Individual PCR reactions consisted of a 2x Platinum® quantitative PCR SuperMix-UDG, 7pmol of the sense and antisense primers, 1pmol of the appropriate dual-labeled probe, 3μL of cDNA, to a final volume of 33μL in sterile water. Reactions were heated to 10min at 95°C, followed by 50 amplification cycles of 95 °C for 20s and 60°C for 60s using a BioRad thermal cycler and an iQ realtime PCR detection system. A standard curve, that was prepared using a serial dilution of a reference sample, was included in each realtime run to correct for possible variations in product amplification. Relative copy numbers were obtained from standard curve values, and these were normalized to the values obtained for our house keeping gene, cyclophilin. Data are expressed as a percentage of the control ± SEM. It should be noted that no significant variations in the housekeeping gene were observed between groups when evaluated using either the threshold cycle (C_T), or using the relative levels of expression, and this is consistent with previous reports (Bond et al. 2002). IFNβ and CEBPα gene expression were analysed in triplicate using the Platinum SYBR Green qPCR kit (Invitrogen) using previously published primers and conditions (Jochheim et al. 2004; Stewart et al. 2005).

Statistics

Data for in vivo ATRA studies were analyzed using an analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparisons test using graphpad PRISM v4.0a. All remaining data were analyzed with the Student's *t-test*. Data from control and treated cells were analysed independently for each time point (24h or 48h). Significance was set at $p < 0.05$.

III. Results

VPA Modulates Gene Expression In N-1 And 3T3-L1 Cells

Treating N-1 cells with VPA reduced *rstn* mRNA slightly at 24h (-26%; $p < 0.01$), but was further decreased by 48h (-47%; $p < 0.01$) (Fig 50A). In contrast *fiaf* mRNA was significantly reduced at 24h (-50%; $p < 0.001$), but had returned to near control levels by 48h (-15%; p = N/S) (Fig 50B). Similarly *socs-3* gene expression was significantly increased at 24h (+25%; $p < 0.001$), but appeared unchanged at 48h (Fig 50C). As seen in Figure 50 (D-F), treating 3T3-L1 adipocytes with VPA failed to significantly modify *rstn*, *fiaf*, or *socs-3* expression at either time point.

The Effects Of TSA On Gene Expression

In contrast to VPA, treating N-1 neurons with 5nM TSA dramatically increased *rstn* mRNA at 24h (300%, $p < 0.0001$), but it had returned to control levels by 48 h (Fig 51A). Conversely, *fiaf* gene expression was decreased at 24h (-45%; $p < 0.0001$), and was further reduced by 48h (-62%; $p < 0.0001$) (Fig 51B). Like *rstn*, *socs-3* mRNA was increased 2-fold at 24h ($p < 0.0001$), but had returned to baseline by 48h (Fig 51C). Like VPA, TSA had no effect on *rstn* mRNA in 3T3-L1 cells (Fig 51D). *Fiaf* mRNA was slightly reduced at 24h (~20%; $p < 0.05$), and was substantially decreased at 48h (-65%; $p < 0.001$) (Fig 51E). In contrast *socs-3* expression was increased by 35% ($p < 0.05$) after 24h, but was decreased by 50% ($p < 0.01$) 48h following TSA treatment (Fig 51F).
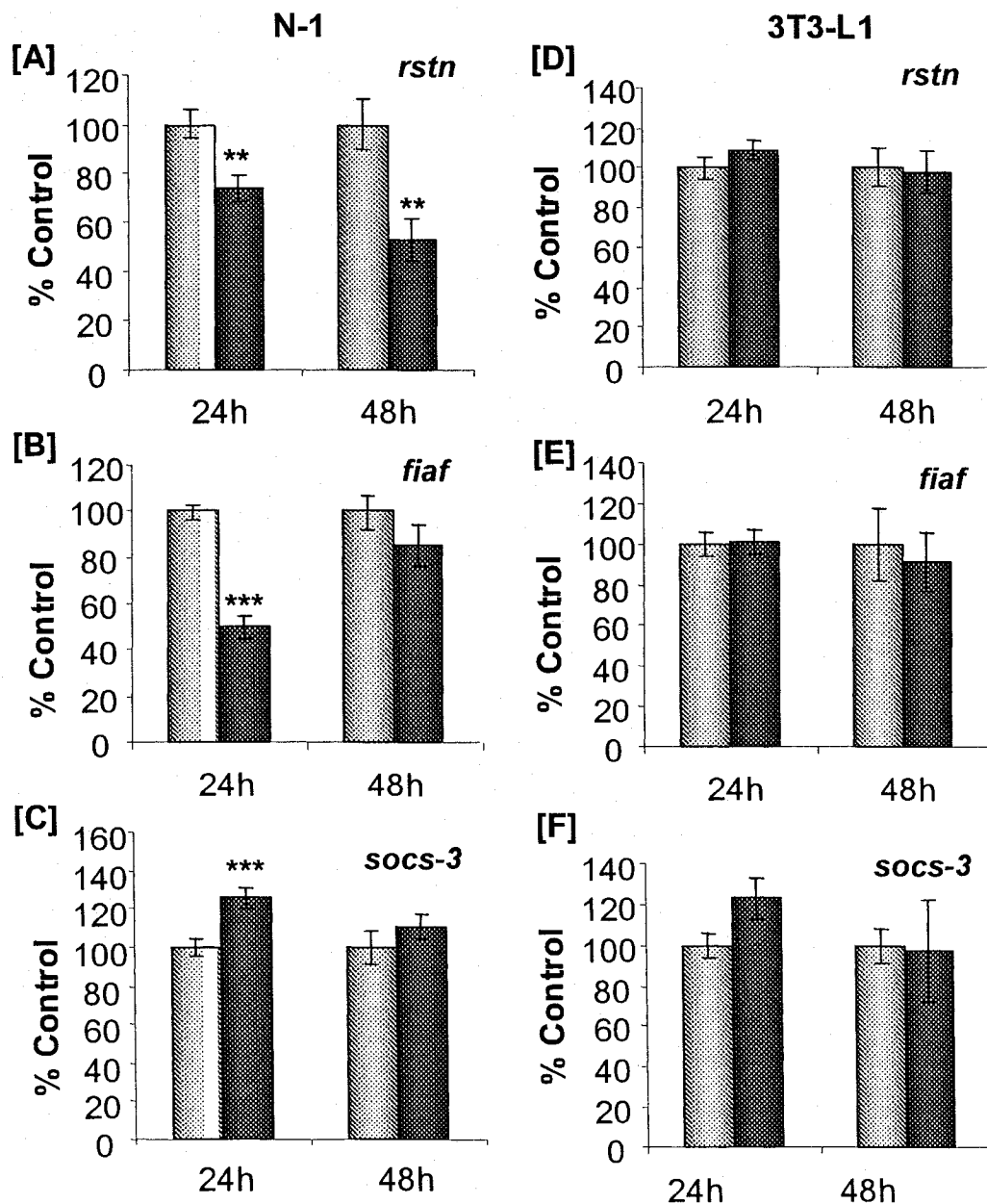
Figure 50: The effects of Valproic acid (VPA) on adipokine gene expression in N-1 and 3T3-L1 cells. N-1 neurons and 3T3-L1 adipocytes were treated for 24h or 48h with 1mM VPA and gene expression was measured using realtime RT-PCR. [A] *Rstn* mRNA was significantly inhibited in N-1 cells 24h and 48h after VPA treatment by 25% and 45% respectively. [B] In N-1 neurons *fiaf* was significantly decreased (-50%) 24h after VPA exposure, but had returned to basal levels by 48h. [C] In contrast *socs-3* expression was increased by 25% 24h after VPA, but had also returned to normal by 48h. [D-F] Unlike N-1 cells, gene expression was unaffected by VPA in 3T3-L1 adipocytes at either time point. Data were obtained from duplicate or triplicate experiments and are expressed as a percentage of the control ± SEM (n=6-10). (**p<0.01, ***p<0.001). (CTL; 1mM VPA)
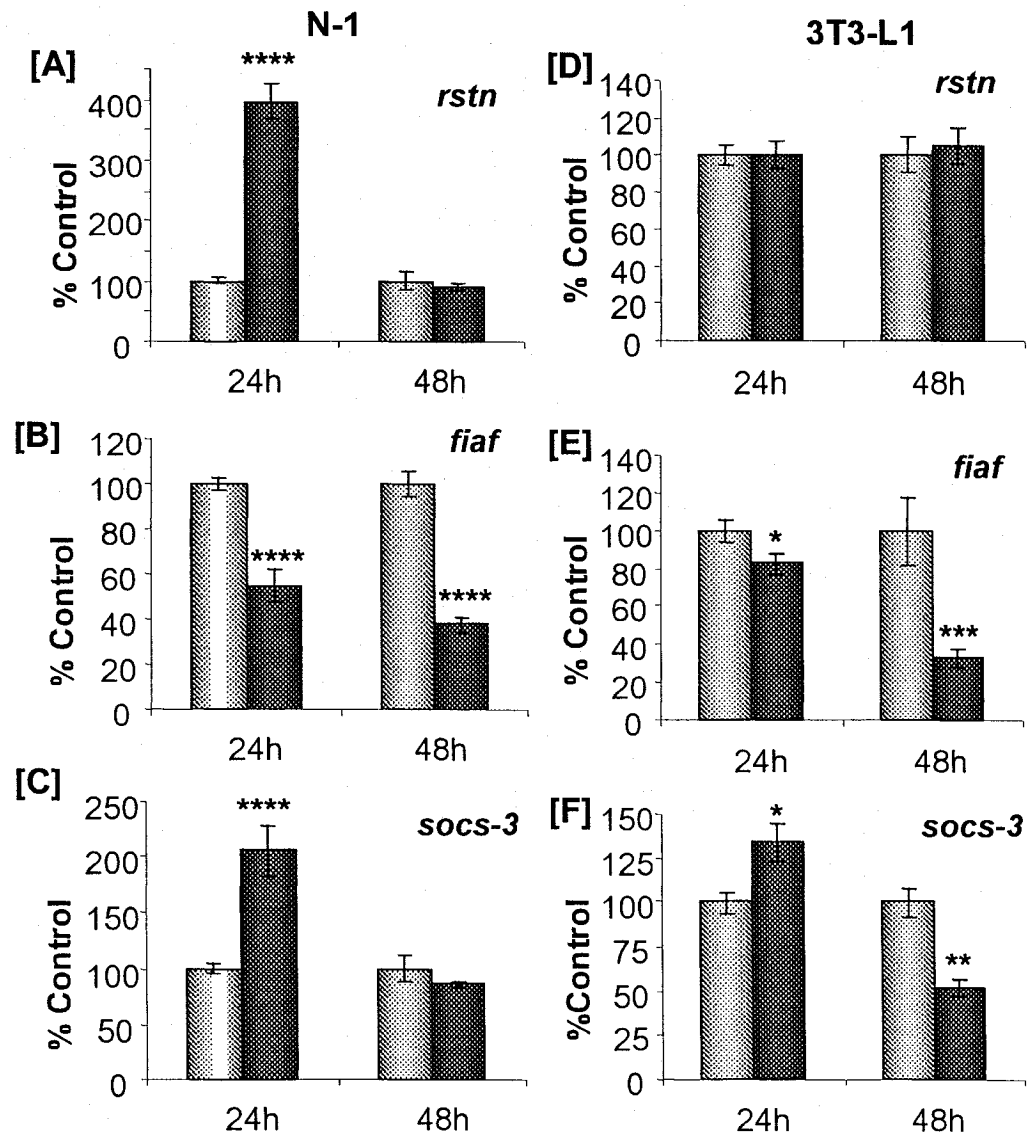
Figure 51: The effects of trichostatin A (TSA) on adipokine gene expression in N-1 and 3T3-L1 cells. Realtime RT-PCR was used to measure gene expression after treating both cell lines with 5nM TSA for either 24h or 48h. [A] In N-1 cells *rstn* gene expression was robustly induced by TSA 24h after treatment (+300%), but had returned to basal levels by 48h. [B] In contrast TSA inhibited *fiaf* expression in N-1 cells by 45% after 24h and by 60% after 48h. [C] After 24h TSA induced *socs-3* in N-1 neurons by 100%, but its expression returned to control levels by 48h. [D] Like VPA, TSA failed to modify *rstn* gene expression in 3T3-L1 adipocytes at either time point. [E] TSA also significantly inhibited *fiaf* expression in 3T3-L1 cells by 20% after 24h and by 65% 48h after treatment. [F] In 3T3-L1 cells *socs-3* was increased by 35% after 24h, but then inhibited by 45% after 48h of TSA exposure. Data were obtained from duplicate or triplicate experiments and are expressed as a percentage of the control ± SEM (n=6-10). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)(CTL; 5nM TSA)

In Vitro Effects Of ATRA On Adipokine mRNA

Rstn gene expression was significantly inhibited in both N-1 (-35%; p<0.05) and

3T3-L1 cells 24h after treatment (-50%; p<0.0001) (Fig 52A). Although ATRA induced

fiaf in N-1 neurons (+35%; p<0.05), it had no significant effect in 3T3-L1 cells (Fig 52B).

As seen in Figure 52C, ATRA increased socs-3 in both N-1 (+22%, p<0.05) and 3T3-L1

cells (+45%; p<0.001).

The PPARγ Agonist Rosiglitazone Induces Adipokine Gene Expression In Vitro

Treating either cell line with ROSI failed to have a significant effect on rstn gene

expression (Fig 53 A). In contrast fiaf gene expression was increased by ROSI in both

N-1 neurons (+80%; p<0.001) and 3T3-L1 adipocytes (+140%; p<0.0001) (Fig 53B).

Although no significant changes in socs-3 were detected, ROSI did induce modest

(~20%) increases in both cell lines (Fig 53C).

Effects Of CEBPα Knockdown In N-1 Cells

As expected, RNAi effectively silenced CEBPα expression in N-1 by more than

90% (p<0.0001) 24h after transfecting cells, and CEBPα mRNA was still 72% (p<0.001)

lower after 48h (Fig 54A). Although rstn mRNA was initially unaffected, it was reduced

by 42% 48h after CEBPα knockdown was initiated (p<0.001) (Fig 54B). In contrast fiaf

gene expression was increased 125% 24h after transfection (p<0.01), and remained

slightly elevated at 48h (+30%, p<0.01) (Fig 54C). Similarly socs-3 expression was

increased by 88% (p<0.05) 24h after cells were treated with STH CEBPα, and remained

increased at 48h (+26%, p<0.05) (Fig 54D). Interferon β (IFNβ) gene expression was

unchanged by STH CEBPα suggesting that the effects on these other genes were

unlikely part of a non-specific RNAi response (data not shown).

Effects Of CEBPα Overexpression In N-1 Neurons

Using a converse approach CEBPα expression was increased in N-1 neurons by

transfecting cells with a CEBPα overexpression vector (pCEBPα). As expected CEBPα
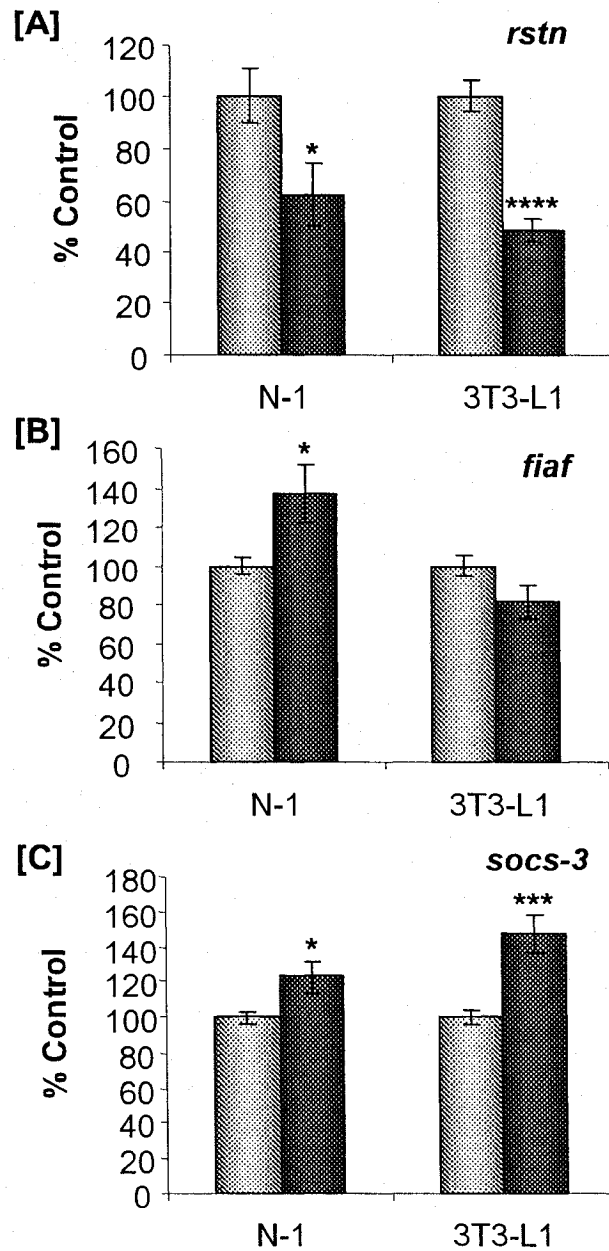
Figure 52: The effects of all-*trans* retinoic acid (ATRA) on gene expression in vitro. Gene expression was analysed by realtime RT-PCR after exposing N-1 hypothalamic neurons and differentiated 3T3-L1 cells to 1μM of ATRA for 24h. [A] ATRA significantly inhibited *rstn* in N-1 (-35%) and 3T3-L1 cells (-50%). [B] In N-1 cells *fiaf* expression was stimulated by ATRA, but was unchanged in 3T3-L1 adipocytes. [C] *Socs-3* was induced by ATRA in both N-1 (20%) and 3T3-L1 cells (45%). Data were obtained from triplicate experiments and are expressed as a percentage of the control ± SEM (n=10-11). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) (CTL; 1μM ATRA)
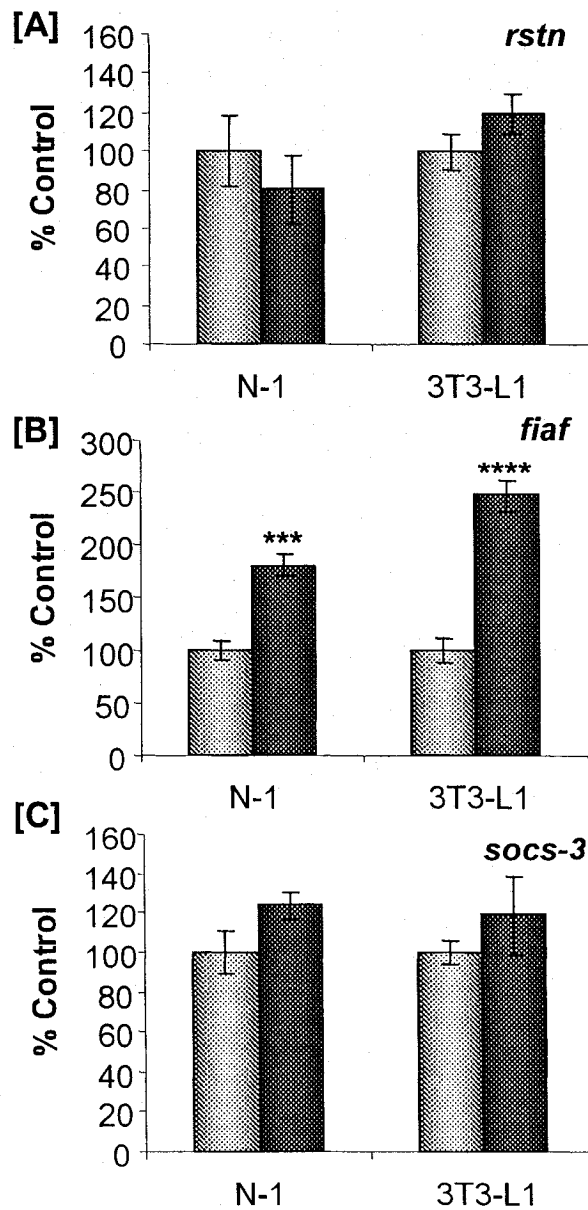
Figure 53: The effects of rosiglitazone (ROSI) on gene expression in N-1 neurons and 3T3-L1 adipocytes. Cells were treated for 24h with ROSI and gene expression was measured using realtime RT-PCR. [A] ROSI failed to modify *rstn* mRNA in either cell line. [B] In contrast *fiaf* gene expression was significantly increased by 80% in N-1 cells and by nearly 150% in 3T3-L1 adipocytes 24h after ROSI treatment. [C] Although no significant changes were detected in *socs-3* expression, it tended to be higher in both cells lines exposed to ROSI. Data were obtained from duplicate experiments and are expressed as a percentage of the control ± SEM (n=6-10). (***p<0.001, ****p<0.0001)(▧ CTL;▓ 1μM ROSI)
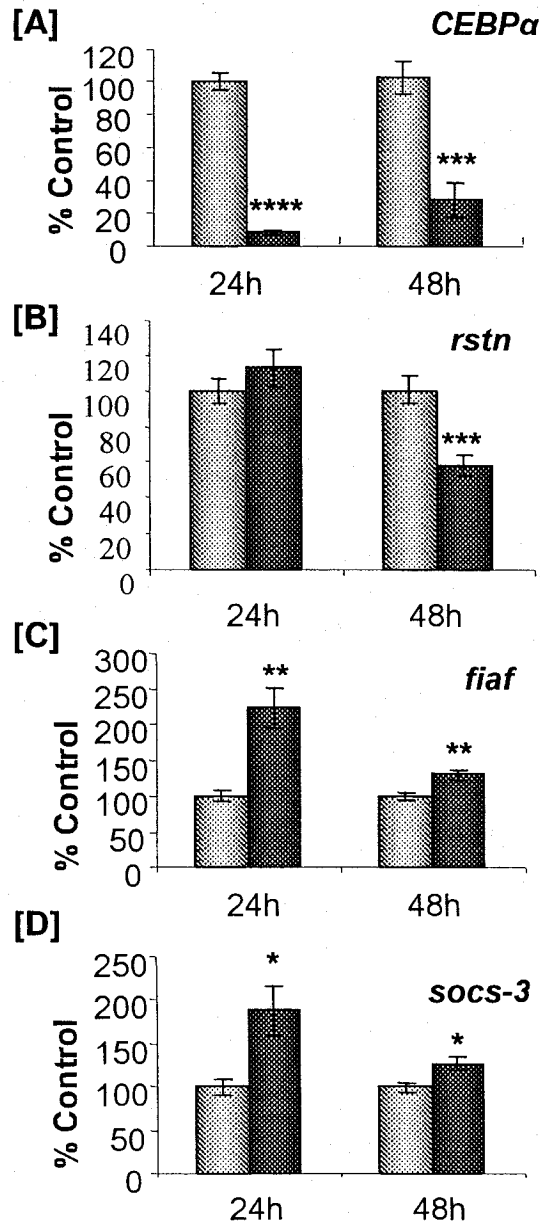
Figure 54: CEBPα knockdown in N-1 neurons

Cells were transfected with a CEBPα-specific siRNA (100nM; STH CEBPα), or the non-specific control (STH CTL), and gene expression was analysed by realtime RT-PCR 24h and 48h later. [A] CEBPα knockdown was greatest in N-1 cells 24h following transfection (-90%), although it was still significantly inhibited after 48h (-70%). [B] Although rstn was unchanged after 24h, its expression was also significantly reduced by 40% 48h after knockdown was initiated. [C] Although the increases in fiaf expression were greatest 24h after CEBPα silencing (+120%), it remained significantly elevated after 48h (+30%). [D] Similarly reducing CEBPα expression induced socs-3 by 85% 24h after transfection, and by 25% after 48h. Data were obtained from duplicate or triplicate experiments and are expressed as a percentage of the control ± SEM (n=7-9). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)(▨ STH CTL; ▩ STH CEBPα)

mRNA was 2×10^5 times higher relative to N-1 cells transfected with the empty vector

(p<0.0001) (Fig 55A). As expected *rstn* expression was robustly increased by 8-fold in

N-1 cells 24h after transfecting them with the CEBPα plasmid (p<0.0001) (Fig 55B). In

contrast *fiaf* and *socs-3* mRNA levels were both inhibited by 45% and 35% respectively

(p<0.0001), relative to cells transfected with the empty vector (pEMPTY) (Fig 55C and

55D).

VPA Treatment In Vivo Has Minimal Effects On Adipokine Expression

At the start of the experiment mice were weighed to confirm that groups were

evenly matched (32.4 ±0.7g CTL vs. 32.5±0.6g VPA), and bodyweights were unchanged

by VPA 24h after injection. Although hypothalamic *rstn* expression was unchanged, VPA

modestly, but significantly, increased the expression of *rstn* (+24%;p<0.05) in adipose

tissue (Fig 56A). VPA also failed to modify *fiaf* or *soc-3* expression in either the

hypothalamus or fat (Fig 56B and 56D).

ATRA Differentially Modulates Adipokine Gene Expression In Vivo

Prior to treatment mice were weighed in each of the 4 groups (control, 20mg/Kg,

50mg/Kg or 100mg/Kg ATRA) to confirm no significant weight variations between

groups. Prior to sacrifice, 24h post-injection, mice were weighed again and no significant

changes in body mass were detected. As expected, ATRA dose-dependently reduced

hypothalamic *rstn* expression, but this effect was only significant at the highest dose (-

25%, p<0.05) (Fig 57A). Although there was a tendency for *rstn* mRNA to be lower in

FAT it failed to reach statistical significance (Fig 57D). In the hypothalamus ATRA

significantly inhibited *fiaf* expression at all doses tested (-20 to -35%) (Fig 57B). In

contrast *fiaf* expression was dose-dependently stimulated by ATRA in FAT (+20-80%)

(Fig 57D), and these changes were the opposite of what was observed in vitro (Fig 52B).

ATRA also inhibited hypothalamic *socs-3* expression, but this was only significant with

the 50mg/Kg dose (-30%; p<0.01) (Fig 57C). Similar to what was detected in 3T3-L1

[A]

CEBPα

% Control

3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00

****

pEMPTY   pCEBPα

[B]

rstn

% Control

1000
800
600
400
200
0

****

pEMPTY   pCEBPα

[C]

fiaf

% Control

120
100
80
60
40
20
0

****

pEMPTY   pCEBPα

[D]

socs-3

% Control

120
100
80
60
40
20
0

****

pEMPTY   pCEBPα
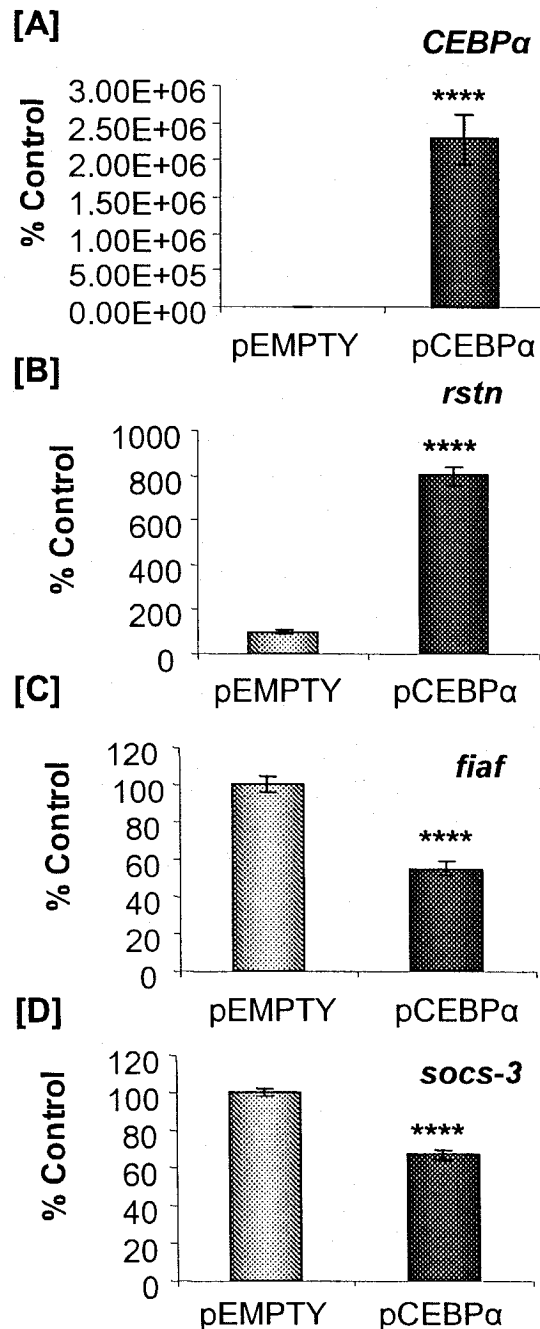
Figure 55: The effects of CEBPα overexpression in N-1 neurons

Gene expression was analysed in N-1 cells 24h after being transfected with a CEBPα expression plasmid, or an empty vector, using realtime RT-PCR. [A] As expected CEBPα mRNA was significantly increased in cells transfected with the CEBPα vector relative to the control values. [B] CEBPα overexpression robustly induced rstn gene expression in N-1 cells (+700%). [C] In contrast fiaf mRNA was significantly reduced in cells overexpressing CEBPα. [D] Socs-3 gene expression was also significantly inhibited by CEBPα overexpression. Data were pooled from duplicate experiments and are expressed as a percentage of the control ± SEM (n=7-8). (****p<0.0001)
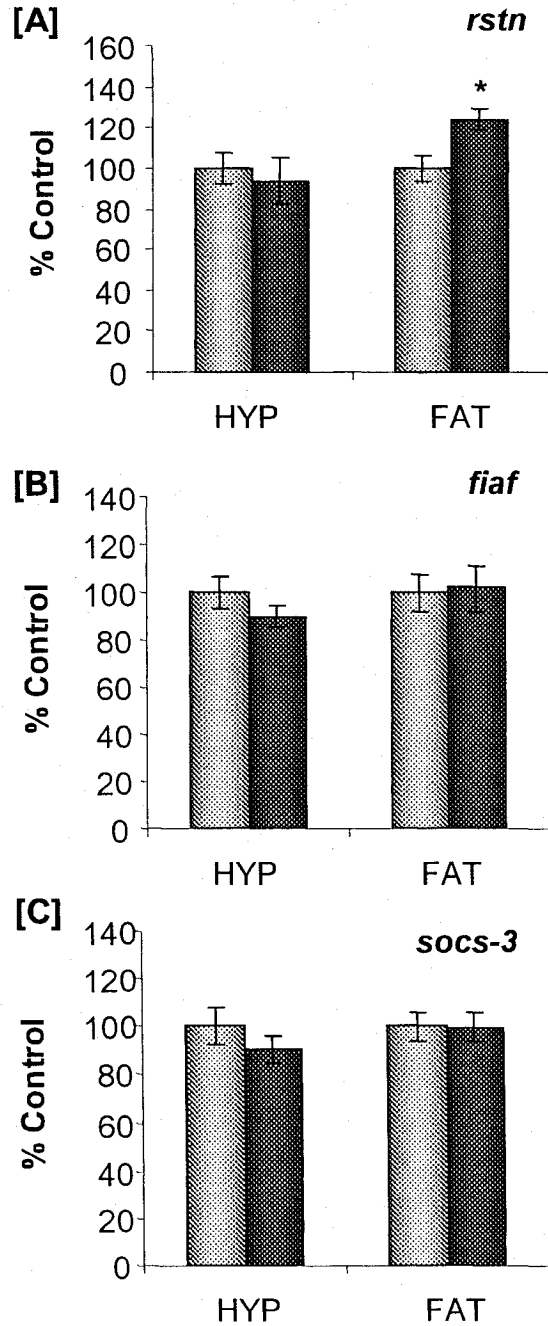
Figure 56: The effects of VPA on gene expression in vivo. Male mice were treated with a single injection of VPA (100mg/Kg) and gene expression was measured 24h later using realtime RT-PCR. [A] Although no changes were detected in the HYP, *rstn* was significantly increased (~25%) by VPA treatment in adipose tissue. [B] VPA failed to modify *fiaf* expression in either tissue. [C] Similarly no changes in *socs-3* mRNA were detected 24h following the injection of VPA. Data are expressed as a percentage of the control ± SEM (n=6). (*p<0.05) (CTL; 100mg/Kg VPA)
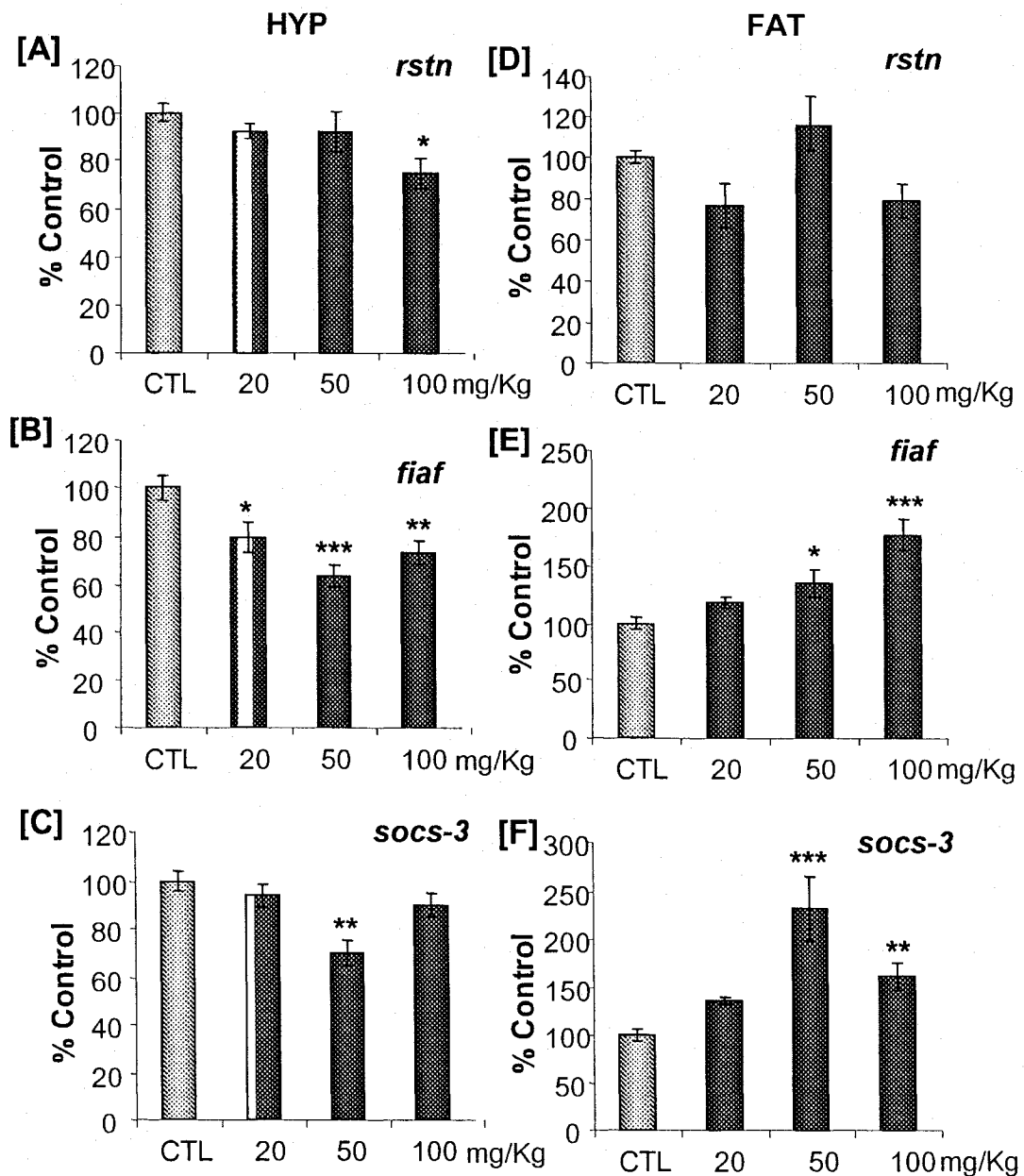
Figure 57: The effects of ATRA on gene expression in vivo

Twenty four hours following treatment with ATRA (20, 50 or 100mg/Kg) gene expression was analysed in the hypothalamus and visceral fat. [A] ATRA dose-dependently inhibited *rstn* expression in the hypothalamus by 25%. [B] *Fiaf* expression was also significantly attenuated in the HYP by ATRA treatment. [C] Only the 50mg/Kg dose of ATRA reduced *socs-3* expression in the HYP. [D] No significant changes in *rstn* were detected with any dose of ATRA in fat. [E] *Fiaf* expression was dose-dependently induced by ATRA in fat. [F] Similarly *socs-3* expression was significantly increased by ATRA treatment. Data were pooled from multiple experiments and are expressed as a percentage of the control ± SEM (n=7-8). (*p<0.05, **p<0.01, ***p<0.001)(▨ CTL; ■ ATRA)
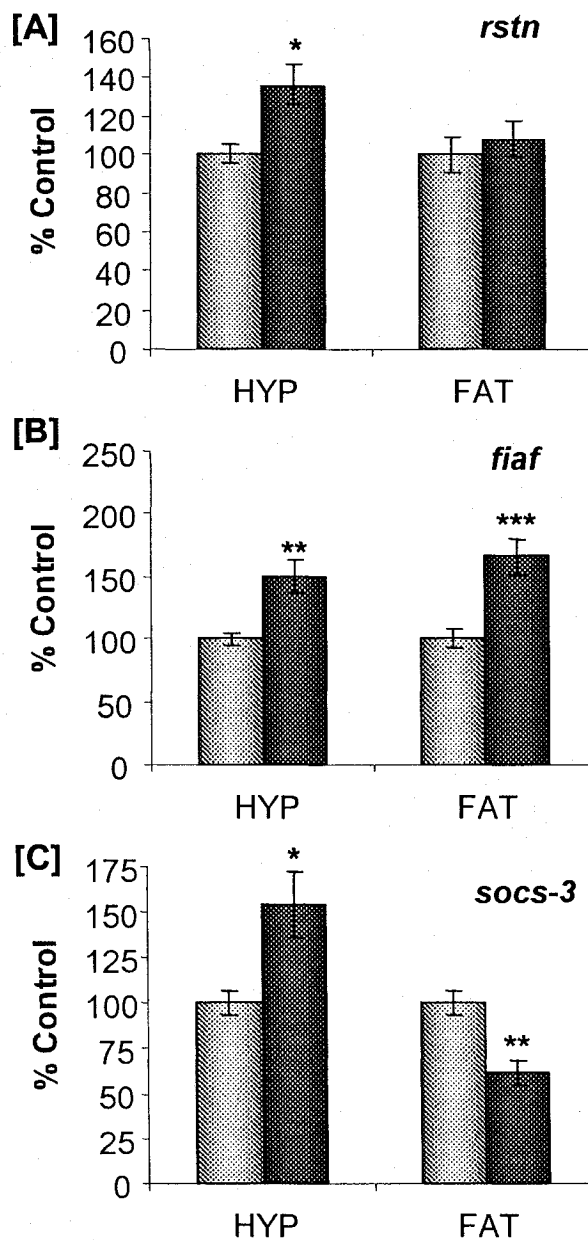
Figure 58: The effects of rosiglitazone on gene expression in vivo.
Realtime RT-PCR was used to measure fat and hypothalamic gene expression in male mice 24h after being injected with rosiglitazone (100mg/Kg). [A] ROSI significantly induced *rstn* gene expression in the hypothalamus (35%), but had no effect in visceral fat. [B] *Fiaf* mRNA was increased by 50% and 65% in the HYP and fat, respectively, following the injection of ROSI. [C] ROSI also increased *socs-3* mRNA in the HYP (+50%), but reduced its expression in fat (-40%). Data are expressed as a percentage of the control ± SEM (n=7). (*p<0.05, **p<0.01, ***p<0.001) (CTL; 100mg/Kg ROSI)

adipocytes, ATRA induced *socs-3* expression in adipose tissue (+35%-135%) but this was only significant with the two highest doses (Fig 57F).

Rosiglitazone Differentially Modulates Adipokines In Vivo

Prior to treatment mice were weighed to confirm there were no significant differences between groups (30.6 ±0.7g CTL vs. 30.6±0.6g ROSI). When mice were weighed 24h later there was a slight, but non significant, variation (29.8±0.76g CTL vs. 31.4±0.0.67 ROSI). Intriguingly hypothalamic *rstn* expression was significantly increased by ROSI (+35%; $p<0.05$), and ROSI failed to modify *rstn* mRNA in adipose tissue (Fig 58A). However, like my in vitro investigations (recall Figure 53B), rosiglitazone significantly stimulated *fiaf* expression in both the hypothalamus (+50%; $p<0.01$) and FAT (+65%; $p<0.001$) (Fig 58B). ROSI also significantly increased hypothalamic *socs-3* mRNA (+54%; $p<0.05$), but suppressed *socs-3* gene expression in adipose tissue (-35%; $p<0.01$).

IV. Discussion

In these experiments I showed that various compounds implicated in the control of CEBPα or PPARγ transcriptional activity also modified adipokine and *socs-3* gene expression in two cell line models, N-1 hypothalamic neurons and differentiated 3T3-L1 adipocytes. Similar experiments in vivo confirmed the effects of these compounds on adipokine expression in the hypothalamus and visceral fat. Treating N-1 cells with VPA, a purported inhibitor of CEBPα, induced a significant reduction in *rstn* and *fiaf*, but modestly increased *socs-3*, in a time-dependent manner. To exclude the possibility that VPA was exerting its effects via a HDAC-dependent mechanism cells were treated with TSA, a more specific HDAC inhibitor. This confirmed that in N-1 cells the changes in *rstn* were HDAC-independent, although the effects on *fiaf* and *socs-3* gene expression might be partially mediated by HDAC activity. Unexpectedly VPA had no effect on gene expression in 3T3-L1 adipocytes suggesting that other transcription factors, besides

CEBPα, are having a more profound effect. Similarly TSA induced only modest changes in *fiaf* and *socs*-3 in 3T3-L1 adipocytes. Thus VPA and TSA induce changes in gene expression that are, at least partially, cell type-specific. Treating N-1 neurons and 3T3-L1 adipocytes with ATRA or rosiglitazone induced similar trends in gene expression; very modest decreases in *rstn* were detected and *fiaf* and *socs-3* tended to be increased. To further asses the role of CEBPα its expression was silenced in N-1 cells using an RNAi based approach. This resulted in the expected decreases in *rstn* and elevations in *fiaf* and *socs-3,* although these effects were time-dependent. Conversely overexpressing CEBPα induced the opposite effects on gene expression in N-1 neurons suggesting that the effects in the previous experiment were not due to off-target RNAi pathways. In vivo VPA only modestly increased *rstn* expression in fat, and had no effects in the hypothalamus, nor did it modify the expression of *fiaf* or *socs-3* 24h after being injected. As expected ATRA dose-dependently inhibited *rstn* gene expression in the HYP, but also inhibited *fiaf* and *socs-3* expression. In contrast ATRA had no clear effect on *rstn* expression in fat, although significant increases in *fiaf* and *socs-3* expression were detected. Similarly ROSI induced all 3 genes analysed in the HYP, whereas in adipose tissue ROSI failed to modify *rstn*, although an increase in *fiaf* and reduction in *socs-3* mRNA were detected. Despite the gene expression patterns in vivo being dissimilar to what was measured in vitro, these drugs induced cell type and tissue-specific changes in gene expression. In summary CEBPα clearly modulates *rstn, fiaf* and *socs-3* gene expression in the N-1 hypothalamic cell line, and might also be the case in the intact adult hypothalamus. However hypothalamic gene expression is also being influenced by peripheral adiposity feedback signals that might also impact hypothalamic adipokine expression. Therefore compounds that modify CEBPα transcriptional activity in N-1 neurons are likely influencing hypothalamic gene expression in vivo, and these changes might impact central energy homeostasis and signaling pathways in vivo.

The compounds used in these studies have been implicated in the regulation of CEBPα transcriptional activity. However they are not very specific and are also capable of modifying gene expression by alternative mechanisms. Thus an RNAi approach was used since it would permit me to specifically interfere with the expression of CEBPα in order to establish whether this transcription factor was even capable of modulating gene expression in N-1 neurons. As expected *CEBPα* knockdown was transient and the greatest reductions in expression were detected 24h after transfection, although its expression remained significantly suppressed even after 48h. The increases in *fiaf* and *socs-3* were greatest 24h after CEBPα knockdown was initiated. Although these increases were still detectable 48h later, they were less robust which might be a consequence of CEBPα levels beginning to increase from their nadir. Like VPA treatment, *rstn* levels were not decreased until 48h after CEBPα silencing was initiated in N-1 neurons. This lag could be due to the time required for CEBPα protein levels to decrease, or perhaps *rstn* mRNA is particularly stable in the N-1 cell line so reductions are not immediately detectable. As noted earlier (see Chapters 2 and 3), concerns have been raised about the specificity of RNAi, thus in addition to silencing CEBPα I used a plasmid based approach to increase its expression in N-1 cells and confirmed that the opposite effects on *rstn, fiaf* and *socs-3* gene expression occurred. To further exclude the possible involvement of off-target RNAi pathways the expression of IFNβ was also analysed, which confirmed that STH CEBPα did not induce an interferon response (Jackson and Linsley 2004). Thus the similarities in the gene expression profiles of N-1 cells treated with VPA, ATRA and ROSI and those obtained from cells transfected with STH CEBPα, are in further support of the hypothesis that these compounds exert their effects on gene expression via a CEBPα-dependent mechanism in N-1 cells.

Although VPA, ATRA and ROSI appeared to inhibit gene expression via a CEBPα-dependent pathway, the precise mechanism(s) are still open for debate. For

example VPA was reported to modify CEBPα-mediated transcription by either reducing

its expression (Qiao et al. 2006), or by modifying its ability to bind DNA (Lagace et al.

2004; Duan et al. 2005). VPA can also inhibit HDAC and when cells were treated with

the more specific HDAC inhibitor, TSA, significant changes in *fiaf* and *socs-3* were also

detected. Therefore alternative mechanisms may have induced the gene expression

changes that were detected in these experiments. Similarly retinoic acid (RA) blocked

CEBPα expression via a RAR-dependent pathway in 3T3-L1 adipocytes, but is also

capable of interfering with other key adipogenic transcription factors such as PPARγ

(Schwarz et al. 1997; Shao and Lazar 1997). Although rosiglitazone is a highly specific

PPARγ agonist, it can also modify CEBPα expression in 3T3-L1 cells since these two

factors are mutually regulated (Shao and Lazar 1997). In vitro investigations in primary

astrocytes suggested that rosiglitazone might also modify glucose metabolism and gene

expression via PPARγ-independent mechanisms (Dello Russo et al. 2003; Park et al.

2003), suggesting that ATRA and ROSI might also modify gene expression via CEBPα-

and PPARγ-independent mechanisms. In Chapter 3 I demonstrated that the RNAi-

mediated knockdown of *rstn* increased the expression of *fiaf* and *socs-3* in N-1 cells

suggesting that there is a certain degree of interaction occurring between the genes

being studied. Therefore directly modifying *rstn* expression might lead to indirect

changes in *fiaf* and *socs-3*. However it seems unlikely that this is the case in these

studies since the decreases in *rstn* expression that were induced by VPA treatment, or

by CEBPα knockdown, were slower to occur than the detected increases in *fiaf* and

socs-3. Promoter analysis has revealed that several potential CEBP binding sites are

located in the proximal DNA regions of the resistin (Hartman et al. 2002), FIAF (Yoshida

et al. 2004), and SOCS-3 (Abbud et al. 2004) genes suggesting that CEBPα might

directly modify their expression. This issue of whether CEBPα directly modifies the

expression of these genes could be resolved by using chromatin immuno-precipitation

(ChIP) to verify whether CEBPα directly binds to their promoters in N-1 neurons or 3T3-L1 adipocytes. If this was found to occur, then the next step would be to verify whether these compounds modify the binding of CEBPα to these target promoters. Although the CEBPα knockdown data strongly correlate with the gene expression profiles obtained from N-1 cells treated with VPA, ATRA and ROSI, suggesting that it is at least partially responsible for mediating the detected changes in gene expression, these experiments do not preclude the existence of other mechanisms that might also induce gene expression changes in N-1 neurons.

Although there are some similarities in gene expression changes detected in N-1 neurons and 3T3-L1 adipocytes, there is also some discordance. For example VPA and TSA modified gene expression in N-1, but not 3T3-L1 cells. These differences might reflect the cell line-dependent effects of CEBPα. For example overexpressing CEBPα induced *rstn* expression by approximately 7-fold in 293T cells (Zhou et al. 2006), an increase that was of similar magnitude to the changes detected in N-1 cells. In contrast overexpressing CEBPα in 3T3-L1 cells was reported to increase *rstn* expression by about 2-fold (Song et al. 2002; Zhou et al. 2006). Although preadipocytes express higher levels of endogenous CEBPα, *rstn* gene expression is lower than in fully differentiated adipocytes (Zhou et al. 2006). Clearly there are other factors and mechanisms in fat cells that are able to interfere with the stimulatory effects of CEBPα on *rstn* expression. Thus CEBPα plays a vital, but not exclusive, role in the regulation of *rstn* in adipose tissue (Zhou et al. 2006). The overexpression of CEBPα also induced *rstn* in L6 myocytes, but it failed to activate the resistin gene in NIH 3T3 fibroblasts or mouse myeloid progenitor 32D cells (Song et al. 2002). However the inability of CEBPα to induce *rstn* is not unexpected since in vivo CEBPα is highly expressed in hepatocytes, yet *rstn* expression was not readily detected in the liver (Song et al. 2002). The inability of CEBPα to induce *rstn* in these other cell types could be due to endogenous inhibitory

mechanisms, or other elusive regulatory factors, that might also be involved in the control of resistin gene expression (Song et al. 2002; Zhou et al. 2006). It also seems plausible that the DNA regions surrounding the resistin gene could be modified during cellular differentiation, favoring *rstn* expression in only certain cell types. For example histones H3 and H4 are hyperacetylated in the resistin promoter region of 3T3-L1 adipocytes, but not preadipocytes (Hartman et al. 2002). This suggests that the resistin promoter might be suppressed, as opposed to turned-off, in non-adipose cells (such as N-1 neurons) that have also been reported to express low levels of *rstn*. Furthermore the failure of VPA to modify adipokine expression in 3T3-L1 adipocytes might reflect the relative insensitivity of this cell line to respond to changes in CEBPα expression or DNA binding. In contrast N-1 neurons might be hypersensitive to changes in CEBPα which is why VPA might have only induced changes in adipokine expression in these cells, and not 3T3-L1 adipocytes. Similarly other compensational mechanisms might be able to overcome the changes in CEBPα in 3T3-L1 cells since multiple factors are known to regulate *rstn* gene expression in adipocytes. Differential adipokine gene regulation has previously been shown in brain (C6 glioblastoma), pituitary (GH3 somatomammotrophs) and fat cells (3T3-L1 adipocytes) using a luciferase reporter assay that was driven by the leptin promoter (Li et al. 2001). Therefore adipokine gene regulation appears cell type-specific, although the causes and consequences of this differential regulation remain unclear.

Rosiglitazone is used clinically as an insulin sensitizing drug that impacts the expression of several adipokine genes, including *rstn* and *fiaf,* through PPARγ-dependent mechanisms. Although treating differentiated 3T3-L1 cells with rosiglitazone was initially reported to inhibit *rstn* expression and secretion (Steppan et al. 2001) I was unable to reproduce these results in either 3T3-L1 adipocytes, N-1 hypothalamic neurons, or within the AtT 20 corticotroph cell lines (Brown et al. 2005b). Treating human

myeloid U937 cells with ciglitazone, another PPARγ agonist, also failed to modify *rstn* expression (Yang et al. 2003a). Perhaps rosiglitazone failed to modify *rstn* expression in these studies based on how 3T3-1L cells were differentiated, and the effects of TZDs on *rstn* are likely cell-type specific. However ROSI induced the expected increases in *fiaf* expression in both N-1 neurons and 3T3-L1 adipocytes confirming the activity of the PPARγ agonists used in these studies. In parallel studies, injecting mice with a single high dose (100mg/Kg) of rosiglitazone also failed to modify *rstn* expression in fat, although *rstn* mRNA was increased in the hypothalamus. The inability of rosigltiazone to inhibit *rstn* expression in fat is consistent with our previous report where CD-1 male mice were treated orally with ROSI over a prolonged period (up to 7 weeks) which also failed to modify pituitary *rstn,* and produced minimal decrease in adipose tissue (Brown et al. 2005b). Decreased *rstn* expression was also reported in various animal models of diabetes and obesity, and rosiglitazone paradoxically increased *rstn* expression in white adipose tissue from ob/ob mice and Zucker diabetic fatty rats (Way et al. 2001), much like the detected increases in hypothalamic *rstn* in my experiments. Moreover transfecting undifferentiated 3T3-L1 cells with expression vectors for PPARγ, and its heterodimeric binding partner RXRα, also increased resistin promoter activity by nearly 2-fold (Chung et al. 2006). However the authors suggested that these changes were probably non-PPARγ dependent since the PPARγ agonist, troglitazone, failed to further stimulate *rstn* gene expression. Perhaps these genetic manipulations modified the expression of other transcription factors implicated in the control of the resistin promoter, such as CEBPα. Although rosiglitazone appears to be neuroprotective, and acts as an anti-inflammatory molecule in the CNS, it was unclear whether these effects are PPARγ-dependent (Dello Russo et al. 2003; Park et al. 2003), or whether TZDs could even cross the blood brain barrier (Maeshiba et al. 1997; Sundararajan et al. 2006). However treating mice with rosiglitazone increased PPARγ binding DNA in cortical nuclear

extracts, and this was associated with decreased expression of several inflammatory cytokines (Luo et al. 2006). In my experiments, injecting mice with the rosiglitazone slurry induced the expected increases in *fiaf* expression in adipose tissue and the hypothalamus further suggesting that the drug used in these studies was a) active and b) could reach the appropriate central nuclear targets. Despite ROSI inducing tissue-dependent changes in gene expression, it remains uncertain whether these effects are PPARγ-dependent.

Although prolonged treatments with VPA or ATRA have been reported to reduce bodyweights in mice (Felipe et al. 2004; Qiao et al. 2006), no significant changes in overnight bodyweight were detected in these studies. Similarly rosiglitazone treatment has also been reported to induce weight gain in rats and mice, but paradoxically improves glucose tolerance (Chaput et al. 2000; Carmona et al. 2005; Sotiropoulos et al. 2006). Although significant changes in overnight bodyweight gain were not seen, ATRA tended to decrease bodyweight (-0.75g; 100mg/Kg) whereas rosiglitazone induced a modest increase in overnight weight gain (+1.6g; 100mg/Kg), but VPA had no effect. Intriguingly ATRA decreased the expression of *socs-3* in the hypothalamus, whereas rosiglitazone induced its expression. Thus changes in *socs-3* appear to correlate with bodyweight changes; increases in *socs-3* mRNA were associated with weight gain and decreases with weight loss. As discussed in Chapter 3, *socs-3* has a profound impact on the regulation of hypothalamic energy sensing pathways. For example *socs-3* haploinsufficiency, or the neuronal-specific deletion of *socs-3*, is protective against the development of diet-induced obesity (DIO) and insulin resistance (Howard et al. 2004; Mori et al. 2004; Kievit et al. 2006). In contrast feeding mice a high fat diet selectively increases hypothalamic *socs-3* (Munzberg et al. 2004). Clearly, modifying hypothalamic *socs-3* gene expression impacts peripheral energy metabolism and bodyweight. Therefore ATRA and rosiglitazone may have induced their modest effects on bodyweight

by modifying the expression of hypothalamic *socs-3*. However it is unclear whether these compounds modify the intensity of feedback signals that are also known to impact hypothalamic *socs-3* expression, or whether these changes could occur directly via a PPARγ or CEBPα-dependent mechanism. As noted above, the *socs-3* promoter does contain several putative CEBP binding sites, and CEBPα is a negative regulator of *socs-3* expression in N-1 neurons, which suggests CEBPα might directly modulate hypothalamic *socs-3* expression in vivo.

Intriguingly VPA failed to have any effect on bodyweight or on gene expression in fat or in the hypothalamus, with the exception of the modest increases detected in fat expression. This lack of effect could be due to our use of a single injection which may have been inadequate to elevate and maintain plasma VPA concentrations, whereas others observed changes when VPA was injected multiple times daily over a period of several days (Qiao et al. 2006). Similarly *rstn* expression was only decreased after 48h in N-1 cells, thus the length of treatment may have been too acute in order to modify gene expression. In addition the doses used here (100mg/Kg) were lower than those reported by others (200mg/kg), and are clearly lower than those used therapeutically (20-30mg/Kg) in humans (Qiao et al. 2006; El-Khatib et al. 2007). However Qiao et al. (2006) justified the use of such a high dose since it reproduced the plasma VPA concentrations that are measured in patients being treated with VPA. This suggests that mice are particularly efficient at metabolizing, and clearing, VPA from their system. Therefore prolonged VPA treatment, and using an increased dosage, might be required in order to induce changes in bodyweight and gene expression.

In summary these studies suggest that brain-derived adipokines are regulated by CEBPα in the N-1 hypothalamic cell line. Although it remains to be resolved whether these effects are occurring directly, all three gene promoters contain putative CEBP response elements. Three of the drugs tested, VPA, ATRA and ROSI, also induced

gene expression profiles that were similar to those obtained when CEBPα expression was silenced in N-1 neurons. However these effects appeared unique to the changes detected in 3T3-L1 adipocytes and reaffirms previous demonstrations that adipokine gene expression is cell-type specific (Li et al. 2001). Although the gene expression profiles obtained in vitro did not match those changes detected in vivo, this discrepancy likely results from the complex feedback systems that are equally capable of modifying gene expression in adipose tissue and the hypothalamus in vivo. Furthermore these data suggest that central adipokine gene expression is tightly regulated by a complex system. Additionally, CEBPα and PPARγ have previously been implicated in various critical brain functions including development, inflammation and neuroprotection, though it remains unclear how they are exerting their effects. Having established that brain-derived leptin impacts C6 cell survival, CEBPα, and PPARγ could potentially be modifying the expression of other brain-derived adipokines that might also prove to be cytoprotective. **In conclusion these studies show that ATRA and ROSI impact central adipokine expression and cellular signaling and might also be affecting central energy homeostasis, although the effects of VPA remain less convincing. Therefore centrally expressed CEBPα might be a relevant pharmacological target that could be used to modulate central adipokine expression and bodyweight via hypothalamic-dependent pathways and should be investigated further.**

Chapter 5: The Effects Of Lipopolysaccharide (LPS) On Adipokine Gene Expression; Implications For Cachexia And LPS-Induced Insulin Resistance?

I. Introduction

Obesity And Inflammation: A Causal Link To Diabetes?

A relatively recent revelation in obesity research is the positive association between expanding fat mass and elevated levels of circulating inflammatory markers. Thus obesity appears to be a state of chronic mild inflammation (Trayhurn and Wood 2005), although it remains unclear whether obesity causes inflammation, or the reverse (Tataranni and Ortega 2005). It has been hypothesized that obesity-mediated increases in adipose mass induce a local hypoxic state, leading to the elevated expression and secretion of several inflammatory adipokines including tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and leptin (Trayhurn and Wood 2004). However it has also been suggested that macrophages infiltrate expanding adipose tissue, and they also synthesize and secrete inflammatory cytokines implicated in the development of diabetes (Fantuzzi 2005). In addition macrophages also appear to induce autocrine/paracrine signaling events, and crosstalk with adipose tissue (Fantuzzi 2005). Regardless of how obesity-associated inflammation occurs, the increases in cytokines that result from chronic inflammation contribute to the progression and development of obesity-associated conditions, most notably insulin resistance and diabetes (Tataranni and Ortega 2005; Tilg and Moschen 2006). Furthermore anti-inflammatory drugs, such as aspirin, appear to alleviate obesity associated insulin resistance (Yuan et al. 2001), further implicating increased cytokine production in the development of insulin resistance.

Adipokines, Diabetes And The Brain

Many, if not all, adipokines appear to influence hypothalamic metabolic pathways that control appetite and energy expenditure (Ahima et al. 2006). Many of these central

pathways have been implicated in the etiology of insulin resistance and the development of diabetes (Schwartz and Porte 2005; Plum et al. 2006). Although insulin modulates glucose uptake in several peripheral tissues including fat, liver and muscle, this does not appear to be the case in the brain (Schwartz and Porte 2005). Instead insulin is thought to act as an adiposity feedback signal in the CNS, and regulates peripheral glucose metabolism by modifying hepatic gluconeogensis via the vagus nerve (Plum et al. 2006). Thus the brain is a critical diabetic target tissue (Schwartz and Porte 2005). Additionally insulin signaling appears to be inhibited by socs-3, an endogenous intracellular negative feedback signal that has been implicated in the development of leptin and insulin resistance (Munzberg and Myers 2005; Howard and Flier 2006). For example adipocytes that are socs-3-deficient have increased levels of phosphorylated insulin receptor substrate 1 (IRS 1) and IRS 2, downstream signaling components of the insulin receptor signaling cascade (Shi et al. 2004). Paradoxically the overexpression of socs-3 in adipose tissue appeared to be protective against the development of systemic insulin resistance in vivo, although it did induce local resistance (Shi et al. 2006). Likewise LPS was hypothesized to induce peripheral insulin resistance by stimulating socs-3 expression in liver, muscle and fat (Ueki et al. 2004). However the liver-specific knockout of socs-3 also led to a state of insulin resistance that mimicked a chronic inflammatory state (Torisu et al. 2007). When samples of peripheral tissues from pre diabetic patients are cultured in vitro, or when adipose tissue from diabetics is removed and grown under euglycaemic conditions, they are fully insulin sensitive (Zierath et al. 1994; Eriksson et al. 1999). This has led to the hypothesis that the development of peripheral insulin resistance, and diabetes, is preceded by hormonal and neuronal perturbations in other tissues such as the CNS (Buren and Eriksson 2005) (see Figure 70, Chapter 7). For example, unlike peripheral tissues, the proopiomelanocortin (POMC) neuronal specific knockout of socs-3 was protective against diet-induced obesity and improved insulin

sensitivity in mice (Kievit et al. 2006).Thus inflammatory molecules may induce gene expression changes in other tissues (i.e. brain or pituitary), that might lead to the subsequent development of peripheral insulin resistance.

LPS-Induced Endotoxinemia

Lipopolysaccharide (LPS), a bacterial antigen, is found in the outer membrane of gram negative bacteria and is an agonist for mammalian Toll-Like receptors (TLR), in particular TLR 4 (Beutler 2000). LPS challenge induces an inflammatory state by increasing the expression and secretion of numerous cytokines by activating a TLR4 signaling cascade that predominantly stimulates nuclear factor kappa B (NFκB)-mediated transcription (Zhang and Ghosh 2001). Under basal conditions NFκB forms homodimeric or heterodimeric complexes that are retained in the cytoplasm by the inhibitor of kappa B (IκB) (See Figure 59, adapted from (Lodish et al. 2000). When LPS is present it binds the pattern recognition receptor, CD 14, where LPS is either transferred, or forms a larger complex, with TLR4 (Zhang and Ghosh 2001). The association of LPS with TLR4 leads to the downstream activation of inhibitor of kappa B (IκB) kinase (IKK), which phosphorylates IκB and targets it for ubiquitination and proteosomal degradation (see Fig 59). Thus NFκB homodimers/heterodimers are then released and are able to translocate to the nucleus in order to mediate transcription of target genes. Moreover the NFκB signaling pathway is thought to be inhibited by parthenolide, an anti-inflammatory compound derived from the medicinal herb Feverfew, since it binds and inhibits IKK (Kwok et al. 2001). However LPS also appears capable of inducing other signaling pathways that might also contribute to some of the associated inflammatory responses (Zhang and Ghosh 2001).

The peripheral administration of LPS is commonly used to induce an endotoxic inflammatory response in laboratory animals. Animals challenged with LPS also lose weight and develop peripheral insulin resistance (Aubert et al. 1997; Ueki et al. 2004).
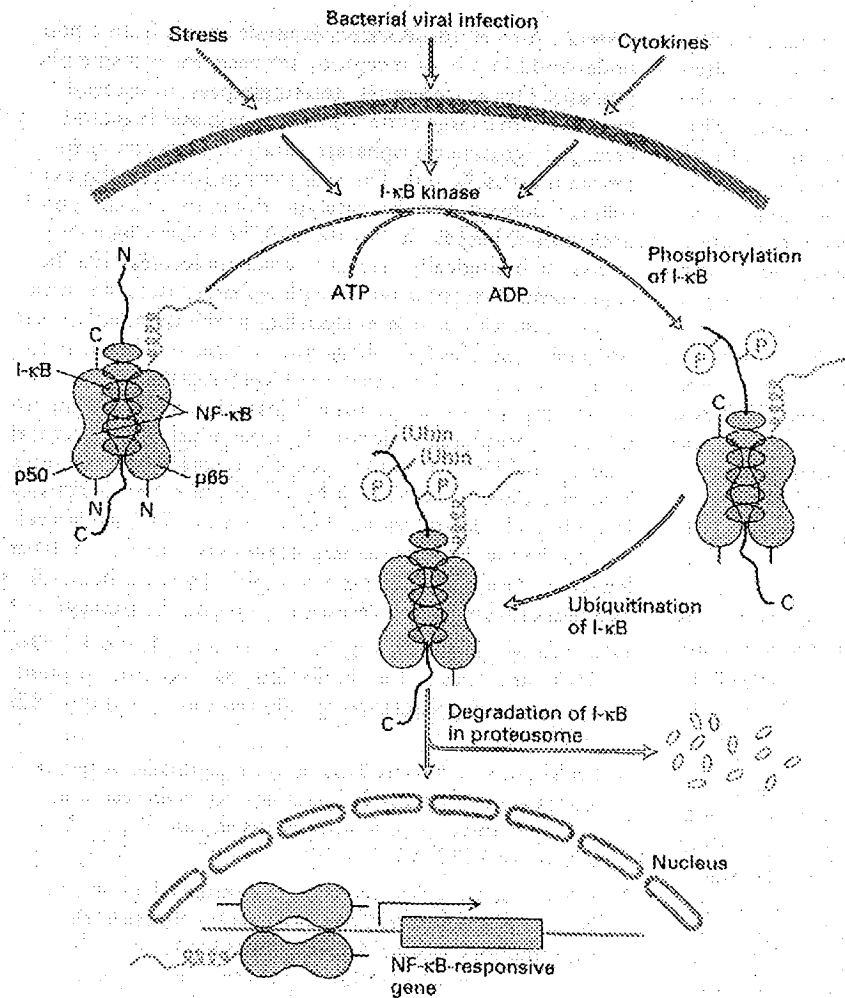
Figure 59: Activating NFκB-mediated transcription
Although NFκB can be activated by various stimuli, for these studies LPS
was of particular interest. LPS induces a TLR4-dependent signal cascade
that results in the activation of IκK (IκB Kinase) which subsequently
phosphorylates the amino terminus of IκB (Inhibitor of kappa B). This
results in the ubiquitination and proteosomal-mediated degradation of
IκB. This allows NFκB dimers to freely translocate into the nucleus where
they can modulate target gene expression. (Figure adapted from Lodish
et al. 2000)

The associated reductions in appetite and increased energy expenditure (i.e. fever) appear to be mediated by hypothalamic melanocortin pathways since the pharmacological blockade of melanocortin 3/4 receptors (MC3/4R) in rats, using SHU 9119, prevented the LPS-induced decreases in appetite (Huang et al. 1999). In agreement, administering LPS to MC4-R knockout mice (-/-) failed to reduce food intake (Marks et al. 2001), but was not the case in MC3-R deficient mice (Marks et al. 2003). In contrast the co-administration of LPS with αMSH, a MC3/4-R agonist, further enhanced the anorectic effects of LPS in rats (Huang et al. 1999). Although LPS modifies appetite via a melanocortin-dependent system, the effects on insulin resistance were not measured. Similarly LPS is thought to increase the blood to brain transport of insulin and leptin, which is partially responsible for inducing an anorectic response to LPS (Banks 2006a). However this fails to explain why leptin deficient (ob/ob) mice are equally responsive to the weight reducing effects of LPS (Faggioni et al. 1997). The peripheral injection of LPS might also induce the unidirectional release of cytokines into the brain interstitium by targeting the polarized endothelial cells that form the blood brain barrier (Rivest et al. 2000). An LPS challenge might also induce the hypothalamic expression of proinflammatory adipokines, such as resistin, leading to altered central energy metabolism by modifying central melanocortin pathways.

What Inflammatory Molecules Are Involved?

As already noted, several inflammatory molecules have already been implicated in the development of insulin resistance following the peripheral injection of LPS, including TNFα, IL-1 and IL-6. However several other novel adipokines also appear to be involved in LPS-induced insulin resistance (Tataranni and Ortega 2005). Resistin (*rstn*) and fasting-induced adipose factor (*fiaf*) are of particular interest since their expression is induced by both hypoxia and inflammation (Belanger et al. 2002; Lu et al. 2002). Additionally these novel adipokines have been implicated in the regulation of lipid

metabolism and glucose homeostasis in rodents (Steppan et al. 2001; Banerjee et al. 2004; Kersten 2005; Xu et al. 2005), though this remains a contentious issue in humans (Arner 2005; McTernan et al. 2006). For example LPS directly stimulated *rstn* gene expression in 3T3-L1 adipocytes and in rat adipose tissue (Lu et al. 2002). Although LPS increased *rstn* gene expression in human blood mononuclear cells, this required the prior induction of TNFα (Kaser et al. 2003). Moreover resistin is itself a potent proinflammatory molecule, and appears to induce chronic inflammation in patients suffering with rheumatoid arthritis by accumulating in synovial fluid (Bokarewa et al. 2005). Although the data on *fiaf* are more limited, its hepatic overexpression was reported to improve glucose tolerance (Xu et al. 2005). Paradoxically *fiaf* knockout mice appear protected against the development of diet-induced obesity (Backhed et al. 2007). Although several other inflammatory molecules also appear to be involved in LPS-induced cachexia, it seems likely that resistin and FIAF are also contributing to the perturbed metabolic state that results following an LPS challenge.

Resistin Influences Central Energy Metabolism

Resistin was reported to be an insulin desensitizing hormone in mice, and knocking out *rstn* lead to reductions in fasting plasma glucose concentrations (Steppan et al. 2001; Banerjee et al. 2004). Although a resistin receptor has yet to be isolated, resistin appears to act on multiple tissues, including the brain. For example the icv administration of resistin peptide fragments stimulated cFOS expression in the hypothalamus of fasted rats, and acutely reduced food intake, but failed to induce any long-term weight changes (Tovar et al. 2005). Likewise the icv injection of resistin has been reported to induce hepatic gluconeogenesis in mice (Ahima et al. 2006; Singhal et al. 2006). My own studies in N-1 hypothalamic neurons suggest that resistin could be impacting central energy homeostasis by modulating *socs-3* expression, and the activity of the novel energy sensor, AMPK (see Chapter 3). Thus the brain appears to be a

resistin target tissue. The metabolic responses induced by the central injection of resistin are strikingly similar to those reported following the administration of LPS; reduced appetite and increased plasma glucose concentrations.

A Role For Brain-Derived Adipokines?

As previously discussed, in addition to adipose tissue, we have provided substantial evidence that *rstn* and *fiaf* are expressed in various regions of the brain and pituitary gland (Morash et al. 2002; Morash et al. 2004; Wiesner et al. 2004). Not only was *rstn* detectable by RT-PCR (Morash et al. 2002), but resistin-immunoreactivity (ir) colocalized with the hypothalamic proopiomelanocortin (POMC) neuronal marker, alpha-melanocyte stimulating hormone (αMSH) (Wilkinson et al. 2005). Thus hypothalamic-derived resistin appears to localize with the central melanocortin signaling system, an essential target implicated in the metabolic consequences that are induced by LPS (Huang et al. 1999; Marks et al. 2003). **I hypothesized that LPS could be modifying adipokine (*rstn* and *fiaf*) and *socs-3* expression in a variety of tissues, including the hypothalamus where it might impact the activity of melanocortin pathways in adult mice. Additional studies were carried out in the N-1 cell line, which coexpresses *rstn* and *fiaf* (Brown et al. 2006b), in addition to numerous other neuropeptides implicated in central energy homeostasis (Belsham et al. 2004). These in vitro studies were performed to establish whether LPS might directly modify hypothalamic *rstn* expression, as reported for 3T3-L1 adipocytes and human blood mononuclear cells (Lu et al. 2002; Kaser et al. 2003). The expression of *fiaf* and *socs-3* was also monitored in N-1 cells, and various antagonists of the TLR4 signaling cascade were used to verify whether they could attenuate the LPS-mediated changes in gene expression.**

II. Materials And Methods

Mice

Male mice (CD-1; postnatal day 30-32) were obtained from Charles River

Breeding farm (St. Constant, QC) and housed in groups in clear plastic cages with free

access to Purina Rat Chow and drinking water, and maintained in a 12h light:12h dark

photoperiod. Following 1 week of acclimatization mice were injected with LPS (5mg/Kg;

s.c.; from *E.coli* O26:B6; #L8274; SIGMA, St. Louis) or saline, and groups of treated or

control mice were sacrificed at 3, 6 or 24h post-injection. Tissues; mediobasal

hypothalamus (HYP), parietal cortex (CTX), whole pituitary (PIT), and epididymal

visceral adipose tissue (FAT) were collected as described in Chapter 2 (section VII) and

snap frozen on dry ice and stored at -70°C until RNA isolation. The experimental

protocol was reviewed and approved by the Dalhousie University Committee on

Laboratory Animals. RNA was isolated from FAT using TRIzol® (Invitrogen; Burlington

ON), and from all remaining tissues and cells using the RNEasy mini kit following the

DNase protocol (Qiagen; Mississauga ON) as described in Chapter 2.

N-1 Cell Culture

Immortalized N-1 hypothalamic neurons were maintained in DMEM containing

10% FBS at 37°C in a humid incubator supplied with 5% CO_2/ 95% air (Belsham et al.

2004). Cells were seeded at 100,000cells/well in Nunc 6 well plates 24h before culturing

overnight in serum-free DMEM prior to experimentation. Cells were pretreated for 30min

with antagonists of the Toll-Like Receptor 4 (TLR4) signaling cascade; IκB antagonist

(Parthenolide; 5μM; SIGMA, Oakville, ON), mitogen-activated protein kinase kinase

antagonist (MAPKK; PD 98,059; 50μM; SIGMA) or p38 MAP kinase antagonist

(SB202190; 10μM; SIGMA). Cells were subsequently treated for 3h with LPS (25μg/mL;

in serum free DMEM) prior to isolating total RNA using the RNEasy Mini kit following the

DNAse protocol (Qiagen) as described previously (See Chapter 2; section III)

Realtime RT-PCR

Total RNA (1.5µg) was reverse transcribed (RT) and PCR amplified using the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's protocol using previously published primers and Taqman™ probes for *rstn*, cyclophilin (Brown et al. 2005b), *socs-3* (Steppan et al. 2005) and *fiaf* (Wiesner et al. 2006) (for primer and probe sequences see Table 2). In brief, RNA was diluted to 12µL and then heat denatured for 5min at 70°C. Samples were returned to ice prior to the addition of 15µL of the 2x reverse transcription master mix and 3µL of the SuperScript™ III RT enzyme master mix (Invitrogen). The RT reaction consisted of 10min incubation at 25°C, 45min incubation at 42°C, followed by a 5min 85°C termination step, and the resulting complementary DNA (cDNA; 30µL) was stored at - 20°C. For PCR amplifications samples were amplified in duplicate and only 1 gene was analysed per reaction. Individual PCR reactions consisted of a 2x Platinum® quantitative PCR SuperMix-UDG, 7pmol of the sense and antisense primers, 1pmol of the appropriate dual-labeled probe, 3µL of cDNA, to a final volume of 33µL in sterile water. Reactions were heated to 10min at 95°C, followed by 50 amplification cycles of 95 °C for 20s and 60°C for 60s using a BioRad thermal cycler and an iQ realtime PCR detection system. A standard curve, that was prepared using a serial dilution of a reference sample, was included in each realtime run to correct for possible variations in product amplification. Relative copy numbers were obtained from standard curve values, and were normalized to the values obtained for our house keeping gene, cyclophilin. Data are expressed as a percentage of the control ± SEM. It should be noted that no significant variations in our housekeeping gene were observed between groups when evaluated using either the threshold cycle (C_T), or using the relative levels of expression, and this is consistent with previous reports (Bond et al. 2002).

Statistics

For the in vivo LPS experiments data for each tissue were analysed using the Student's t-test since values are expressed as a relative percentage of those measured in saline controls for any given time point. For studies in N-1 cells data were subjected to analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparisons test using graphpad PRISM v4.0a. Significance was set at $p < 0.05$.

III. Results

LPS Reduces Bodyweight In CD-1 Mice

Mice were weighed prior to injection to confirm that there were no significant differences between the LPS or saline-control groups. As expected, 24h following the injection of LPS mice lost a significant amount of body weight (27.2±0.4g vs. 24.1±0.5g; $p < 0.001$). In contrast there were no changes in the bodyweights of mice injected with saline.

The Effects Of LPS On Hypothalamic Gene Expression

In the hypothalamus *rstn* gene expression was acutely up regulated by 64% ($p < 0.01$) 3h post-injection, but had returned to control values by 6 and 24h (Fig 60A). Similarly *fiaf* mRNA was rapidly increased by 94% ($p < 0.01$) and 113% ($p < 0.05$) at 3h and 6h postinjection respectively, but had returned to control levels by 24h (Fig 60B). Like *rstn, socs-3* gene expression was markedly upregulated in the HYP 3h following LPS administration (400%; $p < 0.0001$), but had returned to control values by 6h and 24h (Fig 60C).

LPS Modulates Gene Expression In The Cortex

Unlike the HYP, cortical *rstn* gene expression was unchanged 3h following LPS administration, was reduced by 35% ($p < 0.01$) at 6h, and then increased by 24h (40%; $p < 0.05$) (Fig 61A). In contrast, *fiaf* gene expression was increased by 59% ($p < 0.01$),

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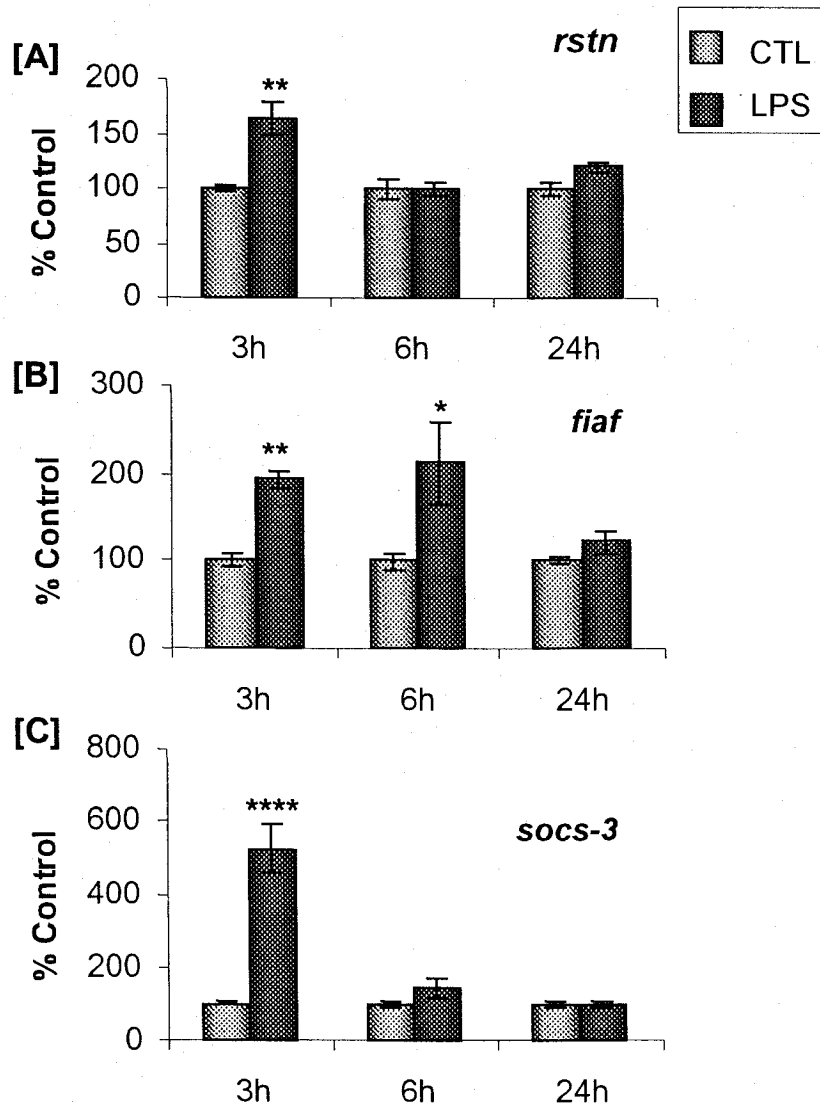
Figure 60: The effects of LPS on hypothalamic gene expression.
Following the injection of LPS (5mg/Kg) into male mice hypothalamic gene expression was analsyed at various time points by realtime RT-PCR. [A] *Rstn* gene expression was increased at 3h, but had returned to basal levels by 6h and 24h. [B] In contrast *fiaf* was significantly increased 3 and 6h post injection, but had returned to basal levels by 24h. [C] Like *rstn*, *socs-3* was strongly induced at 3h, but had returned to control values by 6h. Data are expressed as a percentage of the control ± SEM (n=4-6). (*p<0.05, **p<0.01, ****p<0.0001)
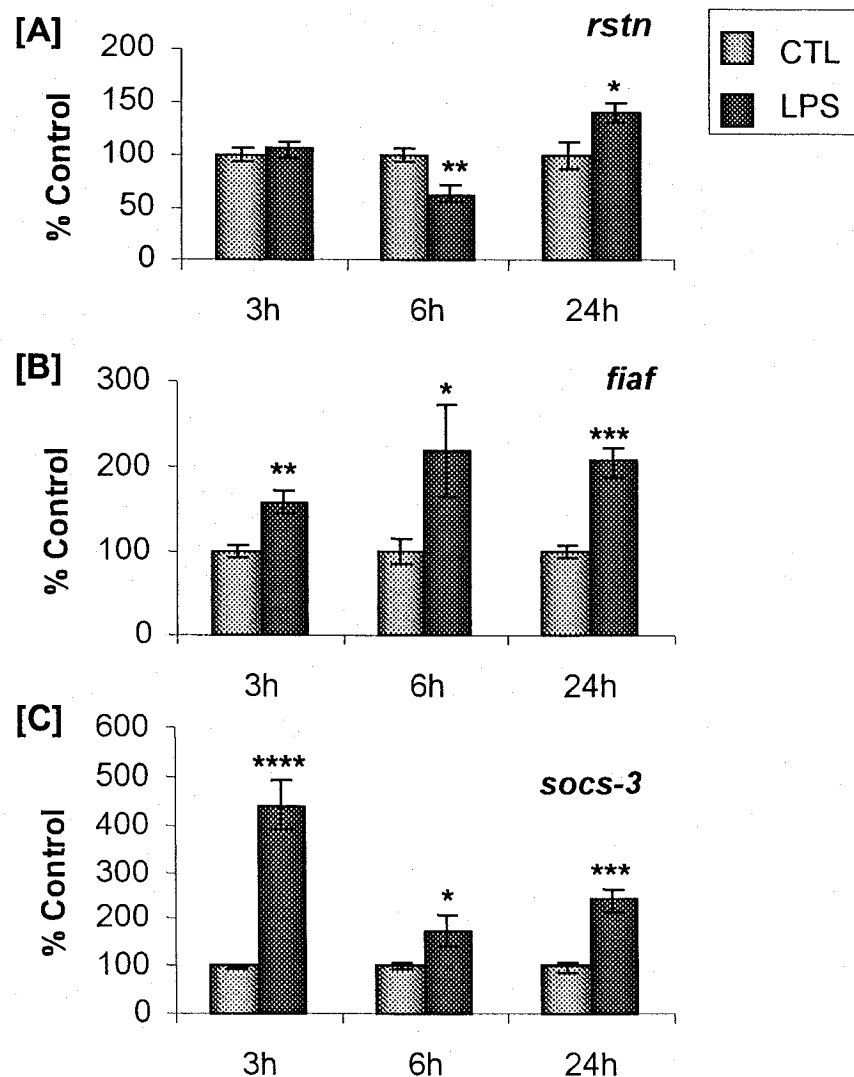
Figure 61: The effects of LPS on gene expression in parietal cortex.
Realtime RT-PCR was used to evaluate gene expression in the CTX at various time points following the injection of LPS (5mg/Kg) in male mice. [A] At 3h *rstn* gene expression was unchanged, was reduced by 35% at 6h, and increased by 40% after 24h. [B] *Fiaf* mRNA was rapidly induced by 3h (+60%), and mRNA levels were 100% higher 6h and 24h following the LPS challenge. [C] Although the increases in *socs-3* were greatest at 3h (+340%), its expression remained significantly elevated for more than 24h (+100%). Data are expressed as a percentage of the control ± SEM (n=4-6). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

119% (p<0.05) and 106%(p<0.001) at 3h, 6h and 24h respectively, relative to saline

treated controls (Fig 61B). Similarly LPS induced *socs-3* expression by 340%

(p<0.0001), 73% (p<0.05) and 140% (p<0.001) at 3h, 6h and 24h postinjection,

respectively (Fig 61C).

Pituitary Adipokines Are Stimulated By LPS

In the pituitary *rstn* was strongly induced 3h postinjection (+110%; p<0.01), but

had returned, and remained, at control levels at 6 and 24h (Fig 62A). *Fiaf* mRNA was

also increased by 107% (p<0.05) and 321% (p<0.01) at 3h and 6h postinjection, but had

returned to basal levels by 24h (Fig 62B). As seen in Figure 62C, LPS induced *socs-3*

expression by 440% (p<0.0001) and 184% (p<0.01) at 3h and 6h, but no changes were

detected after 24h.

LPS Modulates Gene Expression In Adipose Tissue

Unexpectedly no changes in *rstn* gene expression were detected in FAT at any

of the time points tested (Fig 63A). In contrast *fiaf* gene expression was increased by

57%(p<0.05), 144%(p<0.01) and 137% (p<0.01) at 3h, 6h and 24h following LPS

injection, relative to the saline injected controls (Fig 63B). LPS also induced *socs-3* gene

expression in FAT by 700% (p<0.0001) 3h post-injection, and it remained 4-fold higher

(p<0.01) 6h and 24h later, relative to control values (Fig 63C).

LPS Increased Fiaf And Socs-3, But Not Rstn, In N-1 Hypothalamic Cells

As seen in Figure 64A, neither LPS nor any of the signaling inhibitors

(Parthenolide, PD98,059 or SB202190) had any effect on *rstn* gene expression in N-1

cells. In contrast LPS induced a striking 10-fold increase in *fiaf* expression (p<0.001).

LPS-induced increases in *fiaf* expression were only partially reduced by pre-treating N-1

neurons with PD98,059 (-40%, p<0.001) and SB202190 (-35%, p<0.001), and IκB

antagonist, parthenolide, was the most effective at attenuating the increases in *fiaf*

expression (-66%; p<0.001) relative to cells treated with LPS alone. Although treating
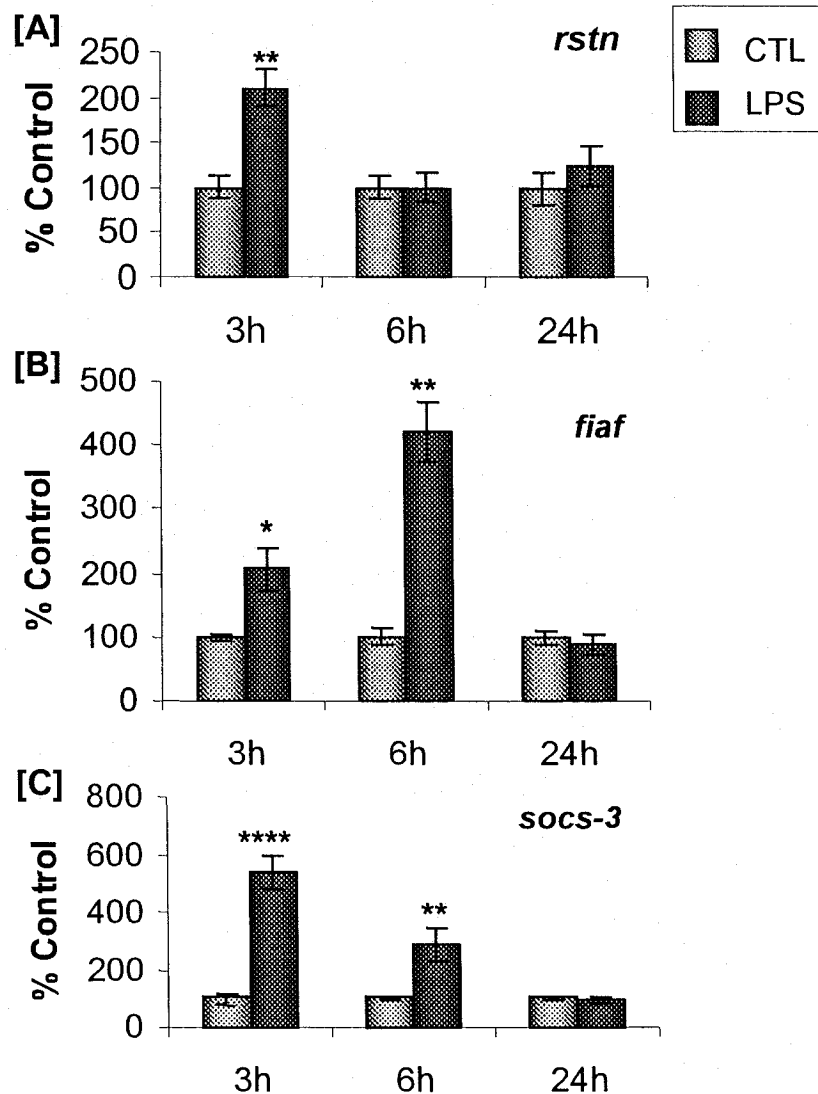
Figure 62: LPS modulates gene expression in the pituitary gland.
Gene expression was analysed in the pituitary 3h, 6h and 24h after an LPS challenge (5mg/Kg) was initiated using realtime RT-PCR. [A] *Rstn* was robustly increased 3h following LPS (+100%), but had returned to control levels by 6h. [B] Although *fiaf* gene expression was increased by 100% 3h after LPS, its expression peaked at 6h (+320%) and had returned to basal levels by 24h. [C] *Socs-3* mRNA was also increased after 3h (+400%), but was still significantly elevated at 6h (+175%), and finally returned to basal levels by 24h. Data are expressed as a percentage of the control ± SEM (n=4-6). (*p<0.05, **p<0.01, ****p<0.0001)
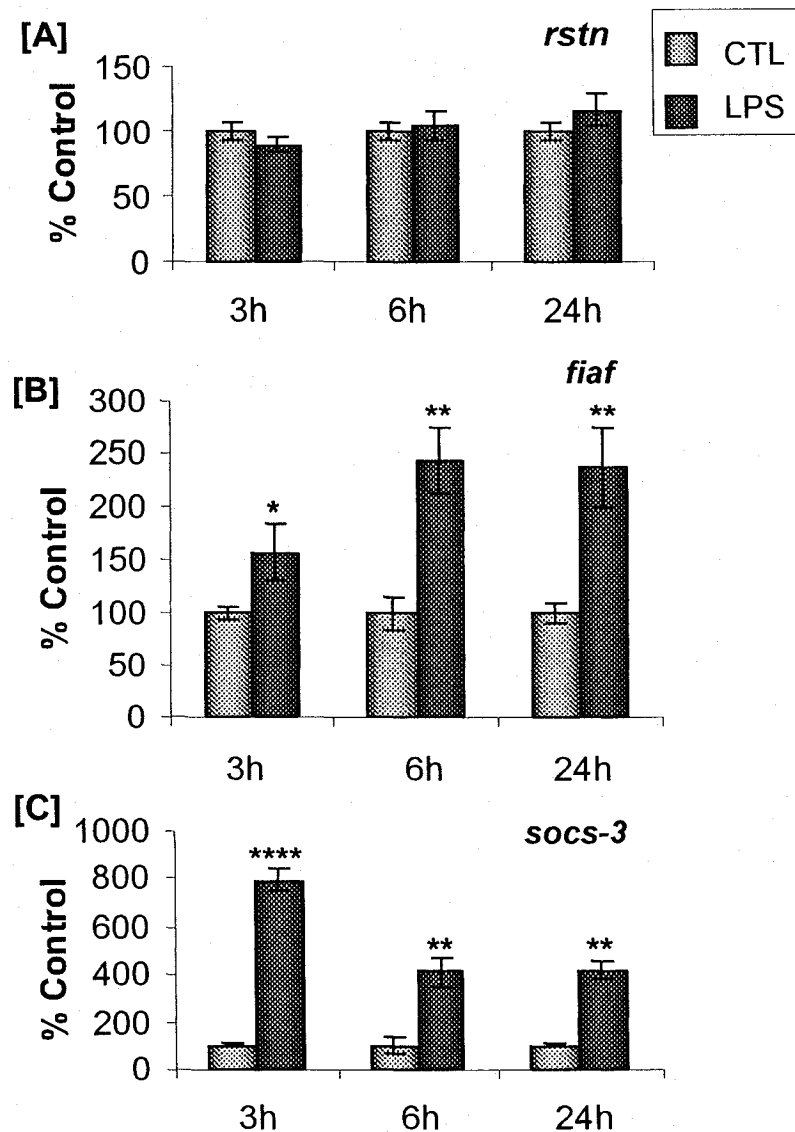
Figure 63: The effects of LPS on gene expression in visceral fat.
Realtime RT-PCR was used to measure gene expression changes in visceral fat following the injection of LPS in male mice. [A] Unexpectedly challenging mice with LPS failed to modify *rstn* gene expression at all time points measured. [B] In contrast, *fiaf* expression was significantly elevated by 3h (+60%), and was further increased 6h and 24h following LPS injection (+140%). [C] *Socs-3* was also induced by LPS, peaked at 3h (+700%), but remained 300% higher at both the 6 and 24h time points. Data are expressed as a percentage of the control ± SEM (n=4-6). (*p<0.05, **p<0.01, ****p<0.0001)
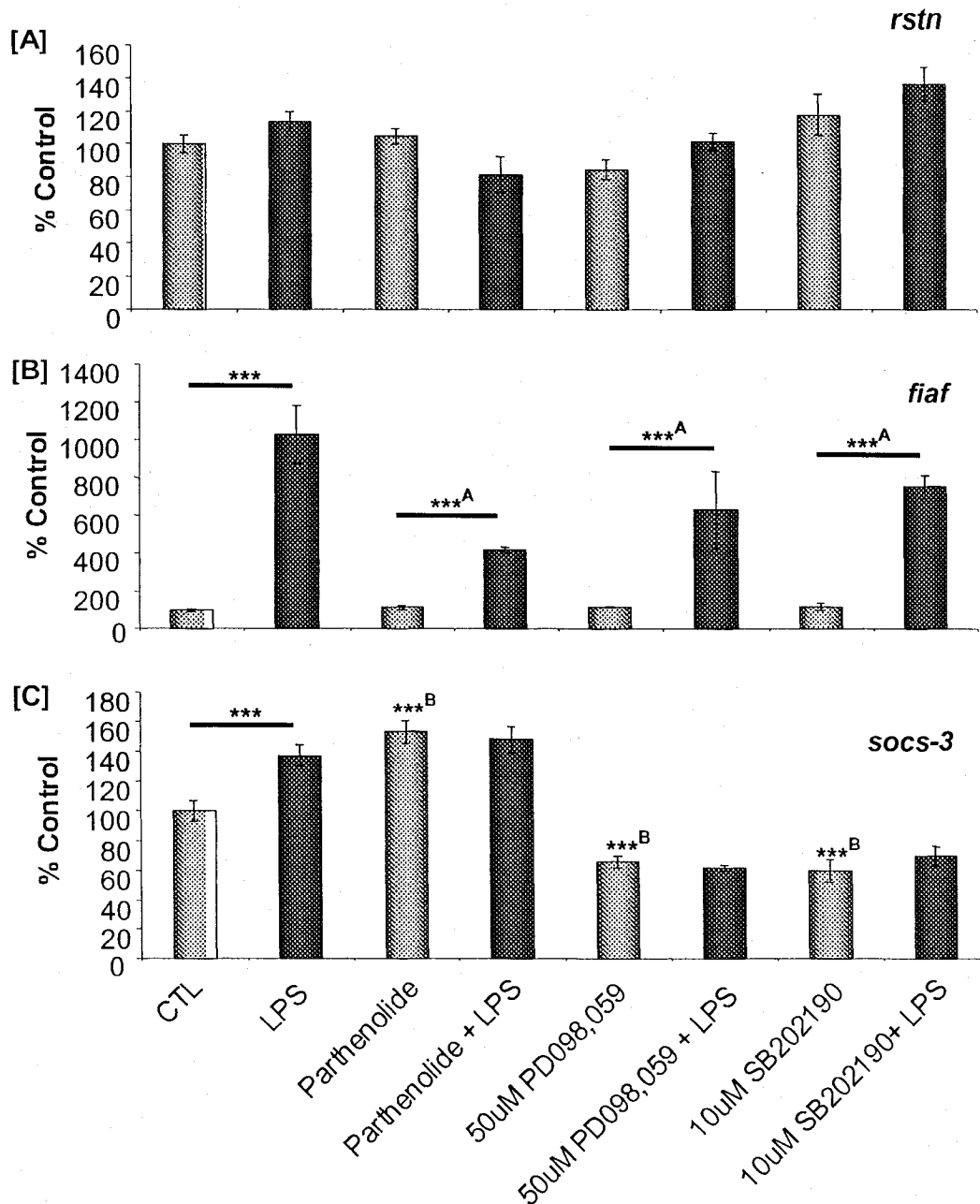
Figure 64: The effects of LPS, and various NFκB inhibitors, on gene expression in N-1 neurons N-1 cells were pretreated (30min) with various NFκB signaling antagonists followed by a 3h exposure to LPS (25μg/mL). [A] *Rstn* gene expression was unchanged by LPS or the NFκB signaling antagonists. [B] LPS robustly increased *fiaf* expression (10-fold), and this was only partially attenuated by the NFκB inhibitors. [C] But LPS induced a modest increase in *socs-3* expression (+40%), treating N-1 cells with the signaling antagonists alone also had significant effects; parthenolide induced *socs-3* (+50%) whereas PD098,059 and SB202190 inhibited its expression (-35%). Data were pooled from multiple experiments and are expressed as a percentage of the control ± SEM (n=7-8). (***p<0.001, ***A p<0.001 relative to LPS-treated cells, ***B p<0.001 relative to untreated cells)

N-1 cells with LPS increased *socs-3* expression (+37%, p<0.001), this was much less drastic than the changes measured in vivo (Fig 64C vs Fig 60C). In addition, pretreating cells with the various signaling inhibitors fully attenuated the stimulatory effects of LPS on *socs-3* expression. However treating the cells alone with the inhibitors also induced significant changes in *socs-3* expression; parthenolide stimulated *socs-3* by 53% (p<0.001), whereas PD98,059 and SB202190 both reduced *socs-3* by 40% (p<0.001) relative to unstimulated cells.

IV. Discussion

These studies clearly show that LPS-induced endotoxinemia results in time- and tissue-dependent increases in *rstn*, *fiaf* and *socs-3* gene expression. Moreover mice lost significant amounts of bodyweight 24h following the injection of LPS, whereas mice injected with saline maintained a stable body mass. Although LPS rapidly induced *rstn* gene expression in the HYP and PIT, *rstn* mRNA was decreased and then increased in the cortex at different time points, and *rstn* remained unchanged in adipose tissue. LPS rapidly induced *fiaf* expression in all tissues analysed, but had returned to normal levels by 24h in the HYP and PIT. In contrast *fiaf* mRNA remained maximally elevated in CTX and FAT at 24h. *Socs-3* gene expression was also increased by LPS treatment, with the greatest increases detected 3h postinjection. Moreover, like *rstn*, *socs-3* mRNA was first attenuated in the HYP, followed by the PIT, but remained significantly elevated in the CTX and FAT even 24h after the LPS challenge. In contrast to the results in vivo, LPS failed to significantly induce *rstn* gene expression in N-1 cells. However LPS treatment modestly increased the expression of *socs-3* in N-1 neurons, but induced a robust increase in *fiaf* mRNA (10-fold) in the same cells. Moreover treating cells with TLR4 signaling antagonists partially attenuated the LPS-induced increases in *fiaf*, suggesting that multiple signaling pathways are responsible for inducing its expression. In contrast

these drugs completely block the stimulatory effects of LPS on *socs-3*. However, as seen in Figure 64B, the drugs alone had significant effects on *socs-3* expression in N-1 cells relative to the control-treated cells. Taken together these data are consistent with my hypothesis that an LPS challenge would modify the expression of centrally-derived adipokines, and *socs-3*, target genes that mighty impact central metabolic pathways that are normally involved in the regulation of appetite, energy expenditure, and glucose homeostasis.

As expected, mice challenged with LPS lost a significant amount of weight 24h post-injection. It has previously been shown that the cachectic effects of LPS are dependent on an intact hypothalamic melanocortin system, but it is unclear how LPS is modulating the activity of this neuronal pathway. Although several adipokines are now implicated in the regulation of hypothalamic energy homeostasis (Ahima et al. 2006), their individual roles in inflammatory-induced anorexia remains uncertain. For example increases in leptin expression, a proinflammatory adipokine, is thought to mediate some of the anorectic responses to LPS (Sarraf et al. 1997). However leptin-deficient mice were not immune, nor protected, from the anorectic effects of LPS (Faggioni et al. 1997). Likewise LPS increases the transport of leptin and insulin across the BBB (Banks 2006a), and this transport reaches maximal levels between 16 and 24h after LPS administration (Xaio et al. 2001). However by 24h mice had already lost a significant amount of bodyweight, suggesting that other mechanisms are responsible for the rapid weight loss. Currently there appear to be no studies investigating the anorectic effects of LPS in *rstn* or *fiaf* knockout mice. Inflammatory-induced systemic insulin resistance might even act as a protective mechanism by blocking peripheral tissues from using the fuel sources (i.e. glucose) that are required for the proper functioning of the CNS (Lazar 2005). Perhaps redundant mechanisms have evolved to preserve the cachectic and

diabetic response induced by bacterial or viral infection, thus disrupting a single gene might not necessarily attenuate the anorectic response to inflammatory cytokines.

Could the increased expression of hypothalamic *rstn* contribute to the LPS-induced weight loss? The changes in hypothalamic *rstn* expression are particularly intriguing since resistin-ir co localizes with the melanocortin system (Wilkinson et al. 2005), a key target implicated in the LPS-induced metabolic disturbances (Marks et al. 2003). Additionally the icv injection of resistin has been shown to inhibit food intake (Tovar et al. 2005), and increase gluconeogenesis (Singhal et al. 2006), in a similar manner to what occurs following an LPS challenge. Although *rstn* is of relatively low abundance in the hypothalamus and in N-1 hypothalamic neurons, this alone should not be equated to lack of function nor preclude it from being able to modify central metabolic pathways (Wilkinson et al. 2007). As demonstrated in Chapter 3, *socs-3* and *fiaf* expression appear to be significantly inhibited by endogenous *rstn* in N-1 cells, despite its relatively low level of expression in this novel cell line model. Moreover the detected changes in N-1 cells are of similar magnitude to those reported to have a profound impact on appetite regulation in mice. For example diet-induced obesity (DIO) increases *socs-3* by 50% in the arcuate nucleus (Munzberg et al. 2004), whereas *socs-3* haploinsufficiency (i.e. 50% reduction in *socs-3*) is protective against DIO (Howard et al. 2004). Similarly hypothalamic AMPK, a novel heterotrimeric cellular energy sensor, is implicated in regulation of appetite and hepatic gluconeogenesis (McCrimmon et al. 2004; Minokoshi et al. 2004; Kahn et al. 2005; McCrimmon et al. 2006). Endogenous *rstn* was also capable of increasing AMPK activation in N-1 neurons, further implicating that it is involved in central energy metabolism (see Chapter 3). Thus along with the novel studies presented in Chapter 3, these data suggest that hypothalamic *rstn* might inhibit appetite, or modify hepatic gluconeogenesis, though this requires confirmation in

vivo. A major limitation to the N-1 cell line is that many of these hypothalamic controlled processes are dependent on changes in specific hypothalamic regions. For example DIO only increased *socs-3* in the ARC (Munzberg et al. 2004), whereas AMPK had to be activated in the ventral medial hypothalamus (VMH) in order to increase hepatic glucose production (McCrimmon et al. 2004). To further elaborate on this potential hypothalamic resistin system the present studies took advantage of the fact that LPS treatment reduces appetite and increases peripheral gluconeogenesis in mice. Thus if hypothalamic-derived resistin were involved in inflammatory induced cachexia then I would expect LPS to induce its expression in the hypothalamus, which proved to be the case. Experimentation is now needed to confirm such a link in vivo. Having established the ideal conditions for RNAi knockdown of *rstn* in vitro (Chapter 3), and having successfully applied this technology in vivo (see section VIII of Chapter 2), it should now be possible to use RNAi to block the LPS-induced increase in hypothalamic *rstn* in vivo and to monitor the effects on appetite, bodyweight and gene expression in all tissues to verify whether hypothalamic-derived *rstn* is contributing to the anorectic responses that occur during inflammation.

LPS induces the expression, and release, of an array of inflammatory cytokines via NFκB- and MAPK-dependant pathways. Promoter analysis revealed the presence of NFκB response elements within the mouse resistin gene, and LPS induced *rstn* gene expression in white adipose tissue of male Wistar rats, in a dose- and time-dependant manner (Lu et al. 2002). LPS unexpectedly failed to increase *rstn* gene expression in visceral adipose tissue of CD-1 mice. Though these studies do not preclude the possibility of increased resistin secretion, this discrepancy might be explained by our use of different time points, or species, than those used by Lu et al. (2002; 12h, rat). Nevertheless, and unlike adipose tissue, LPS rapidly stimulated *rstn* gene expression in

mouse hypothalamus and pituitary 3h post injection. On the other hand, LPS failed to induce *rstn* gene expression when added directly to N-1 hypothalamic neurons. Moreover pre-treating N-1 hypothalamic neurons with various TLR4 signaling antagonists alone had no significant effect on basal *rstn* gene expression, excluding the possibility that the TLR4 signaling pathways are constitutively or maximally activated in N-1 neurons. This lack of response could be accounted for if (a) N-1 neurons are not representative of the in vivo hypothalamic target cells for LPS or (b) LPS stimulates the local expression and release of other cytokines at the blood brain barrier that indirectly induce hypothalamic *rstn* gene expression in vivo (Rivest et al. 2000; Banks 2006a), much like the indirect effects of LPS on *rstn* in human blood mononuclear cells (Kaser et al. 2003). LPS has been shown to induce the expression of CEBPs in the CNS (Saito et al. 1999), and as seen in Chapter 4, CEBPα is capable of modulating *rstn, fiaf* and *socs-3* gene expression in N-1 neurons. However the rapid increase in hypothalamic *rstn* that was induced by LPS suggests that a more direct stimulus exists.

The response of the cerebral cortex to LPS was different to that in the hypothalamus or pituitary since LPS induced delayed fluctuations in cortical *rstn*. At 6h it was significantly reduced, whereas at 24h it was elevated. This shift in cortical *rstn* mRNA might be the a consequence of altered BBB integrity that occurs between 16-18h following LPS administration (Xaio et al. 2001), thus exposing the cortex to novel circulating molecules that could also be modifying *rstn* expression following an LPS challenge. The tissue-dependent changes in *rstn* gene expression that occur following LPS administration suggest that it is modulated by indirect pathways that might be dependent on local tissue integrity and signaling events.

The changes in pituitary gene expression appeared very similar to those detected in the hypothalamus (Figure 62 vs. Figure 60). For example *rstn* gene expression was increased only 3h after the injection of LPS in both tissues. Similarly the

increases in *fiaf* and *socs-3* were no longer detectable 24h after the injection of LPS, unlike the cortex and adipose tissue, although the attenuation of pituitary expression was a little slower to occur than in the hypothalamus. In previous studies pituitary *rstn* gene expression was shown to be dependent on a) leptin since pituitary *rstn* mRNA was lower in ob/ob mice and b) the hypothalamus, since a hypothalamic lesion induced by neonatal MSG treatment drastically reduced its expression (Morash et al. 2002; Morash et al. 2004). There are several possible hypothalamic signals that could be impacting pituitary adipokine genes. Could the changes in hypothalamic adipokine gene expression be driving the expression of pituitary adipokines, as part of a feedforward system, since the measured increases in the hypothalamus appeared to precede those measured in the pituitary? However other peripheral signals might also induce the expression of pituitary adipokines. For example treating AtT 20 corticotrophs with glucocorticoids, or injecting adrenalectomized mice with dexamethasone, induced pituitary *rstn* gene expression (Brown et al. 2005b). Since LPS is also capable of eliciting a stress response (Mazzocchi et al. 1995), it might also be responsible for inducing the expression of pituitary adipokines.

FIAF is thought to modulate lipid metabolism through the inhibition of lipoprotein lipase activity (LPL), although it has also been implicated in the control of body composition and glucose metabolism (Kersten 2005; Xu et al. 2005; Backhed et al. 2007). Although the role(s) of FIAF in the central nervous system remain largely unexplored, we previously speculated that it may participate in local inflammatory responses, tissue repair and angiogenesis (see Chapter 6)(Wiesner et al. 2006). LPS induced the expression of *fiaf* in all tissues analysed, although these increases were quickly attenuated in the hypothalamus and pituitary. Furthermore the studies in N-1 cells suggest that LPS might directly stimulate hypothalamic *fiaf* expression. Pretreating N-1 neurons with TLR4 signaling antagonists partially attenuated *fiaf* expression,

suggesting that these increases are at least partially-dependent on NFκB and MAPK signaling pathways. Although the LPS-mediated increases in *fiaf* were relatively larger in vitro than those measured in vivo (10-fold vs. 2-fold), this might reflect cell-specific variations between the various hypothalamic neuronal populations or possible feedback signals that might limit the LPS-mediated stimulation of hypothalamic *fiaf* gene expression in vivo. Similarly the sustained increase in *fiaf* gene expression that was detected in visceral fat might also be a secondary consequence of the LPS-induced anorexia since fasting is known to induce *fiaf* expression in adipose tissue (Kersten et al. 2000; Wiesner et al. 2004). These data suggest that *fiaf* might be yet another proinflammatory adipokine since its expression is stimulated by LPS, but inhibited by the anti-inflammatory compound parthenolide, though this requires further investigation.

The time and tissue-dependent patterns detected in adipokine and *socs-3* expression might be the consequence of a transition between a prediabetic and diabetic state. For example most of the LPS-induced changes in hypothalamic gene expression occurred by 3h, whereas these effects lingered slightly longer in the PIT until 6h, but even after 24h adipokine gene expression remained significantly elevated in the cortex and adipose tissue. Although LPS modestly induced *socs-3* expression in N-1 neurons, it seems likely that LPS indirectly stimulates *socs-3* expression in vivo since the detected increases were substantially larger (e.g. 40% vs 400%). In addition treating N-1 cells alone with parthenolide induced *socs-3* expression, suggesting it might mediate some of the anti-inflammatory effects associated with this compound. In contrast *socs-3* expression was inhibited by the MAP kinase inhibitors, PD 98,059 or SB 202190, suggesting MAPK signaling pathways are atleast partially activated even under basal conditions in N-1 neurons. The delayed responses in *socs-3* expression are also intriguing since LPS-induced endotoxins were reported to peak 6h after the peripheral injection of LPS in the rat, and were undetectable 24h following the LPS challenge

(Abdulla et al. 2005), further suggesting *socs-3* expression is induced indirectly. Although mRNA stability could vary between tissues, it seems likely that there are other stimulatory signals driving target gene expression in the "downstream" tissues, particularly fat. For example changes in hypothalamic gene expression might activate a feedforward mechanism that induces a cascade of gene expression changes whose effects linger in these downstream target tissues over prolonged periods. The sustained increases of *socs-3* mRNA in adipose tissue is indicative of a state of peripheral insulin resistance (Ueki et al. 2004). These data are consistent with a preexisting model that involves neurochemical changes, such as increased expression of hypothalamic-derived adipokines, which invoke the transition from a prediabetic to an insulin resistant state in peripheral tissues (i.e. fat; see Figure 70)(Buren and Eriksson 2005).

In summary LPS induced significant reductions in bodyweight and stimulated *rstn*, *fiaf* and *socs-3* gene expression in a tissue- and time-dependent manner. The detected increases in hypothalamic *rstn* gene expression further suggest its possible involvement in central energy metabolism and inflammatory-associated cachexia, and this can now be resolved by RNAi studies. Similarly LPS increased the expression of *fiaf*, which might be involved in the local tissue injury and inflammatory responses. Likewise LPS may induce systemic insulin resistance by stimulating tissue-dependent changes in adipokine gene expression which are capable of inducing a shift from a prediabetic, to an insulin resistant, state. In total these data appear to be consistent with my hypothesis that centrally-derived adipokines modulate central energy metabolism and insulin sensitivity.

Chapter 6: The Effects Of Brain Damage On Central Adipokines: Implications For Cachexia Or Brain Repair

I. Introduction

Although brain injury has long lasting effects on cognition and motor function, it can also induce metabolic changes that are deleterious to patient recovery and survival. For example traumatic brain injury (TBI) reduces appetite and increases energy expenditure, thus depleting critical energy reserves which worsens the final outcome for patients (Thompson et al. 2003; McIlvoy 2005; Moinard et al. 2005). Similar metabolic consequences were also observed in the rat brain following traumatic brain injury (Thompson et al. 2003; Otori et al. 2004; Moinard et al. 2005; Morales et al. 2005). Likewise hypoxic-ischemic injury of the neonatal rodent CNS delayed somatic development, induced hyperactivity and had long-lasting effects on weight gain (Balduini et al. 2001; Fan et al. 2005; Lubics et al. 2005). Thus in addition to impaired motor and cognitive function, different types of brain injury appear to modify metabolism and bodyweight regulation. The modulation of cellular energy metabolism is particularly important following brain damage since it can modify the balance between survival and apoptosis, which can have a substantial impact on future brain function (Ramamurthy and Ronnett 2006). For example TBI preconditioning is thought to increase glycogen stores as part of a neuroprotective mechanism (Otori et al. 2004). Likewise drugs such as rosiglitazone are postulated to modify local cellular glucose metabolism in order to improve brain cell survival (Sundararajan et al. 2006). In addition brain injury has been shown to increase the expression of adipogenic transcription factors, such as CEBPα and PPARγ, in the CNS which in turn might regulate central adipokine expression (Walton et al. 1998; Victor et al. 2006). **Having established in previous chapters (2 and 3) that brain-derived adipokines impact cell survival, signaling and energy**

metabolism, I hypothesized that brain injury would also modify the expression of brain-derived adipokines.

Common Types Of Brain Injury

Hypoxic/ischemic encephalopathy (HIE) is a leading cause of neonatal death, is implicated in the development of cerebral palsy and epilepsy, and induces neurological impairment that negatively impacts learning and memory depending on the severity (Rice et al. 1981; Ambalavanan et al. 2006; Perlman 2006). The prenatal/neonatal brain, particularly the cortex, hippocampus and striatum, are especially susceptible to HIE damage (Rice et al. 1981). However the patterns of injury are reported to vary between different strains of mice and rats (Oliff et al. 1995a; Sheldon et al. 1998). Most damage is thought to arise from a shift to anaerobic metabolism which induces a cascade of neurotoxic events, including a potent inflammatory response that is associated with the delayed secondary injury (Perlman 2006). Since anoxia alone was unable to induce neonatal brain damage in rodents, this led to the development of a more effective hypoxia/ischemia (H/I) model that involves occluding the common carotid artery (CCA) followed by a brief exposure to a temperature controlled hypoxic environment (Rice et al. 1981). Although neonatal H/I damage has long-lasting effects in rodents, such as decreased weight gain, it rapidly induces hyperthermia and impairs energy metabolism (Balduini et al. 2001; Fan et al. 2005; Lubics et al. 2005). In addition H/I injury delays somatic development in rat pups and has prolonged effects on learning and memory (Fan et al. 2005). Although animals damaged by neonatal cerebral H/I become hyperactive, their motor function and coordination is also compromised (Fan et al. 2005; Lubics et al. 2005). Various approaches have been tested to treat HIE, although mildly cooling the CNS and controlling blood glucose are currently some of the more effective treatments for offering neuroprotection; better therapies are still being sought (Perlman 2006). Thus understanding what genes are activated in the CNS following H/I, and their

effects on local cellular energy metabolism, might yield suitable therapeutic targets that can be exploited in order to limit further neuronal damage that occurs following reperfusion.

Traumatic brain injury (TBI) has been described as a silent epidemic that is responsible for 50,000 deaths per year in the USA alone, and causes significant disabilities in a further 70,000-90,000 people (Langlois et al. 2004; Morales et al. 2005). In addition to mechanical damage, TBI results in a subsequent hypoxic/ischemic event that leads to further memory loss, reduces motor function and is the primary cause of injury-related death in children (Prins and Hovda 2003). Limiting this delayed injury by preventing excessive brain swelling, reducing hypoxic/ischemic conditions and lowering hyperglycemia could drastically reduce mortality as well as improve the outcome for those that survive (Johan Groeneveld et al. 2002; Dash et al. 2004; Morales et al. 2005). Just following injury TBI patients become hypophagic and cachectic, and the subsequent increases in energy expenditure further compound the underlying brain injury (Thompson et al. 2003; Suz et al. 2006). At present there are no therapeutic interventions capable of counteracting or repairing TBI-induced brain damage despite extensive research. Most studies have focused on a variety of pharmacological strategies including, for example, glutamate antagonists, endocannabinoids and free radical scavengers (Royo et al. 2003). Microarray analysis revealed that TBI increases expression of several genes that are implicated in cell cycle, metabolism, inflammation and signaling to name a few (Matzilevich et al. 2002; Rall et al. 2003; Dash et al. 2004), though their individual roles in the pathogenesis of TBI and their suitability as therapeutic targets remains to be established.

Various rodent experimental models of TBI have been developed. Although none are totally capable of recapitulating the full spectrum of features observed clinically, they do reproduce many of the symptoms associated with neurotrauma including decreased

appetite and hyperthermia (Thompson et al. 2003; Morales et al. 2005). The lateral fluid

percussion (FP) model of brain injury, as used in my studies, involves applying a

unilateral force to mechanically damage the brain, although a bilateral diffuse injury also

occurs (see Figure 65). TBI also disrupts the cortical blood brain barrier, as detected by

the spread of Evans blue dye, which is involved in the induction of post-traumatic edema

(McIntosh et al. 1989; Nimmo et al. 2004). This model of neurotrauma involves striking

the dural surface of the brain using an isotonic saline bolus that is rapidly injected into

the skull using a piston that is connected to a pendulum (see Figure 65) (McIntosh et al.

1989; Morales et al. 2005). The resulting brain injury is considered to be more clinically

relevant than other experimental models of neurotrauma since it results in more cortical

damage and has less of an effect on the brain stem (McIntosh et al. 1989). In addition

the FP model is capable of inducing damage of graded severity by either increasing or

decreasing the pressure applied to the piston (McIntosh et al. 1989). Thus this model of

neurotrauma is highly suited to inducing a clinically relevant brain injury in the rodent

CNS in order to study the effects on the expression of centrally-derived adipokines.

Inflammation And Adipokine Expression

A further critical consequence of brain injury is a neuroinflammatory response. As

noted in the previous chapter, adipokines are implicated in obesity-related inflammation

(Rajala and Scherer 2003; Trayhurn and Wood 2005). White adipose tissue

vascularization is relatively poor under the best of conditions, but blood flow appears to

be further decreased in obese individuals which further exacerbates local hypoxic

conditions (Trayhurn and Wood 2005). Thus this hypoxic milieu that occurs in expanding

adipose tissue is thought to induce the expression of pro-inflammatory cytokines and

adipokines in obese individuals which appear to significantly impact the development of

obesity-related diseases such as type 2 diabetes (Trayhurn and Wood 2004; Juge-Aubry

et al. 2005; Trayhurn and Wood 2005). A key transcription factor implicated in this
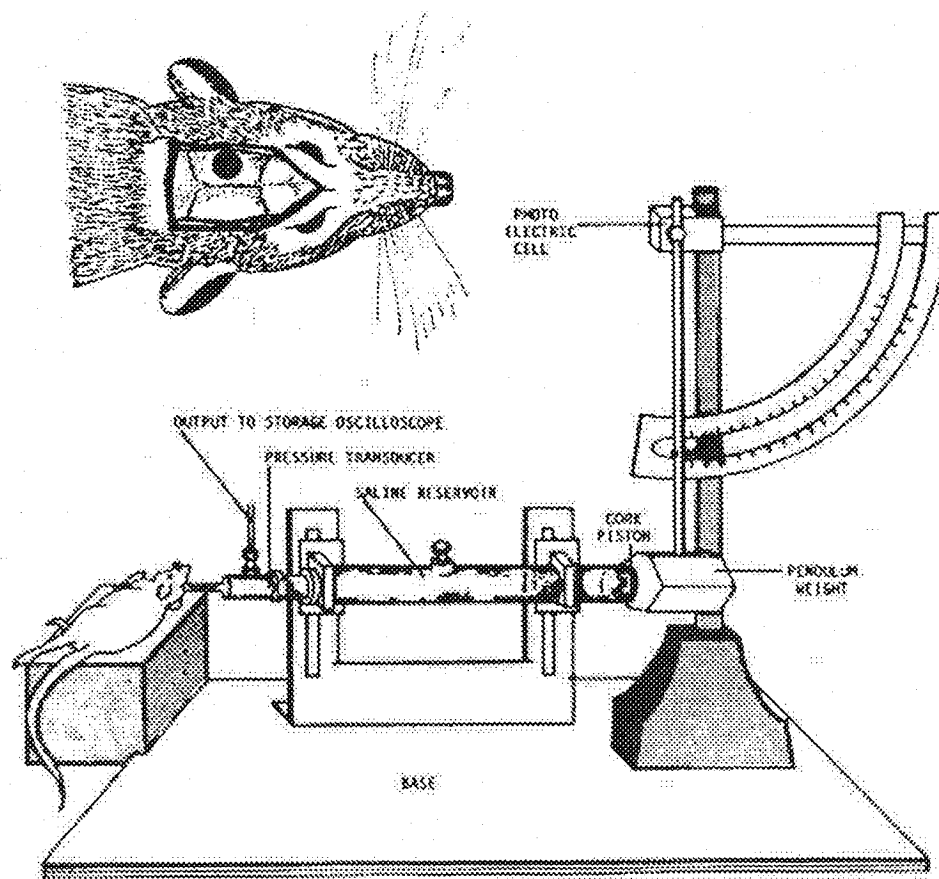
Figure 65: The lateral fluid percussion model of traumatic brain injury.
This model of brain injury involves exposing the rat skull, and following craniectomy, affixing a hollow female Leur-Loc over the parietal cortex using dental cement, as indicate by the grey dot on the rat skull above. Note that the dura mater remains intact. Subsequently a fluid percussion device, containing a male Leur-Loc, is filled with sterile isotonic saline that was prewarmed to 37°C. The Leur Locs are connected between the fluid percussion device, and to the rat, as seen in the cartoon. The affixed pendulum can then be raised to different heights in order to induce brain injury of graded severity. When the pendulum is released and the hammer strikes the cork piston on the fluid percussion device it generates a fluid bolus that induces an intracranial pressure pulse. (Figure adapted from McIntosh et al. 1989)

process is hypoxia-inducible factor-1 alpha (HIF-1α), since its expression and stability are increased by this low oxygen environment (Wang et al. 1995). Moreover HIF-1α appears to modify the expression of proangiogenic adipokines including vascular endothelial growth factor (VEGF) and leptin (Lolmede et al. 2003). Although the association of FIAF with obesity-related hypoxia and inflammation is more speculative, the evidence is suggestive. FIAF expression was induced in human endothelial cells (Kim et al. 2000), human and rat cardiomyocytes (Belanger et al. 2002), and human glioblastoma cells (Lal et al. 2001) following gaseous or chemically-induced hypoxia. I hypothesized that the expression of brain-derived adipokines would also be induced by the hypoxic environment that results following neurotrauma.

Brain injury and neuroinflammation are capable of inducing the expression of several adipogenic transcription factors in the CNS that modulate adipokine gene expression. For example damaging rat brains by cerebral H/I resulted in the increased expression of CEBPα mRNA and protein that were detected 6h and 48h later, respectively (Walton et al. 1998). In addition this was associated with increased DNA binding of CEBPα, as detected by an electrophoretic mobility shift assay (EMSA) (Walton et al. 1998). Likewise, despite the relatively low abundance of PPARγ mRNA in the adult rat brain, its expression was rapidly induced by middle cerebral artery occlusion (MCAO), and remained significantly elevated for up to 14 days following injury (Victor et al. 2006). TBI also induced cortical DNA binding of NFκB for several days, and this could be further increased by the co-injection of LPS (Hang et al. 2004). Microarray analysis also detected increases in hypoxia-inducible factor-1 alpha (HIF-1α) expression following TBI (Dash et al. 2004). It seems likely that the increased expression of these adipogenic transcription factors in the CNS will also lead to the increased expression of brain-derived adipokines following injury.

Although the roles of resistin and FIAF in brain function and repair remain largely

unexplored at this time, leptin is clearly involved in brain growth and development. As previously discussed the leptin deficient (ob/ob) mouse has a smaller brain than its lean littermates (Steppan and Swick 1999), and this can partially be reversed by leptin treatment (Ahima et al. 1999). In addition neonatal leptin treatment stimulated the development of hypothalamic neuronal feeding pathways in ob/ob mice (Bouret et al. 2004b; Bouret et al. 2004a). Leptin is also neuroprotective and guarded brain cells from ibotenate-induced excitotoxicity in vitro and in vivo (Dicou et al. 2001). Likewise treating the human SH-SY5Y neuroblastoma cell line with leptin protected cells from apoptosis when cultured in serum free medium and these effects were blocked by inhibiting leptin receptor signaling pathways (Russo et al. 2004). However as described in Chapter 2 a 2-fold *increase* in cell death resulted when RNAi was used to *silence* endogenous leptin gene expression in rat C6 glioblastoma cells. Therefore brain injury might induce the expression of centrally-derived adipokines, most notably leptin, as part of a protective response. Although the expression of leptin has been detected in the brain of most species, the mouse is a notable exception (recall Table 1), therefore the effects of brain injury on leptin were not analysed in the mouse.

Brain Injury And Central Adipokine Expression

Following our demonstration that the brain is a source of several adipokines, including leptin (Morash et al. 1999), resistin (*rstn*) (Morash et al. 2002; Morash et al. 2004) and FIAF(*fiaf*) (Wiesner et al. 2004), we speculated that brain injury might increase their expression in the CNS. Although a specific role(s) for resistin and FIAF in brain function in vivo is presently uncertain, resistin was capable of modulating cell signaling and metabolism in the N-1 hypothalamic neuronal cell line (Chapter 3). In contrast leptin clearly possess neuroprotective properties in vitro and in vivo (Dicou et al. 2001; Russo et al. 2004). Thus central adipokines might be involved in angiogenesis and

offer some degree of neuroprotection, in addition to inducing metabolic changes that are associated with brain injury.

In the present studies I hypothesized that brain injury, and the associated inflammatory responses that occur, would modulate the expression of brain-derived adipokines in the mouse and rat CNS. This hypothesis was tested in two different systems: (1) a modified model of cerebral hypoxia/ischemia (H/I) in neonatal mice (Rice et al. 1981), and these experiments were carried out in collaboration with Dr. George Robertson and Nathalie Earl (Department of Pharmacology); and (2) inducing TBI in adult rats using the well characterized lateral fluid percussion (FP) model of brain injury (McIntosh et al. 1989), and these experiments were carried out in collaboration with Dr. Hilary Thompson and David Lebold (Deparment of Biobehavioral Nursing and Health Systems, University of Washington, Seattle, WA USA and Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA USA). Since H/I is known to induce long-lasting effects on weight gain and development (Balduini et al. 2001; Fan et al. 2005; Lubics et al. 2005), *rstn* and *fiaf* gene expression were analysed by realtime RT-PCR at 48h, 7days and 21days post injury in the mouse brain. In contrast TBI appears to rapidly modify temperature and bodyweight (Thompson et al. 2003; Otori et al. 2004), thus leptin, *rstn* and *fiaf* mRNA was analysed 12h post injury in the rat brain.

II. Materials And Methods

Neonatal Mouse Cerebral Hypoxia And Ischemia

The experimental protocol was reviewed and approved by the Dalhousie University Committee on Laboratory Animals. Mice (C57BL/6) were obtained as litters, with their dams, from Charles River Breeding Farms (Quebec, Canada), and maintained under a photoperiod of 12h light: 12h darkness and given free access to Purina Rat

Chow and drinking water. Male mouse pups (PD 8-10) were subjected to unilateral

common carotid artery (CCA) occlusion in combination with hypoxia to produce a

cerebral hypoxic/ischemic brain injury (Rice et al. 1981; Han and Holtzman 2000).

Briefly, under isoflurane anaesthesia, a small incision was made to expose the left CCA

which was then electro-coagulated and the wound closed by suture. After 2 hours of

recovery with dams, hypoxia was induced by placing pups in a warm (37°C), humidified

chamber and exposing them to 8% oxygen (nitrogen 92%; 60 min). Pups were returned

to their dams and their recovery monitored for 2 hours. At 2, 7 or 21 days post-H/I insult,

mice were killed by decapitation and samples of both ipsilateral and contralateral

cerebral cortex and hippocampus were dissected and frozen in liquid nitrogen and

stored at -70°C until RNA isolation. Control animals were sham-treated with a small neck

incision without CCA occlusion or hypoxia.

Fluid Percussion Model Of Traumatic Brain Injury In The Rat

All procedures were approved by the University of Pennsylvania's Animal Use

and Care Committee and conformed to standards set by the National Institute of Health

Guide for the Care and Use of Laboratory Animals (1996). Following anesthesia with

sodium pentobarbital (65mg/kg), adult male Sprague-Dawley rats (350-400 g, Harlan,

Indianapolis, In) underwent craniectomy and were subjected to lateral FP brain injury of

moderate severity (2.92-3.17 atmospheres) as previously described in detail (See Figure

65) (McIntosh et al. 1989). Normothermia was maintained throughout all procedures and

for 120 minutes after lateral FP injury. Animals were allowed to recover and returned to

home cages. Sham-injured rats were anesthetized and surgically prepared in the exact

same manner but did not receive FP brain injury. Animals were sacrificed 12h post-

injury, and following decapitation, brains were rapidly removed and a 3 mm coronal

section was cut from the occipital-parietal level, which included the injury site over the

left parietal cortex. First, the different brain regions were dissected out on the ipsilateral

side (side of injury) on a cold glass plate in the following order: (1) hippocampus, (2) cortex, and (3) thalamus, followed by the contralateral sites. Sections were immediately placed in RNAlater® (Ambion, Austin, TX) and stored at room temperature before shipment to Halifax, NS, for RNA isolation.

RNA Isolation And Realtime RT-PCR

For all brain tissues total RNA was isolated using the RNEasy mini kit following the DNAse protocol (Qiagen) as outlined in detail in section VII of Chapter 2. Total RNA (2 µg) was reverse transcribed in a total volume of 40µL and PCR amplified using SuperScript™ III RT enzyme master mix (Invitrogen) according to the manufacturer's protocol. For PCR amplification samples were amplified in triplicate and only 1 gene was analysed per reaction. Reactions were prepared using Platinum® quantitative PCR SuperMix-UDG following the supplied guidelines. They consisted of: 17.5 uL of the 2 X Platinum® Quantitative PCR SuperMix-UDG (Invitrogen), 7 pmol of forward and reverse primers for the relevant mouse or rat genes (See Table 2), 1 pmol of dual-labeled probe, 2-3µL of cDNA in a total volume of 35 µL using Platinum® quantitative PCR SuperMix-UDG, and contained 3µL cDNA, and were amplified according to the manufacturer's protocol. In brief samples were incubated for 10min at 95°C, followed by 60 amplification cycles of 95°C for 20s and 60°C for 60s using a BioRad thermal cycler and an iQ realtime PCR detection system. A standard curve, that was prepared using a serial dilution of a reference sample, was included in each realtime run to correct for possible variations in product amplification. Relative copy numbers were obtained from standard curve values, and were normalized to the values obtained for our house keeping gene, cyclophilin. Data are expressed as a percentage of the control +/-SEM. It should be noted that no significant variations in our house keeping gene were observed between groups when evaluated using either the threshold cycle (C_T), or using the relative levels of expression, and is consistent with previous reports (Bond et al. 2002).

Statistics

For all studies data were analyzed using Student's *t-test*. In the H/I experiments changes were being compared between the ipsilateral and contralateral brain tissues. Similarly for the TBI experiments the ipsilateral and contralateral brain tissues were analysed independently, thus data were only compared between the sham and TBI groups. Significance was set at $p< 0.05$.

III. Results

The Effects Of H/I On Rstn And Fiaf Expression In The Mouse Brain

As expected gross inspection of the dissected brains from mice exposed to unilateral H/I revealed clear damage to the ipsilateral hippocampus and cerebral cortex and is consistent with a previous report (Han and Holtzman 2000). *Fiaf* expression in the mouse ipsilateral cortex and hippocampus was increased compared to contralateral sites at 2 days post-H/I (1.4 fold; $p<0.05$; and 1.9 fold; $p<0.001$ respectively), and at 7 days (1.7 fold; $p<0.01$, and 1.8 fold; $p<0.01$, respectively), but returned to basal levels by 21 days (Fig 66A). It should be noted that *fiaf* mRNA in contralateral brain tissues of H/I animals did not differ significantly from the ipsilateral tissue of sham-operated animals (data not shown), suggesting that the increases in *fiaf* are localised to the site of H/I injury.

In marked contrast to *fiaf* mRNA, *rstn* expression was unaffected by the H/I injury in mice for the seven days following neonatal brain injury. However *rstn* mRNA was significantly increased in the ipsilateral cortex (1.8 fold; $p<0.001$) and hippocampus (1.4 fold; non significant; Fig 66B) 21 days post H/I, coincident with the return of *fiaf* expression to control levels.

The Effects Of TBI On Adipokine Expression In The Rat Brain

Twelve hours following brain injury, leptin mRNA was markedly increased in the ipsilateral cerebral cortex and thalamus (Fig 67A and 67B; 2.5-fold, $p<0.001$ and 2–fold,
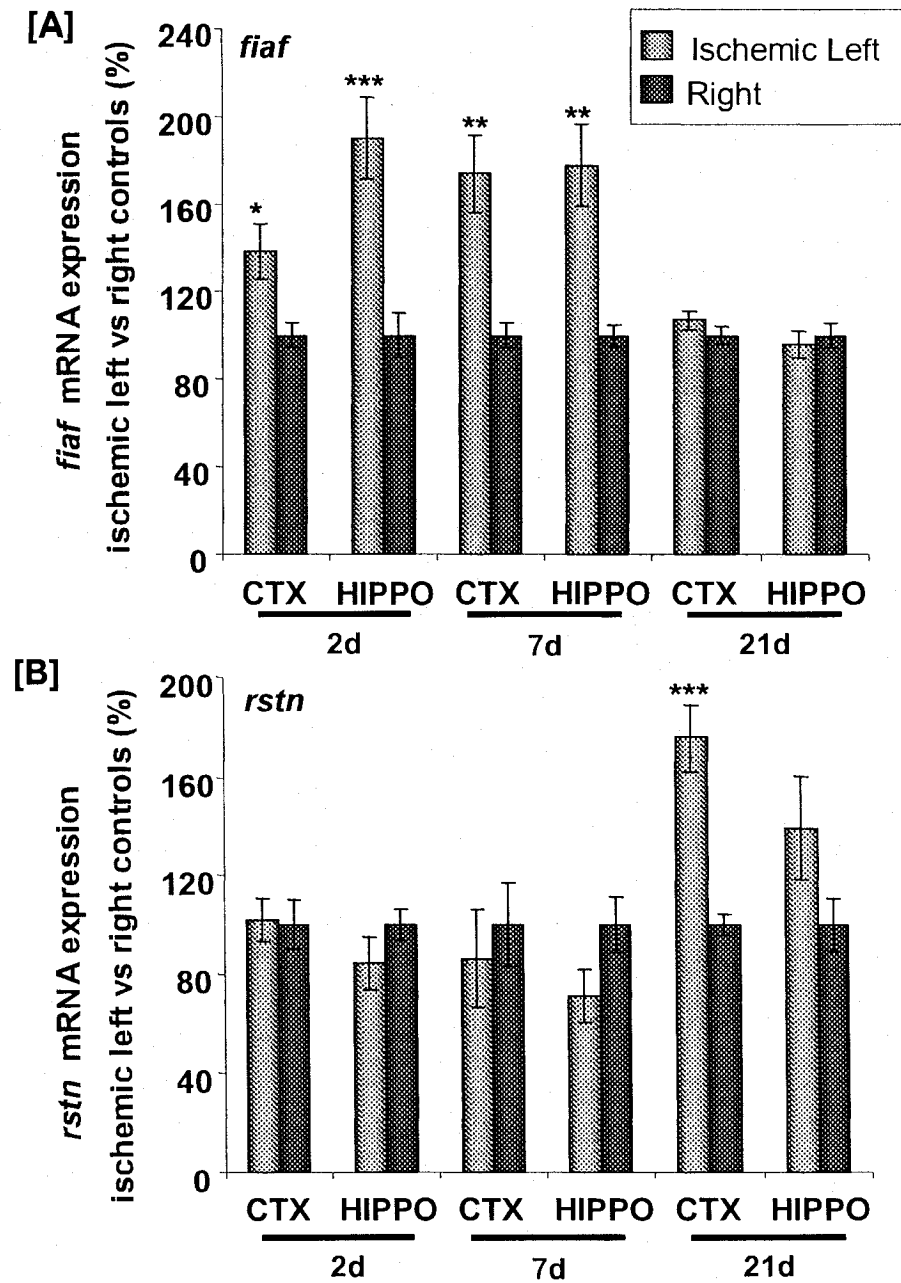
Figure 66: The effects of cerebral H/I on neonatal mouse brain adipokine gene expression. Neonatal mice were subjected to unilateral cerebral hypoxic/ischemic brain injury and gene expression was analysed in the parietal cortex (CTX) and hippocampus (HIPPO) using realtime RT-PCR at 2, 7 and 21 days following damage. [A] *Fiaf* gene expression was induced in the CTX and HIPPO 2 days after H/I, and remained elevated for up to 7 days before returning to basal levels after 21 days. [B] In contrast *rstn* mRNA was unchanged during the first 7 days, but was significantly increased in the CTX 21 days post-H/I. Values are expressed as percentage control ± SEM (n=7-10). (*p < 0.05, **p < 0.01, ***p < 0.001)
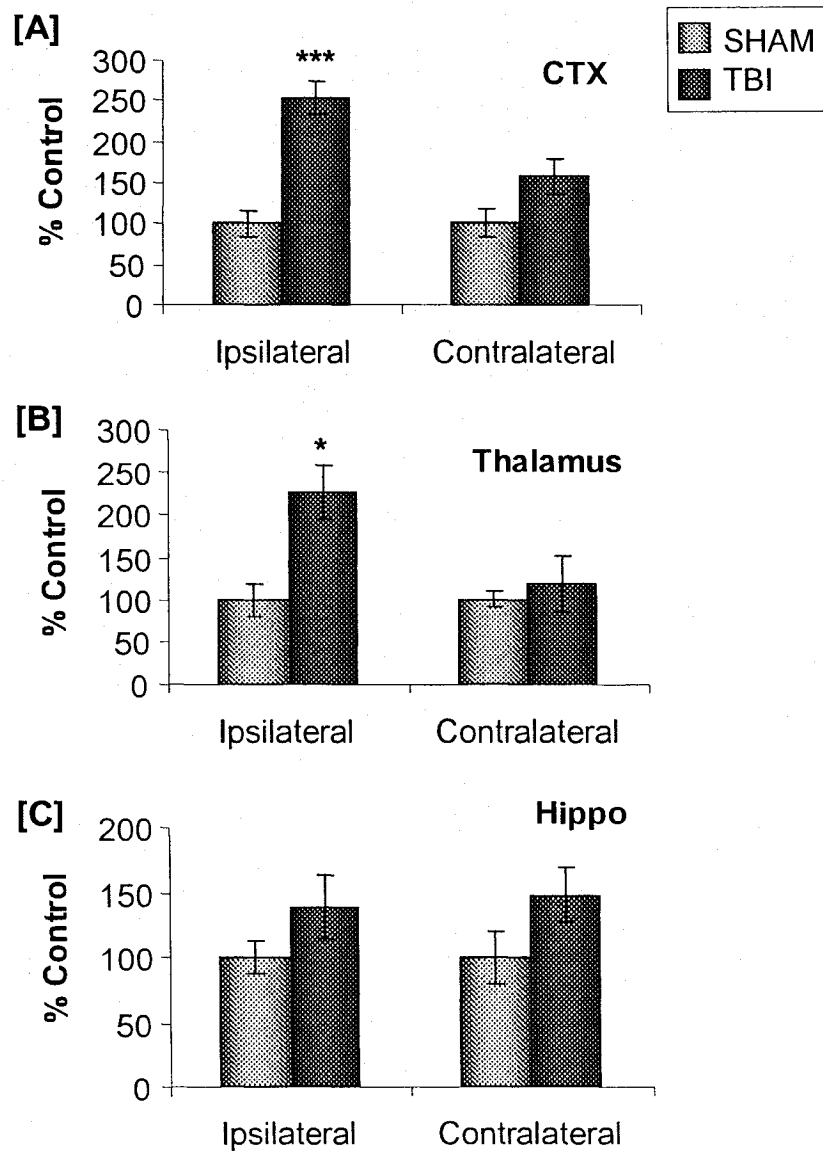
Figure 67: The effects of TBI on leptin gene expression in the rat brain. Significant increases in leptin (*ob*) gene expression were detected in the ipsilateral cortex (2.5-fold) and thalamus (2-fold) 12h following lateral FP-induced brain injury using realtime RT-PCR [A and B]. In contrast *ob* gene expression was unaffected in the hippocampus (HIPPO), or in contralateral brain tissues. Values are expressed as a percentage of the control ± SEM obtained from duplicate experiments (N= 6-8). (*p<0.05, ***p<0.001)
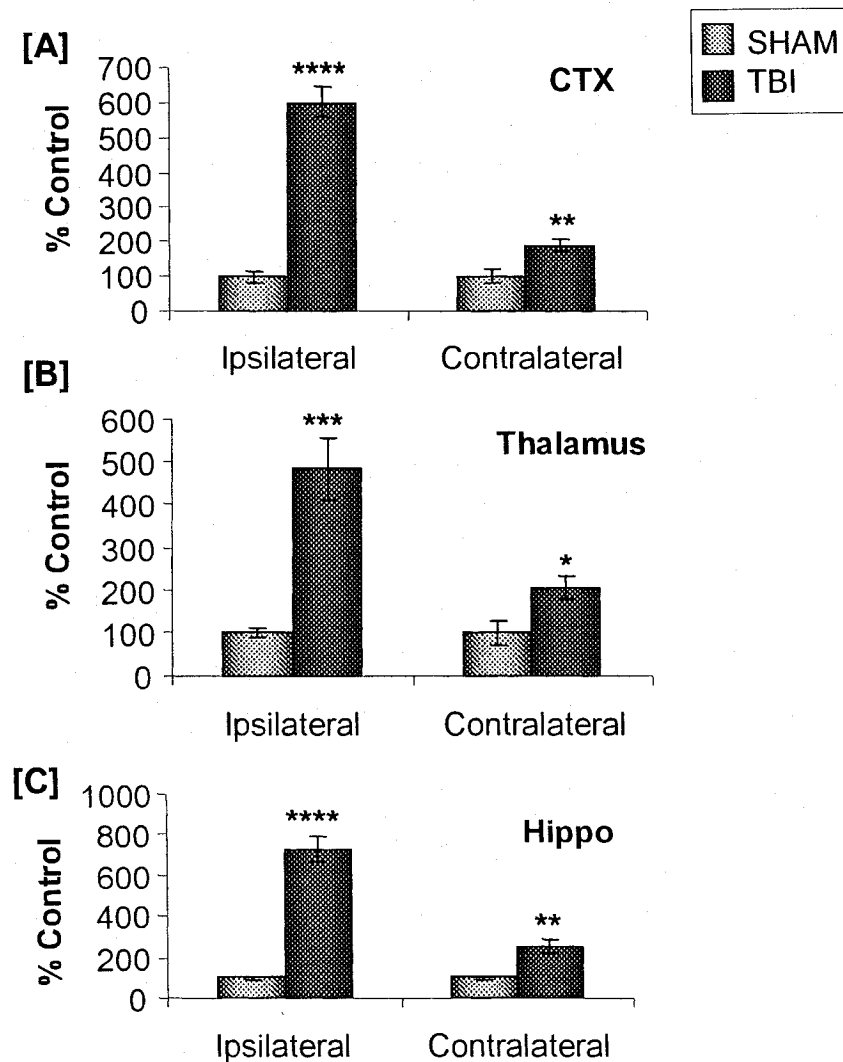
Figure 68: The effects of TBI on *fiaf* gene expression in the rat CNS. Realtime RT-PCR detected significant increases in *fiaf* mRNA in the ipsilateral cortex (6-fold), thalamus (5-fold) and hippocampus (7.5-fold) 12h following TBI. Although *fiaf* expression was significantly upregulated in the contralateral tissues, the increases were not as robust (Cortex; 89%, thalamus; 103%, hippocampus; 150%). Values are expressed as a percentage of the control ± SEM obtained from duplicate experiments (N= 6-8). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)
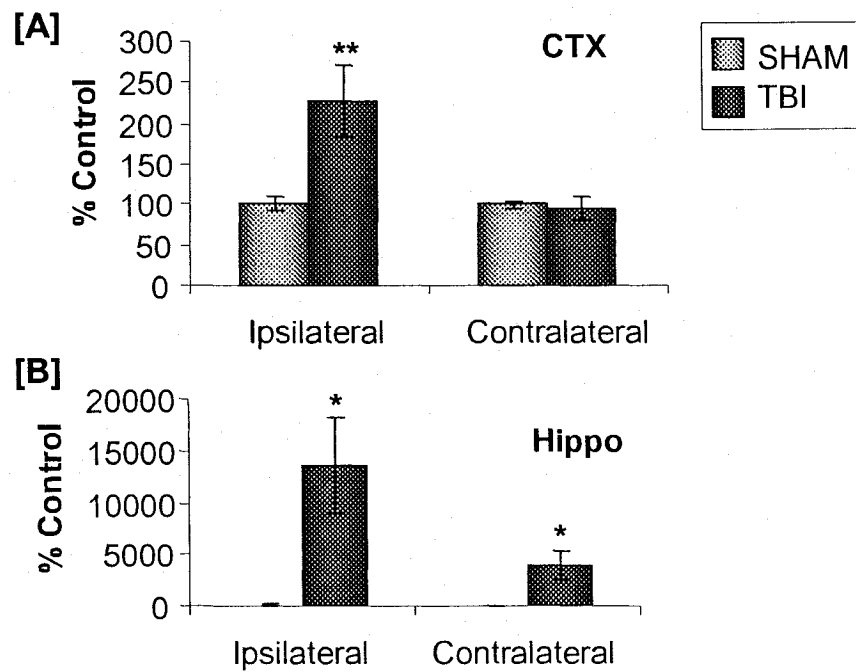
Figure 69: The effects of TBI on *rstn* gene expression in the rat brain. TBI increased *rstn* mRNA in the ipsilateral cortex (128%), but had no effect on the contralateral tissue, as detected by realtime RT-PCR. In contrast, *rstn* was barely detectable in the hippocampus of sham controls, but was strikingly induced following TBI in the ipsi- and contralateral hippocampus (150-fold and 50-fold respectively). Values are expressed as a percentage of the control ± SEM obtained from duplicate experiments (N= 6-8). (*p<0.05, **p<0.01)

p<0.05, respectively) relative to sham controls. Note that leptin expression was not significantly affected in the contralateral cortex and thalamus. In contrast, FP brain injury did not alter leptin mRNA levels in the ipsi- or contralateral hippocampus (Fig 67C).

Fiaf gene expression was more sensitive to TBI than leptin or *rstn* in all brain regions studied (Fig 68). Increases in *fiaf* mRNA were detected in the ipsilateral cortex (6-fold; $p<0.0001$), thalamus (5-fold; $p<0.001$) and hippocampus (7.5-fold; $p<0.0001$)12h post-injury. Another notable difference was the significant, though smaller, increase in *fiaf* mRNA in the contralateral rat brain including the cortex (2-fold; $p<0.01$), thalamus (2-fold; $p<0.05$) and hippocampus (2.5-fold; $p<0.01$).

Significant increases in *rstn* mRNA were detected in the ipsilateral, but not the contralateral, rat cortex (~2.5-fold; $p<0.01$, Fig 69A). Although *rstn* gene expression was barely detectable in the rat thalamus (data not shown) or hippocampus of sham controls, a striking induction of *rstn* was detected in both the ipsi and contralateral hippocampus (150-fold and 50-fold respectively), but was highly variable between animals (Fig 69B).

IV. Discussion

These studies clearly demonstrated that adipokine gene expression is induced in the rat and mouse CNS following brain injury. More specifically *fiaf* and *rstn* gene expression were differentially upregulated in the hippocampus and cerebral cortex following hypoxic/ischemic brain injury in the neonatal mouse brain. *Fiaf* mRNA was rapidly increased and remained elevated over a period of at least 7 days. In contrast *rstn* expression was only significantly increased in the damaged cortex 21 days post-injury, although it tended to be slightly higher in the hippocampus as well. The prompt response in *fiaf* expression, compared to the delayed changes in *rstn* mRNA, suggests that *fiaf* might be involved in the acute response to neonatal brain injury, especially since the changes were confined to the site of injury. Similarly TBI dramatically induced the expression of *leptin, fiaf* and *rstn* in several regions of the adult rat brain only 12h after

injury. Although the largest changes occurred at the site of injury, some significant increases in *fiaf* and *rstn* mRNA were also detected in contralateral brain regions, relative to sham-operated controls. In contrast, elevations in leptin gene expression were confined to ipsilateral cortex and thalamus, and no changes in leptin were detected in the hippocampus. Taken together these data suggest that centrally-derived adipokines could be part of a general response in the pathology of brain damage.

Two different species and models of brain injury were used in these studies to verify that damaging the CNS was capable of inducing the expression of brain-derived adipokines. One common feature was the rapid increase in *fiaf* expression in both the mouse and rat. However there were also some notable differences. TBI rapidly induced *rstn* mRNA in the rat, whereas the upregulation of *rstn* was delayed in the hypoxic ischemic mouse brain (>7 days). This conflicting result may have occurred because of species, age or type of injury that was induced. In addition certain mouse strains appear more vulnerable than others to neonatal H/I brain injury. For example the damage detected in the CD-1 strain was greater than that detected in either the C57BL/6 or 129Sv strains following a similar brain injury (Sheldon et al. 1998). Although hybrid mice that were generated by crossing mouse strains appeared to be even more resistant to brain damage, those crossed with the CD-1 background appeared to have a smaller degree of protection (Sheldon et al. 1998). In addition the pattern of injury varied between mouse strains, and it was suggested that molecular or anatomical differences between them might alter the severity of the injury (Sheldon et al. 1998). Similar differences have also been reported between Wistar and Sprague-Dawley rats, however variations were also detected in the same strains that were purchased from different rat vendors (Oliff et al. 1995a; Oliff et al. 1995b).

Unlike cerebral H/I damage, where gene expression changes were only detected at the site of injury, TBI also induced modest increases in *fiaf* and *rstn* in the

contralateral brain. This likely reflects the bilateral diffuse injury that results following TBI, whereas cerebral H/I appears to induce a more localized and unilateral response in mice (Rice et al. 1981; Morales et al. 2005). Moreover these localized patterns of elevated adipokine expression suggest that the detected increases were directly stimulated by the injury itself as opposed to a secondary inflammatory cascade. Although the mouse brain does not appear to express leptin, it seems likely that if neonatal rats were subjected to cerebral H/I it would also up regulate *ob* mRNA in the rat brain. Despite the apparent differences between the severity of brain injury reported between different rodent strains and species, brain damage modifies adipokine gene expression in the CNS.

Rats, and human patients, often experience a period of wasting following TBI since they become anorectic and hyperthermic, further compounding the effects of the initial neurotrauma (Thompson et al. 2003; Moinard et al. 2005; Suz et al. 2006). In rats TBI rapidly induced anorexia and significant weight loss was readily detected within 24h of injury, and these effects persisted for up to 10 days following injury (Otori et al. 2004; Moinard et al. 2005). However the mechanism(s) by which TBI induces these profound metabolic consequences remains unclear. It is well established that the intracerebroventricular (icv) injection of leptin reduces appetite and increases energy expenditure in rodents (Campfield et al. 1995; Gullicksen et al. 2002). Similarly the icv injection of resistin peptide fragments acutely reduced appetite in rats (Tovar et al. 2005). Perhaps the increases in central adipokine gene expression can elevate local adipokine concentrations to levels that are able to activate hypothalmic pathways that modulate appetite and energy expenditure. Further experiments are required to investigate the effects of brain damage on the levels of central adipokine proteins. Note that TBI also compromises the integrity of the blood brain barrier (BBB), though this appears to be localized to the site of injury (McIntosh et al. 1989; Nimmo et al. 2004).

Thus it's unclear whether circulating adipokines might cross the leaky cortical BBB in order to reach hypothalamic feeding pathways.

There are several potential mechanisms responsible for increasing central adipokine gene expression following brain injury. As already noted, inflammation and brain injury is capable of modifying the expression of several transcription factors implicated in adipokine gene regulation both in the rat and mouse CNS. Although LPS appears to acutely (i.e. hours) increase adipokine gene expression in the mouse brain (see Chapter 5), neonatal LPS treatment failed to reproduce the prolonged (i.e. days) increases in gene expression that were seen following H/I (Wiesner et al. 2006). This suggests that the upregulation of adipokines was unlikely to be the result of an associated inflammatory response alone. In contrast H/I increased the expression of CEBPα, a key modulator of brain *rstn* and *fiaf* expression (Chapter 4), for more than 7 days in the rat brain following H/I (Walton et al. 1998). Thus it might be responsible for stimulating adipokine gene expression in the injured brain over prolonged periods. Although CEBPα increased the expression of *rstn* and inhibited *fiaf* in N-1 hypothalamic neurons (recall Chapter 4), the paradoxical results obtained in the injured mouse cortex (i.e. increased *fiaf* and slightly decreased *rstn*) might reflect the cell type-dependent regulation of adipokine genes in the CNS. Similarly microarray analysis has revealed that certain transcription factors known to be involved in adipokine gene expression are rapidly increased in the rat brain following injury, most notably nuclear factor-κB (NF-κB) and hypoxia inducible factor-1α (HIF-1α) (Matzilevich et al. 2002; Raghavendra Rao et al. 2002; Rall et al. 2003). Resistin promoter analysis revealed the presence of numerous NF-κB binding sites, and the activation of NF-κB increased *rstn* gene expression in adipocytes (Lu et al. 2002). The hypoxic conditions that often result following brain injury not only increase *hif-1α* mRNA, but would also enhance the stability and transcriptional activity of HIF-1α (Wang et al. 1995; Rall et al. 2003). Additionally

HIF-1α is known to transactivate the leptin promoter (Grosfeld et al. 2002), and the *fiaf* promoter contains both NF-κB and HIF-1α promoter elements suggesting these factors might be partially responsible for detected increases in gene expression that occurred following TBI (Belanger et al. 2002). In addition to these nuclear mechanisms, TBI or alterations in cerebral blood flow may induce local increases of several inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), that might also modulate local adipokine gene expression (Kaser et al. 2003; Prins and Hovda 2003). Thus increased cytokine secretion, or modifications in cerebral blood flow, might be partially responsible for inducing the bilateral changes in adipokine expression that occurred following TBI (Morales et al. 2005). Taken together it appears that there are several potential mechanisms, both direct and indirect, that are responsible for inducing central adipokine gene expression following brain injury.

It remains unclear whether increased expression of centrally-derived adipokines has beneficial or deleterious consequence on brain function following cerebral H/I or TBI. *Fiaf* upregulation might be a common marker of brain damage and it is associated with inflammation in several tissues. It has also been reported to exert strong pro-angiogenic effects in a renal carcinoma (Le Jan et al. 2003). For example hypoxia induced the expression of *fiaf* in endothelial cells (Kim et al. 2000), human cardiomyocytes (Belanger et al. 2002) and in human glioblastoma (Lal et al. 2001). In addition the time-course of *fiaf* up-regulation observed in our experiments coincides with that of cerebrovascular angiogenesis following focal brain ischemia in adult mice (Hayashi et al. 2003) or cortical cold injury in adult rats (Nag 2002). Thus *fiaf* could be impacting central angiogenesis following brain injury. A potentially damaging effect of FIAF following TBI could occur via the inhibition of lipoprotein lipase activity (LPL)(Koster et al. 2005), since increased LPL activity is hypothesized to be neuroprotective (Nunez et al. 1995; Paradis et al. 2003).

Although no studies have directly looked at the effects of resistin in brain injury, the intracerebroventricular (icv) injection of resistin peptide fragments did decrease appetite and induce hypothalamic c-fos expression in the adult rat (Tovar et al. 2005). Similarly the icv injection of recombinant resistin was shown to stimulate hepatic gluconeogenesis (Muse et al. 2005; Singhal et al. 2006). This is particularly intriguing since increased brain glucose metabolism appears to be part of the neuroprotective mechanism provided by PPARγ agonists (Garcia-Bueno et al. 2006). Similarly TBI preconditioning increases glycogen stores and this appears to protect rats against a secondary transient forebrain ischemic assault (Otori et al. 2004). Thus the TBI-induced increases in central resistin expression might be impacting glucose metabolism as part of a neuroprotective mechanism. In addition the acute upregulation of resistin suggests that, like FIAF, it may participate in immediate responses to cerebral damage following TBI and induce a local inflammatory response (Pang and Le 2006). Therefore increases in central resistin expression could be affecting appetite or glucose metabolism, which may influence the severity of brain injury, and subsequent recovery.

The effects of leptin have been well studied in the CNS, including its neuroprotective properties (Dicou et al. 2001; Zhang et al. 2006). For example leptin protected glutamate-stimulated mouse cortical neurons, rat C6 glioblastoma cells, and human neuroblastoma cells from death (Dicou et al. 2001; Russo et al. 2004; Brown et al. 2005a). Leptin was also reported to protect against ischemic damage in the mouse brain (Zhang et al. 2006). It is conceivable that leptin aids in brain repair following injury through stimulatory effects on neuronal proteins, such as growth associated protein 43 (GAP-43), that have been implicated in axonal regeneration following TBI (Christman et al. 1997; Ahima et al. 1999; Emery et al. 2000; Valerio et al. 2006). Furthermore the local increases in leptin expression occurred rapidly following brain injury, suggesting it might be part of the first line of defense that protects the CNS against further damage.

This could be especially important since damage has been detected in contralateral hippocampal neurons as early as 10min post-injury following a FP model of TBI (Carbonell and Grady 1999). However brain injury is also known to disrupt the integrity of the BBB (McIntosh et al. 1989; Nimmo et al. 2004), thus leptin of peripheral origin might also access the damaged brain regions in order to increase brain cell survival. Although leptin has clearly been implicated in brain development in the ob/ob mouse (Ahima et al. 1999; Steppan and Swick 1999), I am unaware of any studies looking at the consequences of brain injury in this model system. Similarly there appear to be no reports on the effects or neurotrauma in leptin receptor-deficient mice (db/db) or rats (fa/fa). The use of RNAi to block the local induction of leptin, as done in Chapter 2 for inhibiting the feeding-induced increase in hypothalamic leptin, might discriminate between the effects of peripheral and central leptin following brain trauma in the rat.

As discussed in Chapter 3, AMPK is a novel energy sensing enzyme that has recently been implicated in the hypothalamic control of appetite and hepatic gluconeogenesis (Kahn et al. 2005; McCrimmon et al. 2006). In addition AMPK regulation is impacted by adipokines, although their effects on AMPK activation are tissue-dependent. For example leptin stimulates AMPK in peripheral tissues, but inhibits its activity in the hypothalamus (Kahn et al. 2005; Kola et al. 2006). Thus local changes in adipokine expression, and changes in brain cell glucose metabolism, could be modulating the activity of AMPK in the CNS. Additionally activating hypothalamic AMPK following brain injury might also modify appetite and increase peripheral energy expenditure (i.e. hyperthermia). Recently AMPK was implicated in neuronal survival, although it remains controversial whether increases or decreases in its activity are protective (Ramamurthy and Ronnett 2006). For example activating AMPK offered cytoprotection against glucose deprivation in isolated hippocampal neurons, although there have been conflicting reports in different cell types (Blazquez et al. 2001; Garcia-

Gil et al. 2003; Ramamurthy and Ronnett 2006). Thus the effects of AMPK on cell survival might even be cell-type specific (Ramamurthy and Ronnett 2006). In addition cerebral ischemia induced AMPK in the intact brain, and pharmacologically enhancing its activation using AICAR, appeared to be detrimental to brain cell survival (McCullough et al. 2005). In contrast blocking AMPK activation, using the fatty-acid synthase inhibitor C75 or compound C, reduced infarct volume and appeared cytoprotective (McCullough et al. 2005). However neuronal AMPK activation appears to be a "double edged sword" since modestly increasing its activity (40% caloric restriction) appeared protective (i.e. 2-3 fold) and induced neurogenesis when caloric intake was modestly restricted in mice (Dagon et al. 2005). Conversely when caloric intake was severely restricted (60% restriction), further increases in AMPK activation were detected (i.e. 6-fold), which positively correlated with brain cell death (Dagon et al. 2005). However leptin treatment could reduce these further increases in neuronal AMPK activity and offered protection against apoptosis (Dagon et al. 2005). Therefore the magnitude of AMPK activation in the CNS could be delicately balancing the apoptotic and survival pathways, and suggests it could have a substantial impact on cell survival following neurotrauma. In Chapter 3 I provided evidence that endogenous resistin modulated AMPK activity in the N-1 hypothalamic cell line, suggesting that endogenous adipokines are impacting cellular energy homeostasis. It seems plausible that other endogenous adipokines (i.e. leptin) might also be impacting AMPK activity in brain cells. Thus brain-derived adipokines might precisely modulate the delicate balance in local AMPK activity that could lead to substantial differences in brain function following neurotrauma, and this can now be tested.

Our experimental results clearly demonstrate that brain injury, either in rats or mice, can modify the expression of adipokine genes in the rodent brain. In addition our TBI data suggest that the increases in central adipokine gene expression might be

associated with some of the clinical features of TBI, in particular neuroinflammation and cachexia. I speculate that brain-derived adipokines mediate their effects via an autocrine or paracrine mechanism to modulate local cytokine signaling and inflammatory responses following brain injury. For example local adipokines might modify the local activity of AMPK in order to modulate the delicate balance between cell survival and apoptosis, in addition to impacting cellular energy homeostasis following brain injury. Having established that RNAi can effectively block the induction of leptin gene expression in the intact rat brain (Chapter 2), I can now use this approach to block the injury-induced increases in central adipokine expression as done for brain derived neurotrophic factor (BDNF) and signal transducer and activator of transcription 3 (STAT3) (Baker-Herman et al. 2004; Satriotomo et al. 2006). This approach would help elucidate the individual roles of brain-derived adipokines in the pathology of brain injury by assessing the effects on infarct size and severity of brain injury in their absence, in addition to monitoring the effects on appetite and body temperature. Thus central adipokines could prove to be a relevant therapeutic target to modify central inflammatory pathways and local glucose metabolism in order to minimize the post-ischemic damage that often results following brain injury, and warrants further investigation.

Chapter 7: Conclusions

In previous investigations our laboratory established that the brain and pituitary, in addition to adipose tissue, are also loci of leptin gene expression as detected by RT-PCR and immunohistochemistry (Morash et al. 1999; Morash et al. 2002). Although it was initially hypothesized that brain-derived leptin would be an endogenous ligand for centrally expressed leptin receptors, and act as an autocrine/paracrine signal that might impact local energy metabolism and the normal functioning of the brain, the evidence supporting such a mechanism was limited (Morash et al. 1999; Morash et al. 2003). Leptin gene expression has also been detected in the brain of several species (please see Table 1), although the mouse brain is a notable exception, it seems likely that this is an anomaly or exception to the rule. Subsequently our laboratory has also shown that the brain expresses other adipokine genes including *rstn* and *fiaf* (Morash et al. 2002; Wiesner et al. 2004). In addition leptin mRNA transcripts have been detected in a variety of tissues including placenta (Hoggard et al. 1997), stomach (Bado et al. 1998), skeletal muscle (Wang et al. 1998) and bone (Reseland et al. 2001), to name a few. The leptin receptor is also ubiquitously expressed, and has been detected in a variety of tissues (Tartaglia et al. 1995; Lee et al. 1996). Thus in addition to fat, adipokines appear to be produced throughout the body, suggesting their involvement in several processes that extend beyond the regulation of appetite and bodyweight. For example leptin has been implicated in the regulation of reproduction and fertility (Chehab et al. 1996), haematopoiesis (Bennett et al. 1996), lactation (Mounzih et al. 1998), brain development (Ahima et al. 1999; Steppan and Swick 1999), neuroprotection (Dicou et al. 2001), synaptic plasticity (Shanley et al. 2001), and this list continues to expand.

The primary focus of my studies was to investigate the potential physiological roles of brain-derived adipokines. This was accomplished in three ways: a) using RNAi to determine the possible effects of brain-derived adipokines on cell survival and energy

metabolism in vitro and in vivo; b) by elucidating the potential molecular mechanism involved in the regulation of central adipokines and c) further elaborating on their potential involvement in inflammation and brain injury. RNAi studies established that endogenous brain-derived adipokines were impacting cell death, cellular signaling (i.e. *socs-3*) and energy sensing pathways (i.e. AMPK). Subsequent studies revealed that the regulation of adipokine genes is cell-line dependent since differential changes were detected between N-1 neurons and 3T3-L1 adipocytes following treatment with valproic acid (VPA), all-*trans* retinoic acid (ATRA) or rosiglitazone (ROSI). Moreover the effects in N-1 neurons appeared to be CEBPα-dependent. These compounds also modified adipokine gene expression in vivo and their effects were also tissue-specific. Likewise triggering a neuroinflammatory response, either by the peripheral injection of LPS or by inducing brain injury, elevated the expression of centrally-derived adipokines in a tissue-dependent manner. The data obtained from these studies support a role for centrally-derived adipokines in mediating local energy metabolism, cellular signaling, and cell survival in the intact CNS.

Implications For The RNAi Studies

RNA interference (RNAi) has proven to be one of the most useful tools to study gene function since it allows for the specific silencing of target gene expression both in vitro and in vivo. Proof-of-principle studies in C6 glioblastoma cells revealed that RNAi is an effective means to silence leptin gene expression in a time- and dose- dependent manner. Furthermore the use of a chemically modified siRNA, with enhanced stability, prolonged leptin silencing and limited the risk of inducing non-specific RNAi pathways. In addition, 72h after transfecting cells with the leptin-specific siRNA, I detected a 50% *reduction* in leptin mRNA and protein, but a 2-fold *increase* in C6 cell death. Thus endogenous brain-derived leptin also appears to modulate cell survival, and is consistent with a previous report in which treatment of human neuroblastoma cells with leptin was

protective against apoptosis (Russo et al. 2004). Further studies are now warranted to determine the mechanisms and signaling pathways by which endogenous leptin could impact brain cell survival (see Figure 70).

Preliminary in vivo studies also confirmed that RNAi could be used to acutely block the nutritionally-mediated induction of brain leptin. The icv injection of siSTABLE L7 into the dorsal 3rd ventricle (D3V) of fasted rats was capable of attenuating the feeding-induced increase in hypothalamic leptin mRNA by more than 70% ($p<0.01$). Additionally this acute knockdown (24h) resulted in a slightly greater overnight weight gain, relative to rats that received the non-specific control molecule. However the site of injection greatly influenced the effectiveness of the RNAi targets since siSTABLE L7 had no effect on hypothalamic leptin gene expression when it was injected into the lateral ventricle (LV), although cortical leptin mRNA was reduced in both studies. Similarly the continuous infusion of the 'naked' leptin-specific siRNA into the LV resulted in a modest reduction in leptin mRNA (-30%) in the cortex and hypothalamus, although no increases in bodyweight were detected. Leptin knockdown also *reduced* the expression of cortical resistin, but paradoxically *increased* hypothalamic and pituitary resistin mRNA by 2-fold, as measured by realtime RT-PCR. Thus brain-derived leptin appears to regulate the expression of other centrally derived adipokines, although these effects were dependent on the cell-type or brain region being analyzed. However an important technical goal remains to improve the magnitude and length of silencing achieved under basal conditions in vivo. This might be accomplished either by increasing the dose of siRNA delivered or attempting to use alternative delivery methods (i.e. viral-based shRNA). Perhaps achieving a greater brain leptin knockdown will result in a more pronounced phenotype.

To further investigate the novel interaction between brain adipokines, RNAi studies on resistin and fiaf were also carried out in the novel N-1 hypothalamic cell line
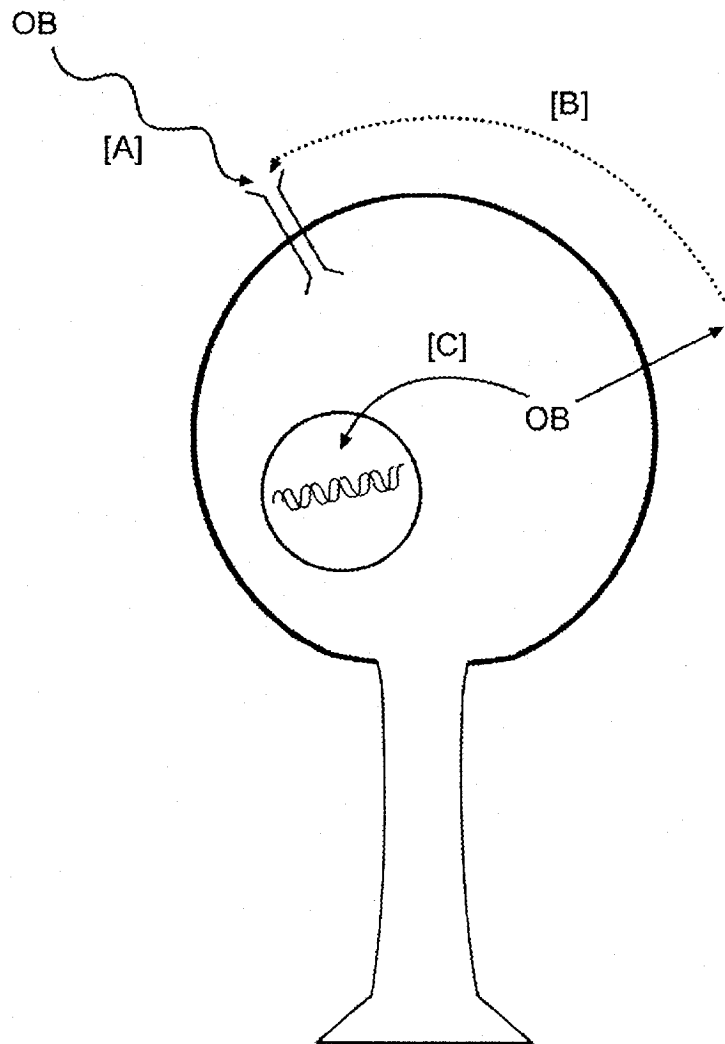
Figure 70: Leptin could be mediating its effects on brain cell survival by various pathways. [A] Exogenous leptin favors brain cell survival by activating leptin receptors (OBR) located on the plasma membrane as shown by Russo et al. (2004) in the human neuroblastoma cell line, and by Ahima et al. (1999) in the mouse brain. [B] Although leptin might be secreted from brain cells to activate OBR via an autocrine/paracrine mechanism in order to prevent cell death, we failed to detect leptin secretion from C6 cells suggesting that this might not be the case (Wilkinson et al. 2007). [C] In contrast brain leptin might be activating an unidentified nuclear leptin receptor, via an intracrine pathway. This would be consistent with the nuclear localization of leptin protein that was observed in C6 cells (Morash et al. 2000), and is supported by the recent speculation that at least one other leptin receptor exists (Farooqi et al. 2007).

that was derived from the embryonic mouse brain (Belsham et al. 2004). Although *fiaf* expression was significantly reduced using a chemically modified siRNA, it failed to significantly modify the expression of other genes being analysed. In marked contrast the RNAi-mediated silencing of *rstn* induced significant increases (>50%) in *fiaf* and suppressor of cytokine signaling-3 (*socs-3*) when N-1 cells were cultured in serum-deprived medium. Moreover resistin knockdown reduced the ratio of phosphorylated to total AMP-activated protein kinase (AMPK), a novel energy sensor implicated in the hypothalamic control of appetite and hepatic gluconeogenesis (Minokoshi et al. 2004; Kahn et al. 2005; McCrimmon et al. 2006). Additionally the effects on *fiaf* and *socs-3* expression, or changes in AMPK activation, were reversed when N-1 cells were treated with resistin, or transfected with a *rstn*-expressing plasmid. Therefore endogenous adipokines, in particular resistin, appear to modify cellular energy metabolism, cell signaling, as well as modulate the expression of other adipokines in this novel N-1 hypothalamic cell line model. These effects now need to be confirmed in vivo.

Molecular Regulation Of Brain Adipokines

Having revealed several potential roles for centrally-derived adipokines in brain function, further investigations focused on elucidating the underlying molecular mechanisms that were responsible for regulating their expression. Most peripheral adipokines are regulated by 'adipogenic' transcription factors, such as CEBPα or PPARγ, which are also expressed in the CNS (Williams et al. 1991; Moreno et al. 2004). Valproic acid (VPA), all-*trans* retinoic acid (ATRA) and rosiglitazone (ROSI) modified adipokine gene expression in N-1 cells, although these changes were independent of those detected in 3T3-L1 adipocytes. Further studies suggested that many of these effects were CEBPα-related since blocking its expression, using RNAi, induced similar gene expression profiles in N-1 neurons. However it is currently unknown whether these drugs modify the expression of CEBPα, or if they directly inhibit the binding of CEBPα

dimers to the various adipokine promoters. An answer to this question will be an important goal in future investigations. Likewise the gene expression profiles detected in vitro did not match what was detected in mice. Therefore other transcription factors are likely involved in the regulation of brain-derived adipokines in vivo. Potential candidates include the glucocorticoid receptor (GR) or PPARα. For example I have shown in previous studies that glucocorticoids stimulate *rstn* gene expression in the AtT 20 corticotrope cell line and in the mouse anterior pituitary (Brown et al. 2005b). Likewise the injection of WY 14643, a PPARα agonist, induced *fiaf* expression in the cortex, although multiple injections inhibited *fiaf* expression in the pituitary and fat (Dr. G. Wiesner- personal communication). Although subsequent studies are needed to further characterize the effects of CEBPα on central adipokine gene regulation, similar experiments should also investigate other possible factors (i.e. GR or PPARα) that might also be modulating the expression of brain-derived adipokines. Since centrally-derived adipokines appear to impact various brain functions, an understanding of their underlying regulatory mechanisms might help identify therapeutic targets that can be exploited to protect the brain, or maintain normal CNS function, following damage or during disease states.

Central Adipokines And Inflammation

Obesity is commonly associated with a chronic low-grade inflammatory state (Trayhurn and Wood 2004; Trayhurn and Wood 2005). Therefore I investigated whether brain-derived adipokines might also be induced during central inflammatory responses. The subcutaneous injection of LPS is known to result in an inflammatory cascade that modifies central energy metabolism by decreasing appetite and stimulating peripheral insulin resistance (Huang et al. 1999; Ueki et al. 2004). In these studies LPS clearly showed time and tissue-dependent changes in adipokine gene expression. Moreover the gene expression changes in the hypothalamus appear to precede those in pituitary,

followed next by the cortex and adipose tissue, suggesting that the hypothalamus might provide feedforward signals to these other tissues to impact gene expression. These data are also consistent with the hypothesis that peripheral insulin resistance can be initiated by elusive neurochemical events (see Figure 71) (Buren and Eriksson 2005). Thus LPS might trigger the expression of hypothalamic-derived adipokines which may lead to changes in appetite or modifications in peripheral gene expression. This hypothesis can now be tested by injecting our *rstn-* or *fiaf*-specific siRNA molecules into the mouse ventricular system just prior to the injection of LPS and monitoring the effects on food intake, bodyweight and the time-dependent changes in peripheral gene expression.

In addition to the debilitating effects on cognition and motor function, brain injury also modifies appetite and body temperature which can have detrimental consequences on patient recovery and survival (Thompson et al. 2003; Moinard et al. 2005). When the rodent CNS was damaged, either by cerebral hypoxic/ischemic (H/I) or traumatic brain injury (TBI), robust increases in adipokine gene expression occurred. For example the neonatal model of H/I damage induced long lasting effects on central adipokine expression; *fiaf* was increased for up to 7 days following H/I, whereas *rstn* was increased only 21days following damage. Likewise TBI rapidly increased the expression of *rstn, fiaf* and *leptin (ob)* in the rat CNS. Although the largest changes were detected near the site of injury, some increases were also detected in some contralateral brain regions following TBI. These studies suggest that brain-derived adipokines might also impact cell survival and participate in local tissue repair via an autocrine/paracrine mechanism (see Figure 70). In addition centrally-derived adipokines might also be mediating some of the metabolic consequences associated with brain injury and neuroinflammation. However further studies are needed to investigate the potential roles of brain-derived adipokines in the pathology of brain injury, and whether they offer any neuroprotection. One method
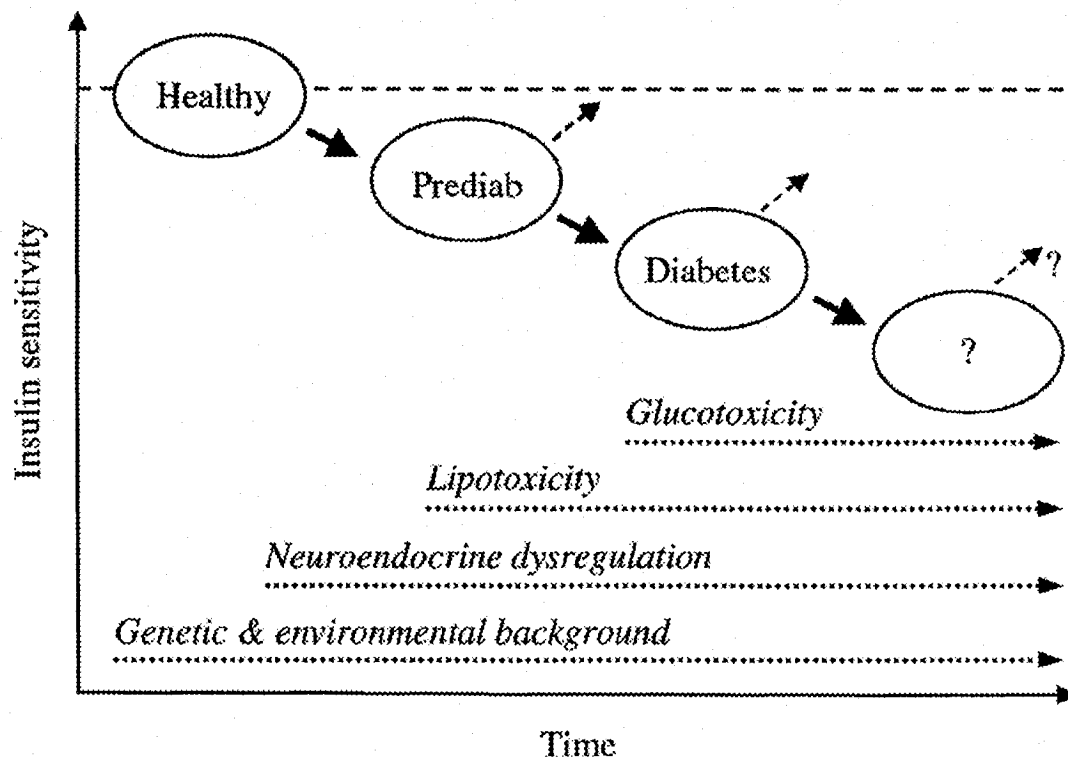
Figure 71: Neurochemical changes might lead to the induction of peripheral insulin resistance. It has been hypothesized that the progression from a healthy to a prediabetic state might involve neurochemical changes that send feedforward signals which lead to the development of systemic insulin resistance and impaired glucose homeostasis. However it is unclear what triggers these events, and how they can manifest into a diabetic state. Perhaps the induction of neuroinflammation is capable of stimulating the necessary cascade of events that involves both centrally and peripherally-expressed adipokines, although other gene targets are also likely involved. (Figure adapted from by Buren and Eriksson 2005)

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to discern the individual roles of locally-derived adipokines in the pathology of brain injury would be to use the optimized siRNA targets to specifically block their expression prior to damaging the CNS and monitoring the outcome either by testing motor function or monitoring bodyweight, food intake and temperature.

Brain Adipokines And An Endogenous Metabolic Tuning Mechanism

These proof-of-principle studies established that RNAi is an effective means to silence endogenous brain-derived adipokines both in vitro and in vivo. Furthermore the data obtained in these investigations suggest that centrally expressed adipokines impact cell survival, cellular signaling and energy sensing pathways. As such brain-derived adipokines could have a significant influence on the normal functioning and maintenance of the CNS. For example hypothalamic-derived adipokines might contribute to the complex system that is implicated in the control of appetite and bodyweight. Although peripheral tissues, such as fat, appear to provide the hypothalamus with adiposity signals that are a gross indication of the available energy stores (Ahima 2005; Badman and Flier 2005; Schwartz and Porte 2005; Ahima et al. 2006), it seems unlikely that this adipostatic system alone is capable of providing the hypothalamus with the signals it requires to delicately control bodyweight with a 99.97% degree of efficiency (Hervey 1969). Similarly brain glucose metabolism is also known to be finely regulated (Penicaud et al. 2002). Perhaps the adipokine crosstalk that is occurring in vivo between centrally derived adipokines forms a fine-tuning mechanism that aids in the precise regulation of hypothalamic metabolic pathways (see Figure 72). In addition centrally-derived adipokines appear to impact the expression of *socs-3* and AMPK activity in the N-1 hypothalamic cell line, targets that have been implicated in normal weight regulation and glucose homeostasis in vivo (Kahn et al. 2005; Howard and Flier 2006). As already noted such a system is not without precedent; recently it was shown that nesfatin-1, a novel satiety hormone, is expressed in fat and the hypothalamus (Oh et al. 2006).
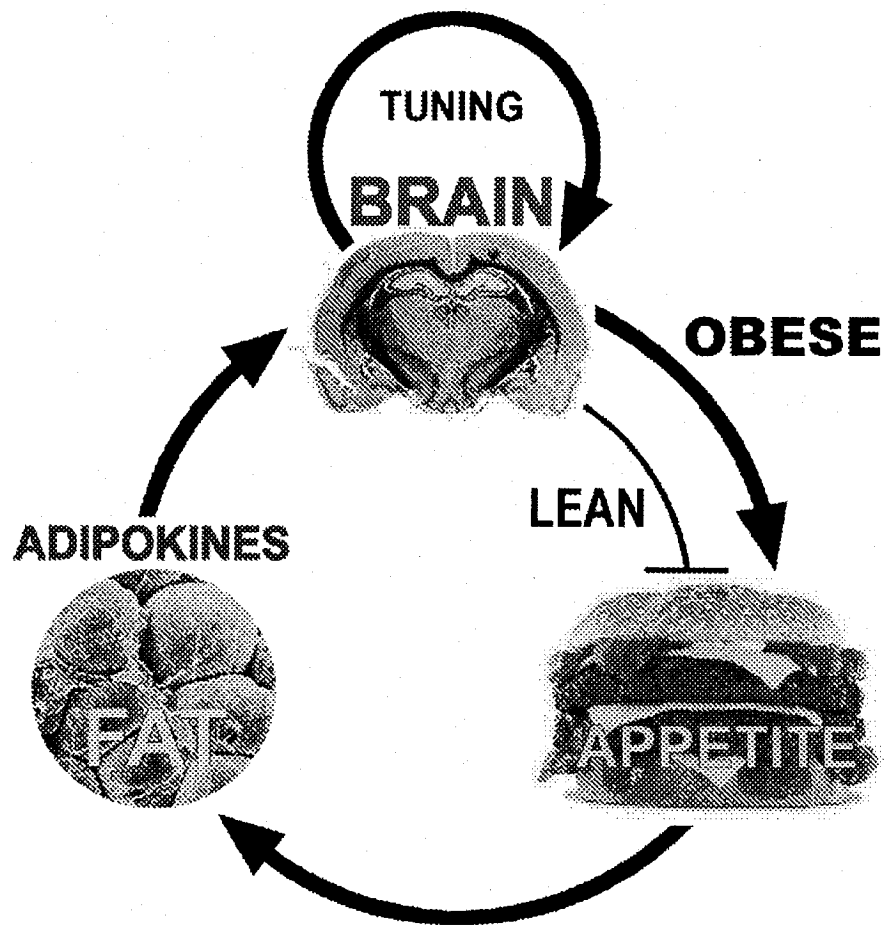
Figure 72: Brain adipokines and a modified lipostatic loop

It has been more than 50 years since Kennedy first proposed the existence of a lipostatic loop in which body fat controls energy metabolism via a hypothalamic-dependent mechanism. Subsequently it has been shown that bodyweight regulation is extremely precise and efficient in humans (99.97%), and that adipose tissue is an endocrine organ that helps control these central metabolic pathways by secreting adipokines. However obesity is characterized by a resistance to these peripheral adiposity signals, and normal satiety mechanisms fail to shut off appetite, which leads to further food consumption and weight gain. Since brain-derived adipokines appeared to modify cell signaling (i.e. *socs-3*) and energy sensing (i.e. AMPK) in N-1 hypothalamic neurons, I hypothesize that centrally expressed adipokines might form an endogenous tuning mechanism that helps mediate the accurate balance between food intake and energy expenditure. Thus disrupting brain adipokine expression might induce a hypothalamic metabolic mismatch that favors a cycle of weight gain.

Similarly the icv injection of nesfatin decreased food intake, whereas the knockdown of hypothalamic nesfatin-1 gene expression had the opposite effect (Oh et al. 2006). Therefore slight perturbations in this hypothetical central adipokine system might lead to a metabolic mismatch that increases the risk of gaining weight or developing insulin resistance by impacting the hypothalamic sensitivity and receptivity to peripheral adiposity signals. Similarly modifying this central adipokine system may induce the necessary neurochemical changes that have been implicated in the initiation of a pre-diabetic state (see Figure 71)(Buren and Eriksson 2005), and might increase the risk of developing metabolic syndrome or diabetes. One way to test this in vivo would be to use our recently optimized RNAi conditions to silence brain-derived adipokines and providing animals with a high fat diet, or a representative equivalent to the average North American diet, and verifying whether this disrupts the regulation of bodyweight or energy metabolism.

Obesity is clinically characterized by a state of leptin resistance (Heymsfield et al. 1999; Munzberg and Myers 2005), thus the obese brain appears incapable of properly receiving or interpreting peripheral adiposity signals. Although other mechanisms have also been suggested, increases in hypothalamic socs-3, an intracellular inhibitor of leptin and insulin signaling, appears to be involved in the etiology of diet-induced obesity (DIO) and insulin resistance (Munzberg et al. 2004; Howard and Flier 2006; Kievit et al. 2006). As demonstrated in vitro, endogenous resistin inhibits the expression of socs-3 in N-1 hypothalamic neurons. As mentioned above, modifying central adipokine expression might also alter the activity or expression of genes implicated in hypothalamic adipokine resistance. In addition the icv injection of leptin has been shown to modify the expression of several hypothalamic target genes that are thought to be responsible for mediating its effects on appetite and energy metabolism. These includes inhibiting the expression of NPY and increasing POMC and corticotrophin-releasing factor (CRF)

mRNA levels, although other neuronal genes also appear to be affected by leptin treatment (Schwartz et al. 1996b; Schwartz et al. 1997). Therefore local adipokine knockdown might also modify the expression of other hypothalamic neuropeptides implicated in the control of appetite and energy expenditure.

Centrally-derived adipokines might also impact metabolism and bodyweight regulation by modifying brain cell number and structure. For example the continuous infusion of ciliary neurotrophic factor (CNTF) induced longterm reductions in bodyweight in adult rodents, and this was attributed to the stimulation of hypothalamic neurogenesis (Kokoeva et al. 2005). Similarly the injection of leptin during critical neonatal periods also modified hypothalamic organization by promoting neurite outgrowth and the development of central feeding circuits (Bouret et al. 2004b; Bouret et al. 2004a). However leptin also induced the rapid 'rewiring' of the leptin-deficient ob/ob adult hypothalamus and might also have a significant impact on weight regulation (Pinto et al. 2004). Having shown that the RNAi-mediated silencing of leptin *induces* cell death in rat C6 glioblastoma, perhaps inhibiting local brain leptin expression during critical developmental periods might have an equally deleterious effect on brain cell survival in neonatal rats. Furthermore the increased expression of adipokines following brain injury could suggest their involvement in brain repair, but this requires further confirmation. Therefore modifying endogenous leptin expression might also impact the neuroanatomical organization of the hypothalamus, or other brain structures, which could lead to further changes in metabolism and bodyweight regulation.

Are Other Adipokines Expressed In The Brain?

Adipose tissue is now regarded as an endocrine gland and appears to express and secrete more than 50 different gene products (Trayhurn and Wood 2004). Thus another logical approach in the ongoing investigations on brain adipokines is to identify other candidate genes that might also be expressed in the CNS. Several possible novel

adipokine transcripts that could be tested were revealed by a brief literature search including visfatin (Fukuhara et al. 2005), apelin (Kawamata et al. 2001), vaspin (Hida et al. 2005), and adipsin genes (Platt et al. 1989). Similarly the converse, that brain transcripts are expressed in fat, also appears true. For example nerve growth factor (NGF), long-accepted as a central neurotrophin (Barde 1990), is now regarded as an adipokine that is secreted from white adipose tissue (Peeraully et al. 2004). Similarly midkine, a peptide previously implicated in neurite out growth and neuronal survival, has recently been shown to participate in 3T3-L1 differentiation during the mitotic clonal expansion phase (Cernkovich et al. 2007). In fact gene expression profiling has revealed a plethora of novel and unexpected transcripts, including several appetite-regulating peptides and receptors, that are expressed in fat including NPY, CRF, NPY receptors, MCH and cholecystokinin (Friedberg et al. 2003; Yang et al. 2003b; Gomez-Ambrosi et al. 2004; Seres et al. 2004; Guan et al. 2005). Nevertheless Trayhurn and Wood (2004) failed to detect some of these transcripts using RT-PCR, thus raising questions about the data and interpretation of gene expression profiling done in fat. Similar doubts have been raised with respect to adipokine expression in the brain, however I believe further investigations are now merited based on the evidence presented in these studies.

In summary, these studies implicate brain-derived adipokines in the control of cell signaling, metabolism and survival. In addition there appears to be a complex interrelationship between centrally-derived adipokines both in vitro and in vivo. Although the expression of central adipokines also appears dependent on adipogenic transcription factors, such as CEBPα, their regulation is tissue and cell-type dependent. In addition these transcripts are induced in the CNS by inflammation and neurotrauma suggesting their potential involvement in the pathology of brain injury and repair. These data also raise the possibility of a hypothalamic autocrine/paracrine system that might be involved in the fine tuning of metabolic output, but this requires further investigation. Another

issue that remains to be resolved is whether 'adipokine' is an appropriate term to label brain (or pituitary) derived peptides such as leptin. By definition an *adipokine* is 'a protein that is secreted from, and synthesized by, adipocytes' (Trayhurn and Wood 2004), which is clearly unsuitable for those adipokine transcripts and protein products that are expressed in non-adipose sites such as the brain. For example muscle-derived cytokines have recently been defined as 'myokines' (Pedersen et al. 2004). With this precedent in mind, and the inappropriateness of the word adipokine, we suggest that cytokine transcripts expressed in the brain, brain tumor cells, and pituitary gland, be more suitably termed *cephalokines* (Wilkinson et al. 2007).

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Appendix I: Primer and siRNA Sequences

Table 2: PCR Primers And Dual-Labeled Probes

NAME		Sequence (5' to 3')	ACCESION	START POSITION	intron spanning?	reference
RAT SEMI-QUANTITATIVE PCR SEQUENCES						
Leptin	sense	5' GGATGACACCAAAACCCTCA 3'	D45862	144	yes	Morash et al. (1999)
	antisense	5' CATGAGCTATCTGCAGCACG 3'		358		
Cyclophilin	sense	5' ATGGTCAACCCCACCGTGTTCTTCG 3'	BC059141	48	yes	Ma et al. (1994)
	antisense	5' CGTGTGAAGTCACCACCCTGGCACATG 3'		253		
RAT REAL TIME PCR						
Leptin	sense	5' AAGACCATTGTCACCAGGATCAA 3'	D45862	166	yes	
	antisense	5' CACGTTTTGGGAAGGCAAGC 3'		199		
	probe	5' CACACGCAGTCGGTATCCGCCAGG 3'		342		
Resistin	sense	5' AGCATGTCACTGTGTCCCATGGAT 3'	NM_144741	144	yes	
	antisense	5' TGGAGGAGACTGACCAGCAATGTA 3'		261		
	probe	5' TCCCTACTGCCAGCTGCAATGAAGAA 3'		204		
FIAF	sense	5' AGAGCAACAGGAAACAAATGGCCC 3'	NM_199115	1562	no	
	antisense	5' TGGACTGAAATGGTCCCTCTGCAT 3'		1669		
	probe	5' ACTTCTCGCCTACCAGAGAAGTTGGA 3'		1615		
Cyclophilin	sense	5' TGTGCCAGGGTGGTGACTT 3'	M19533	224	yes	Bond et al. (2002)
	antisense	5' TCAAATTTCTCTCCGTAGATGGACTT 3'		293		
	probe	5' CACACGCAGTCGGTATCCGCCAGC 3'		244		
SOCS-3	sense	5' GCGGGCACCTTTCTTATCC 3'	NM_053565	210	no	(Steppan et al. (2005)
	antisense	5' TCCCCGACTGGGTCTTGAC 3'		285		
	probe	5' CTCGGACCAGCGCCACTTCTTCA 3'		236		

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NAME		Sequence (5' to 3')	ACCESION	START POSITION	intron spanning?	reference
MOUSE						
Resistin	sense	5' TTCCTTGTCCCTGAACTGCT 3'	BC051196	107	yes	
	antisense	5' TGCTGTCCAGTCTATCCTTG 3'		382		
	probe	5' CACGAATGTCCCACGAGCCACAGG 3'		312		
FIAF	sense	5' ATCCTGGACGAGATGAACTTGCT 3'	AF278699	226	no	
	antisense	5' TCGGAGTCTTTGAAGGGTGCATCT 3'		399		
	probe	5' ATGGCTGCCTGTGGTAACGCTTGTCA 3'		335		
SOCS-3	sense	5' GCGGGCACCTTTCTTATCC 3'	NM_007707	569	no	Steppan et al. (2005)
	antisense	5' TCCCCGACTGGGTCTTGAC 3'		644		
	probe	5' CTCGGACCACCAGCGCCACTTCTTCA 3'		595		
Cyclophlin	sense	5' TGTGCCAGGGTGGTGACTT 3'	X52803	223	yes	Bond et al. (2002)
	antisense	5' TCAAATTCTCTCCGTAGATGGACCT 3'		292		
	probe	5' ACACGCCATAATGGCACTGGCGG 3'		243		
CEBPalpha	sense	5' AAGAAGTCGGTGGACAAGAACAG 3'	BC058161	942	no	Jochheim et al. (2004)
	antisense	5' GTTGCGTTGTTTGGCTTTATCTC 3'		1040		
IFNbeta	sense	5' GGAGATGACGGAGAAGATGC 3'	NM_010510	203	no	Stewart et al. (2005)
	antisense	5' CCCAGTGCTGGAGAAATTGT 3'		305		

Table 3: siRNA Target Sequences

NAME	Sequence (5' to 3')	Accession number	Start position
RAT			
siRNA L1	GACCAUUGUCACCAGGAUCdTdT	NM_013076	108
	GAUCCUGGUGACAAUGGUCdTdT		
siRNA L7	CGUGCUGCAGAUAGCUCAUdTdT	NM_013076	279
	AUGAGCUAUCUGCAGCACGdTdT		
siRNA L9	GACCUGCUCCCUGCCGCAGdTdT	NM_013076	345
	CUGCGGCAGGGAGCAGCUCdTdT		
siRNA L12	CAGUUGGACCUUAGCCCUGdTdT	NM_013076	478
	CAGGGCUAAGGUCCAACUGdTdT		
siRNA SCR	GUCACUGUGACUAGCUCGUdTdT		
	ACGAGCUAGUCACAGUGACdTdT		
MOUSE			
STH CTL	GUGGAAGAUAGUCGGCAGACACAGC		
	GCUGUGUCUGCCGACUAUCUUCCAC		
STH FIAF	GCACCCUUCAAAGACUCCGAGGAUA	nm_020581	376
	UAUCCUCGGAGUCUUUGAAGGGUGC		
STH FIAF 2	UCAAUGGACAAUACUUCCACUCUAU	nm_020581	1202
	AUAGAGUGGAAGUAUUGUCCAUUGA		
STH R12	GUGCAAGGAUAGACUGGACAGCAGC	nm_022984	355
	GCUGCUGUCCAGUCUAUCCUUGCAC		
STH R4	GGCUCGUGGGACAUUCGUGAAGAAA	nm_022984	312
	UUUCUUCACGAAUGUCCCACGAGCC		
STH CEBPα	CAUCUGCGAGCACGAGACGUCUAUA	34849752	278
	UAUAGACGUCUCGUGCUCGCAGAUG		